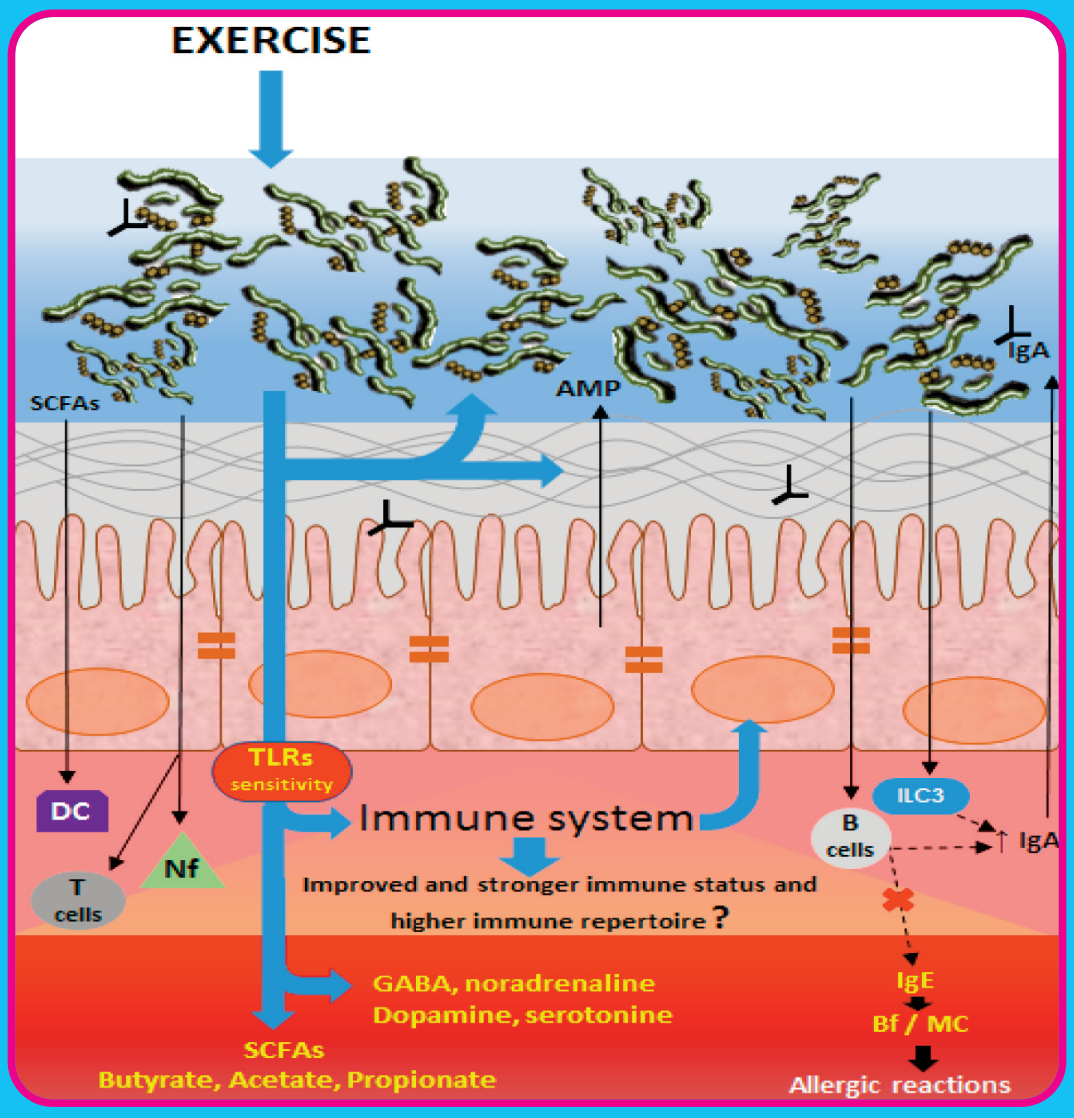
EXERCISE IMMUNOLOGY REVIEW



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EXERCISE IMMUNOLOGY REVIEW

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VOLUME 21 • 2015

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LETTERS

Editorial Statement

Exercise Immunology Review, an official publication of the International Society of Exercise Immunology and of the German Society of Sports Medicine and Prevention, is committed to developing and. enriching knowledge in all aspects of immunology that relate to sport, exercise, and regular physical ativity. In recognition of the broad range of disciplines that contribute to the understanding of immune function, the journal has adopted an interdisciplinary focus. This allows dissemination of research findings from such disciplines as exercise science, medicine, immunology, physiology, behavioral science, endocrinology, pharmacology, and psychology.

Exercise Immunology Review publishes review articles that explore: (a) fundamental aspects of immune function and regulation during exercise; (b) interactions of exercise and immunology in the optimization of health and protection against acute infections: (c) deterioration of immune function resulting from competitive stress and overtraining; (d) prevention or modulation of the effects of aging or disease (including HIV infection; cancer; autoimmune, metabolic or transplantation associated disorders) through exercise. (e) instrumental use of exercise or related stress models for basic or applied research in any field of physiology, pathophysiology or medicine with relations to immune function.

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From the Editors

EIR21 is a larger issue than usual containing 6 reviews, 6 original research articles and one letter. This is the result of an increase in the number of interesting manuscripts offered to us. The high number of submissions last year made it difficult to focus on special topics. Instead, it is a multifaceted compilation of articles on topics that are sufficiently novel and interesting for our readership.

The review of Peake et al. addresses the capacity of skeletal muscle cells to produce cytokines and examines potential other sources of circulating cytokines during exercise. The review of Horsburgh et al. is about the actual topic of epigenetics and focusses on DNA methylation in the context of inflammation and exercise. The article of Munz et al. discusses the role of inflammation in skeletal muscle adaptation to exercise with a focus on ARE-binding proteins. Ringseis et al. present an article about metabolic signals and innate immune activation for a better understanding of immunologic signaling in obesity prevention or therapy by exercise. The review of Bermon et al. intends to shed some light upon the interaction between the gut microbiota, exercise and immunomodulation. The last review article of EIR21 is a comprehensive article about effects of exercise on graft-versus-host disease.

The original research section starts with an article by Gill et al. reporting that a multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses. The research article of Sugama et al. deals with changes in circulating thioredoxin, oxidative stress markers, andinflammation following intensive endurance exercise.The following article by LaVoy et al. demonstrates that exercise can enhance the ex vivo expansion of tumour-associated-antigen-specific cytotoxic T-cells from healthy adults without compromising cytotoxic function. Halper et al. report that age affects TGF-β signaling in leukocytes by altering the expression levels of its receptors. Tug et al. investigated the exercise-induced increase in circulating cell free (cf) DNA and suggest, that cells from the haematopoietic lineage are the main source of cfDNA released during acute bouts of exercise. Perandini et al. analyzed the inflammatory cytokine kinetics to single bouts of exercise in women with active and inactive systemic lupus erythematosus. Finally, the letter of Abbasi et al. presents some evidence of exercise-induced bronchoconstriction in endurance runners and discusses some genetic aspects and gender differences.

This year's issue of EIR is the first one to appear under the new Editor Team with me (Karsten Krüger), closely supported by Mike Gleeson and Jonathan Peake. For EIR22 and the future we want the majority of contributions to be topical review articles. In the case of original research articles we encourage the authors to embed their new data into review articles. Please note that the submission deadline is 31st July 2015 for EIR22 which we aim to publish in early January 2016. We hope you enjoy reading the new issue and appreciate the new format. In the future EIR will be only published online. Only for special demands (like academic libraries) will there be printed versions available.

Personally, I thank you (all ISEI members), and all members of the Editorial Board for the confidence you have placed in us. We hope to see you at the 12th Symposium of the International Society of Exercise and Immunology at 6-9 July 2015 in Vienna.

Thank you, Mike Gleeson and Jonathan Peake, for the close and friendly teamwork and Hinnak Northoff for the honour to be his follower.

Thank you all for your ongoing support of EIR.
A special thanks to all the authors of EIR21.

On behalf of the Editors,

Karsten Krüger

Cytokine expression and secretion by skeletal muscle cells: regulatory mechanisms and exercise effects

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ABSTRACT

Cytokines are important mediators of various aspects of health and disease, including appetite, glucose and lipid metabolism, insulin sensitivity, skeletal muscle hypertrophy and atrophy. Over the past decade or so, considerable attention has focused on the potential for regular exercise to counteract a range of disease states by modulating cytokine production. Exercise stimulates moderate to large increases in the circulating concentrations of interleukin (IL)-6, IL-8, IL-10, IL-1 receptor antagonist, granulocyte-colony stimulating factor, and smaller increases in tumor necrosis factor-α, monocyte chemotactic protein-1, IL-1β, brain-derived neurotrophic factor, IL-12p35/p40 and IL-15. Although many of these cytokines are also expressed in skeletal muscle, not all are released from skeletal muscle into the circulation during exercise. Conversely, some cytokines that are present in the circulation are not expressed in skeletal muscle after exercise. The reasons for these discrepant cytokine responses to exercise are unclear. In this review, we address these uncertainties by summarizing the capacity of skeletal muscle cells to produce cytokines, analyzing other potential cellular sources of circulating cytokines during exercise, and discussing the soluble factors and intracellular signaling pathways that regulate cytokine synthesis (e.g., RNA-binding proteins, microRNAs, suppressor of cytokine signaling proteins, soluble receptors).

INTRODUCTION

Cytokines comprise a large family of polypeptides or proteins. This family includes interleukins, interferons, growth- and colony-stimulating factors, chemokines, members of the tumor necrosis factor group and transforming growth factors. Cytokines play an integrative and regulatory role as universal intercellular messengers. Once secreted, they can mediate intercellular communication locally or systemically. Alternatively, they can mediate intercellular contact even when bound to cell membranes (200). These characteristics distinguish cytokines from other intercellular messengers such as adhesion molecules, which require direct cell-to-cell contact, and hormones, which are produced by specialized endocrine organs and circulate throughout the body to exert their actions. Most cytokines are inducible mediators, are transported through the systemic circulation and are synthesized rapidly by multiple cell types in response to various stimuli. Individual cell types can express and secrete several cytokines simultaneously in response to a single stimulus (200). Cytokines are pleiotropic because they influence several cell types, and elicit different effects, depending on the type of target cells. They exert their pleiotropic actions in two phases. First, they bind to specific receptors expressed on cells with different origins and/or functions. Second, they mediate signal transduction through various intracellular messengers and transcription factors. The biological effects of cytokines depend on the presence and concentrations of other cytokines with synergistic, additive or counter-regulatory actions (200). Cytokines can act in an autocrine, paracrine or endocrine fashion to induce or suppress their own synthesis and regulate the production of other cytokines and their receptors. They possess an important characteristic of self-limiting synthesis through various auto-regulatory mechanisms (e.g., RNA instability) and negative feedback pathways. These pathways include synthesis of eicosanoids and corticosteroid hormones, expression of soluble receptors, and induction of intracellular transcription factors that block signal transduction (200).

The main function of cytokines is to regulate immune function. However, their wide-ranging effects on cell proliferation, differentiation, migration, survival and apoptosis allow them to play a role in homeostatic control of various tissues, organs and systems. For example, together with hormones and neuropeptides, cytokines mediate interactions between the nervous, endocrine and immune systems. Some cytokines also control body temperature, fatigue, appetite and metabolism. A link between cytokines and skeletal muscle was first established almost 50 years ago when researchers identified that an endogenous pyrogen was present in skeletal muscle (203). Cannon and Kluger (32) subsequently made another important discovery that endurance exercise induces the systemic release of a pyrogenic compound. These findings have since stimulated considerable interest in the biological significance and regulation of cytokine production in muscle during exercise and as a result of sepsis, aging, cancer cachexia and chronic inflammatory diseases.

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Initially, exercise research focused on the role of cytokines in mediating inflammatory responses to exercise-induced muscle damage. Evidence has accumulated over the past decade that cytokines play a much broader role during exercise. We now know that cytokines act in a hormone-like manner during exercise, mediating metabolism in working skeletal muscle, the liver and adipose tissue, angiogenesis and neurobiology (166). Following exercise, there is an increase in the circulating concentrations of assorted cytokines. Gene expression for some of these cytokines also increases within skeletal muscle. However, there appears to be a dissociation between local gene expression in skeletal muscle and the systemic concentration of other cytokines. For example, after exercise, tumor necrosis factor (TNF)-α and interleukin (IL)-1β expression in skeletal muscle increases, but the circulating concentration of these cytokines does not change (or only increases slightly). Conversely, the circulating concentrations of IL-1 receptor antagonist (IL-1ra) and IL-10 increase markedly, but these cytokines are not expressed in skeletal muscle after exercise.

Several excellent reviews have discussed the evidence that skeletal muscle is a secretory organ (167, 168, 183, 232). Yet relatively few studies have specifically examined whether skeletal muscle cells themselves are the main source of cytokine gene expression in skeletal muscle during exercise. In this review, we examine this notion in more detail by (1) reviewing the findings from studies of cytokine expression and secretion in cultured skeletal muscle cells, and (2) summarizing the results of studies that have used histological staining of cytokine expression in cross-sections of muscle tissue. We also discuss other potential sources of cytokines both in skeletal muscle, other tissues and the systemic circulation. Most research to date has investigated the systemic factors and intracellular signaling pathways that stimulate cytokine secretion by skeletal muscle cells. By contrast, much less is known about the factors that restrict or inhibit cytokine translation in skeletal muscle cells, and cytokine release into the circulation during exercise. We propose some potential negative regulatory mechanisms that may govern cytokine expression and secretion by skeletal muscle cells. Considering the important role of cytokines as local and systemic mediators of various aspects of health and disease, we contend that continuing research is needed to determine the dominant sources and regulation of cytokine production in the body.

LOCAL AND SYSTEMIC CYTOKINE RESPONSES TO EXERCISE

Numerous studies have investigated changes in the circulating concentrations of cytokines following exercise. Cytokine responses are generally dependent on the combination of mode, intensity, and duration of exercise. In the case of IL-6, prolonged running produces the greatest increase in plasma IL-6 concentration (167, 184). Indeed, circulating IL-6 can increase up to 120× following endurance exercise. IL-1ra (up to 90×), IL-10 (up to 80×), IL-8 (15×) and monocyte chemotactic protein (MCP-1) (up to 3×) also consistently increase in the circulation following exercise (39, 64, 68, 102, 151-154, 164, 165, 208, 212, 219, 225) (Table 1). There is substantial individual variability in the magnitude of changes in these cytokines in plasma after exercise (Figure 1).

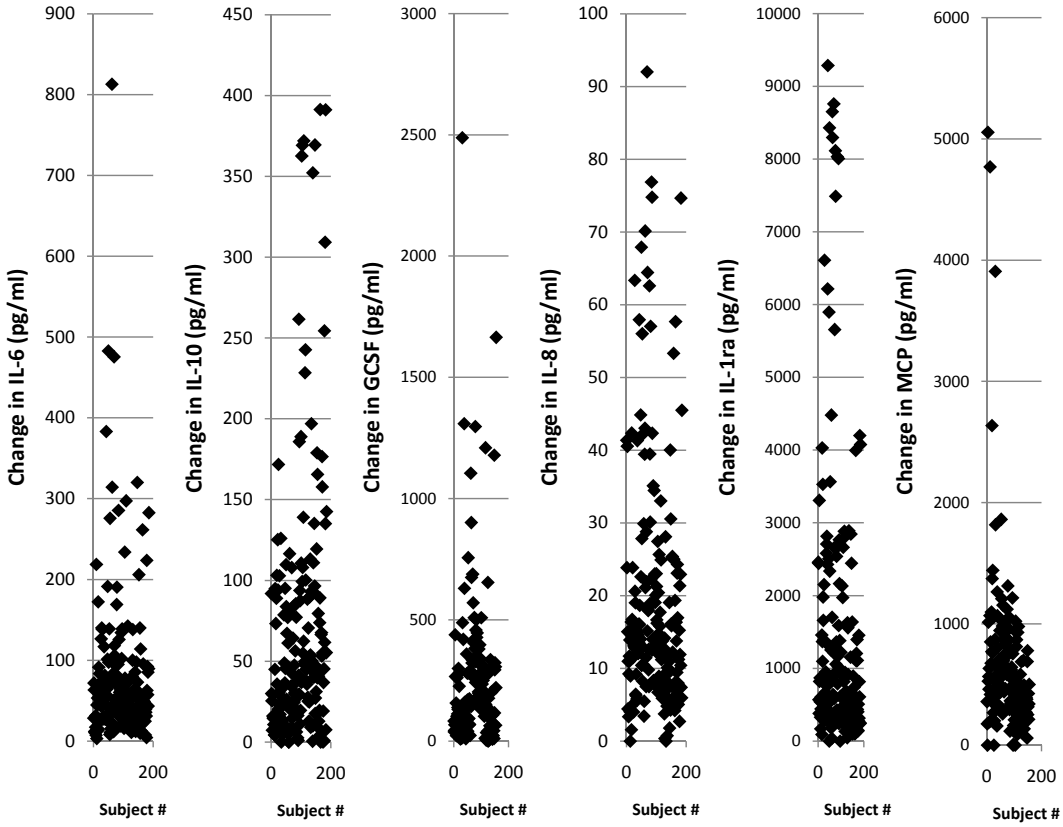

Figure 1. Individual variability in plasma cytokine responses to the Western States 160 km Endurance Run. Data indicate the change (in pg/ml) from pre- to post-exercise. Data are combined from *Int J Sports Med* 24:541-547, 2003; *Brain Beh Immun* 19:398-403, 2005; *Brain Beh Immun* 20:578–584, 2006.

Table 1. Cytokine and chemokine expression in muscle and responses to mechanical strain and exercise.

	Expression in human muscle		Responsive to *in vivo* cyclic strain and EPS of C2C12 or human myotubes		Responsive to exercise		Change in plasma	
	mRNA	protein	mRNA	protein	mRNA	protein		
IL-1β	✓	✓	✓	✓	✓		↑	↔
IL-1ra			×	×			↑↑↑	
IL-2		✓		✓				↓
IL-4	✓			✓				↔
IL-5				✓/×				↔
IL-6	✓	✓	✓	✓	✓	✓	↑↑↑↑	
IL-7	✓	✓		✓/×	✓			
IL-8	✓		✓	✓	✓		↑↑	↔
IL-10	✓ (low)			×	✓		↑↑↑	
IL-12p35	✓			×	×		↑	
IL-13				✓				
IL-15	✓	✓		✓/×	✓	×	↑ ↓	
IL-18	✓	✓						
IFN-γ	✓	✓		×				↔
TNF-α	✓	✓		✓/×	✓/×		↑	↔
MCP-1	✓	✓		✓	✓	✓	↑	
LIF	✓	✓		✓	✓	×		↔
VEGF	✓	✓		✓/×	✓	✓		
BDNF	✓	✓		✓	×	✓	↑	↔
TGF-β	✓	✓			✓			↔
uPA	✓				✓			

✓ or × indicate positive or negative evidence (respectively) of cytokine mRNA or protein expression/secretion in muscle and muscle contraction.
↑ or ↓ indicate an increase/decrease in plasma cytokine concentrations (more arrows denotes a greater increase). ↔ indicates no increase in plasma cytokine concentrations. (Inclusion of two symbols indicates conflicting data.)
N.B. Blank cells indicate no data are currently available. EPS, electromagnetic pulse stimulation. IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemotactic protein; LIF, leukemia inhibitory factor; VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; TGF, transforming growth factor; uPA, urokinase plasminogen activator.

Some of this variability appears to be related to variation in exercise intensity (156) and the extent of exercise-induced muscle damage (157, 228). By contrast, the plasma/serum concentrations of TNF-α (4×), brain-derived neurotrophic factor (BDNF) (2.5×), IL-1β (2×), IL-12p40 (0.3×), IL-15 (0.05×) increase to a smaller extent, whereas leukemia inhibitory factor (LIF) and transforming growth factor (TGF)-β remain unchanged following exercise (25, 29, 39, 137, 153, 154, 186, 190, 220, 225).

The gene expression of IL-1β, IL-6, IL-8, IL-10, IL-15, TNF-α, MCP-1, LIF and TGF-β in skeletal muscle increases following endurance exercise (39, 64, 68, 82, 102, 124, 147, 151, 152, 154, 208, 212) and resistance exercise (25, 50, 94, 124, 149, 150, 192) (Table 1). BDNF mRNA expression also tends to increase (non-significantly) in response to endurance exercise (137). Similar to plasma cytokine responses, exercise-induced changes in cytokine gene expression in muscle can also be highly variable, possibly due to variation in single nucleotide polymorphisms within cytokine genes (52, 186). However, less is known about changes in the protein abundance of these cytokines in skeletal muscle after exercise. IL-6 and BDNF protein expression increases in working skeletal muscle following endurance exercise (68, 89, 137), while the protein abundance of IL-6, IL-8 and MCP-1 also increases after resistance exercise (50, 51) and eccentric exercise (94). Changes in IL-1β are inconsistent (66, 129, 130), whereas IL-15 and LIF protein content does not change following exercise (25, 26, 130, 149).

In contrast with pro-inflammatory cytokines, exercise-induced changes in the anti-inflammatory cytokines IL-4 and IL-13 are less well characterized. We recently reported that IL-4 protein expression in muscle tended to decrease 2 h after resistance exercise in young men. However, following 12 weeks of resistance training, IL-4 protein expression increased when the same individuals performed another bout of resistance exercise (51). This finding suggests that IL-4 may play a role in muscular adaptations to training. In the same study, we observed that IL-13 protein expression in muscle did not change significantly after either bout of exercise (51).

CYTOKINE EXPRESSION AND SECRETION BY SKELETAL MUSCLE CELLS

Most of the evidence for cytokine expression in skeletal muscle is derived from the analysis of isolated RNA or protein extracts from homogenized muscle. However, muscle homogenates reflect contributions from intracellular, sequestered and interstitial sources of cytokines (23). This makes it difficult to identify specific cellular sources of cytokines in vivo. An extensive body of research has explored

Table 2. Expression of cytokines by skeletal muscle cells.

	Constitutive expression in myoblasts		Constitutive expression in myotubes		Change with differentiation	Stimulatory agents	Dose effect	Time effect
	mRNA	protein	mRNA	protein				
IL-1β	H ✓(low) / ✗	H ✓		H ✓(low)	↑ (late)	TNF-α		
IL-1ra	C ✓			H ✗		LPS		+
IL-4	H ✗			H ✓(low)				
IL-6	H ✓/ ✗	H ✓(low)	C ✓ (low)	H ✓	↑ (early)	LPS	+	+
	C ✓ (low)	C ✓		C ✓ (low)				
						TNF-α	+	+
						IL-1β	+	+
						IFN-γ	↔	↔
						TGF-β		
						HSP60	+	
						H_2O_2	+	
						pyrogallol	+	
						X/XO	+	
						epinephrine	+	+
						MG-132		
						AP-1		
						HSF-1		
						Dithiothreitol		
						Thapsigargin		
						Tunicamycin	+	
						Castanospermine	+	
						ATP		+
IL-7			H ✓(low)	H ✓(low)	↑	LPS		
IL-8	H ✓	H ✓(low)	H ✓	H ✓		TNF-α		
						IFN-γ		
						IL-1β		
						'Hyper IL-6'		
						LPS		
						HSP60	+	
IL-10	H ✗			H ✓(low)		TNF-α		
IL-12				H ✓(low)		LPS		+
IL-13				H ✓(low)		IGF-1		
IL-15				H ✗	↑	TNF-α		+
IL-17				H ✓(low)				
IL-18	C ✓							
TNF-α	H ✓/ ✗	H ✗		H ✓(low)	↑ (early)	LPS	+	+
	C ✓ (low)							
						TNF-α		
						IFN-γ		
MCP-1	H ✓/ ✗	H ✗			↑ (early)	LPS		
						TNF-α		
						IFN-γ		
						IL-1β		
						'Hyper IL-6'	+	+
						HSP60	+	
LIF			H ✓(low)	H ✓	↑ (early)	ionomycin		
IFN-γ	H ✓			H ✓				
BDNF			C ✓	C ✓	↓			
VEGF-A				H ✓				
TGF-β	C ✓			H ✓	↑	LPS		↔
						IL-1α		+
						TNF-α		+
						IFN-γ		

✓ indicates constitutive expression. ✗ indicates no constitutive expression. H, human muscle cells. C, C2C12 muscle cells. + indicates positive/additive response. ↔ indicates no effect. N.B. Blank cells indicate no data are currently available. MCP-1, monocyte chemotactic protein-1; LIF, leukemia inhibitory factor; IFN, interferon. BDNF, brain-derived neutrophic factor; VEGF, vascular endothelial growth factor; TGF, transforming growth factor. LPS, lipolysaccharide; HSP, heat shock protein; X/XO, xanthine/xanthine oxidase; hyper IL-6, IL-6 covalently linked to soluble IL-6 receptor; MG-132, proteasome inhibitor; AP-1, activator protein-1; HSF-1, heat shock factor 1;

the capacity of skeletal muscle cells to produce cytokines in vitro by culturing skeletal muscle cells with various agents, and by subjecting skeletal muscle cells to cyclic strain and electromagnetic pulse stimulation (Table 2).

Evidence from cell culture studies

C2C12 myoblasts, L6 muscle cells and human myoblasts constitutively express IL-8 (47, 133), IL-12 (72), IL-15 (180) and TGF-β (72). Some, but not all studies report that myoblasts

also constitutively express IL-1β, IL-1ra, IL-6, TNF-α and MCP-1 (17, 47, 70-73, 106, 133, 144). Myoblasts do not express IL-10 or interferon (IFN)-γ (144). The constitutive expression of cytokines is generally stronger in differentiated myotubes compared with myoblasts (83, 85, 86, 102, 107, 134, 144, 235) (Table 2).

Myoblasts and myotubes express and secrete cytokines *in vitro* in response to a wide range of stimuli (Table 2). ATP, H_2O_2, xanthine/xanthine oxidase, nitric oxide and epinephrine are produced during exercise through redox reactions and autonomic nervous activity. These agents directly regulate the synthesis of cytokines by skeletal muscle cells by activating various signaling pathways (6, 70-72, 96, 107, 128, 235). The regulatory influence of TNF-α, IL-1β, IL-6, IFN-γ, TGF-β and HSP60 is probably less direct, and depends on their relative concentration within the local microenvironment of skeletal muscle. Many of the agents listed above induce a concentration-dependent increase in the secretion of IL-6, IL-8, TNF-α and MCP-1 (70, 72, 73, 85, 107, 134, 144). LPS, TNF-α, IL-1β, IL-6, IFN-γ and epinephrine also induce a time-dependent increase in the secretion of IL-6, TNF-α, TGF-β and MCP-1 for up to 48 hours (70-73, 133, 144). Human myotubes that undergo cyclic mechanical strain release IL-6, IL-8, MCP-1 and G-CSF (172). Mouse (62, 146, 235), rat (31) and human (25, 182, 195) myotubes that contract in response to electromagnetic pulse stimulation release a wide array of cytokines (Table 2). In addition to well characterized cytokines, the list of 'contraction-responsive' cytokines continues to grow (182, 195).

The findings from cell culture studies provide important information on skeletal muscle cell secretion of cytokines in the context of trauma, sepsis or chronic inflammatory conditions. However, these findings are not necessarily applicable to understanding how skeletal muscle cells generate cytokines during exercise, for several reasons. The concentrations of most of the agents are most likely lower in skeletal muscle during exercise compared with the concentrations used in cell culture studies. The period for which skeletal muscle is exposed to these agents during exercise is also typically shorter than the incubation periods used in cell culture studies. Although epinephrine stimulates IL-6 mRNA expression and secretion (70), epinephrine appears to play a relatively minor role in regulating systemic changes in IL-6 during exercise (214). Last, cyclic strain and electromagnetic pulse stimulation of myotubes do not fully reflect the dynamic conditions in skeletal muscle during exercise, where both stimulatory and inhibitory agents are present.

Evidence from in vivo muscle analysis

Human biopsy studies reveal that skeletal muscle expresses mRNA for numerous cytokines (Table 1) (25, 29, 83, 91, 94, 109, 149, 152, 154, 212). Mature myofibers make up most of the cellular mass within skeletal muscle, and as described in the previous section, myotubes express mRNA for various cytokines. Nevertheless, muscle homogenates represent a mixture of different types of cells; other inflammatory and stromal cells in skeletal muscle also secrete cytokines. Some research has used immunohistochemistry and immunofluorescence staining to examine the cellular sources of cytokines in healthy human muscle at rest (68, 89, 149, 175). Malm et al. (129) found that IL-6 and IL-1β are localized to both muscle and non-muscle cells. The same group subsequently reported that IL-6 expression is low within skeletal muscle cells themselves, whereas it is expressed in the epimysium (130). In muscle of patients with inflammatory myopathies, cytokines are highly abundant, but are mainly localized to other cell types such as inflammatory T cells and macrophages, and in proximity to blood vessels in the endomysium and perimysium (46, 194). Collectively, these findings demonstrate that cytokines are likely secreted by various cell types present in skeletal muscle, and not exclusively by skeletal muscle cells themselves.

Surprisingly little research has used RNA *in situ* hybridization, immunohistochemistry or immunofluorescence staining to identify which fibers and resident cell types (e.g., endothelial cells, infiltrating leucocytes, satellite cells) in skeletal muscle produce cytokines in response to exercise. Table 3 summarizes the results of studies that have attempted to identify where cytokines are expressed in skeletal muscle after exercise or muscle contractions. Hiscock et al. (89) report that following endurance exercise, both IL-6 mRNA and protein are expressed mainly in type II fibers with high muscle glycogen content. They propose that the greater IL-6 expression in type II fibers may result from greater release of Ca^{2+} from the sarcoplasmic reticulum into the cytosol of the type II fibers (89). Following eccentric exercise, Hubal et al. (94) observed that MCP-1 is mainly expressed within the interstitial space between myofibers, and localizes with macrophages and Pax7+ satellite cells. Two studies have demonstrated that IL-6 also localizes with Pax7+ satellite cells in the basal lamina of muscle fibers after eccentric exercise (138) and compensated hypertrophy (197). Following downhill running, Malm et al. (130) reported that IL-1β is mainly localized to non-muscle cells, whereas Fielding et al. (66) found that IL-1β is localized to the pericellular space. Using immunofluorescence staining for MCP-1 and IL-8, we recently reported that these chemokines are not present within mature myofibers after exercise. Rather, they are localized within the endomysium between muscle fibers and in close proximity to a number of cell types including macrophages, satellite cells and blood vessels (50). Hoier et al (90) investigated the subcellular localization of VEGF in muscle 2 h after cycling. They discovered that VEGF was located in the subsarcolemmal regions, between the contractile elements within the muscle fibers, and in pericytes positioned on the skeletal muscle capillaries. Lauritzen et al (116) conducted an elegant study in which they incorporated an enhanced green fluorescent protein tagged for IL-6 into muscle fibers isolated from the quadriceps muscles of mice. At rest, the fluorescent tag was localized in vesicular structures at the surface and in the interior of the transfected muscle fiber. Following *in situ* contractions of the fibers, the number of vesicles expressing the fluorescent tag decreased in both locations, indicating vesicular transport of IL-6 out of the fiber. These diverse findings highlight the need for more research to gain a better understanding of the local regulation and secretion of cytokines in muscle during exercise.

Table 3. Cytokine localization in skeletal muscle after exercise.

Cytokine	Species	Exercise mode	Muscle	Located within muscle fibers	Located outside muscle fibers	Ref.
IL-6	Humans	Endurance	Vastus lateralis	✓ (type II fibers)		(89)
IL-6*	Rats	Endurance	Plantaris	✓ (type I and IIa fibers)		
IL-6	Humans	Eccentric	Vastus lateralis		✓ (satellite cells)	(138)
IL-6	Humans	Eccentric	Vastus lateralis	low	✓ (fibroblasts)	(130)
IL-6	Humans	Eccentric	Vastus lateralis	low	low	(129)
IL-6	Mice	Eccentric	Gastroc.	✓	✓ (inflammatory and satellite cells)	(226)
LIF	Humans	Eccentric	Vastus lateralis		✓ (endothelial cells)	(130)
IL-1α	Humans	Eccentric	Vastus lateralis	✓	✓ (endothelial cells)	(129)
IL-1β	Humans	Eccentric	Vastus lateralis	✓	✓	(129)
IL-1β	Humans	Eccentric	Vastus lateralis		✓ (pericellular space)	(66)
MCP-1	Humans	Eccentric	Vastus lateralis		✓ (macrophages and satellite cells)	(94)
MCP-1	Humans	Resistance	Vastus lateralis		✓ (macrophages, satellite cells, blood vessels)	(50)
IL-8	Humans	Resistance	Vastus lateralis		✓ (macrophages, blood vessels)	(50)
VEGF	Humans	Endurance	Vastus lateralis	✓ (subsarcolemmal sarcoplasm)	✓ (pericytes)	(90)
VEGF*	Rats	Endurance	Plantaris	✓ (type IIb fibers)		(22)
VEGF*	Rats	Endurance	Gastroc.	✓ (type I and IIa fibers)		(28)
TGF-β_1	Rats	Eccentric	Gastroc.	✓ (injured myofibers)		(201)

* mRNA. Gastroc, gastrocnemius.

DISSOCIATION BETWEEN LOCAL AND SYSTEMIC CYTOKINE RESPONSES TO EXERCISE

Curiously, although many cytokines are expressed in skeletal muscle following exercise, with the exception of IL-6, they are not released into the circulation—at least in large amounts (65, 68, 211, 212). In explanation of these observations, it has been suggested that some cytokines are produced locally by interstitial cells, and may not enter the circulation (130, 191). Catoire et al (37) recently conducted a systematic comparison of cytokine gene expression in muscle and plasma cytokine concentrations after one-legged 1 h cycling at 60% heart rate reserve. They discovered that mRNA expression of IL-6, MCP-1, CXCL2 (macrophage inflammatory protein-2α) and CX3CL1 (fractalkine) was significantly upregulated. By contrast, mRNA expression of other cytokines including IL-7, IL-8, IL-15 and BDNF did not change significantly after exercise. Within plasma, MCP-1 and fractalkine increased after exercise, whereas IL-6 remained unchanged. This is the only study to report simultaneous changes in MCP-1 and fractalkine within muscle and plasma following exercise. The finding that plasma IL-6 concentration did not change despite local expression in muscle may reflect the relatively small muscle mass and low intensity of exercise (37). An alternative explanation for the dissociation between local and systemic cytokine responses is that skeletal muscle may not secrete sufficient quantities of cytokines to increase their concentration in the systemic circulation.

Although distinct from exercise, Borge et al. (23) conducted an elegant study to investigate whether cytokines are released systemically from skeletal muscle in response to lipopolysaccharide (LPS). Plasma and interstitial fluid were collected from mice 0.5, 1.5 and 3 h after intravenous administration of 3.5 mg/kg LPS. The concentrations and kinetics of changes in cytokines were markedly different between interstitial fluid and plasma. The findings from this study by Borge et al. (23) provide important information about skeletal muscle as a source of circulating cytokines. The higher concentration of IL-1β in interstitial fluid compared with plasma suggests that although skeletal muscle cells produce IL-1β, the systemic release of IL-1β from skeletal muscle is probably tightly regulated. The higher concentrations of TNF-α, IL-10, MCP-1 in plasma compared with interstitial fluid 1.5 h after LPS infusion suggests that skeletal muscle is not a major source of these cytokines in the circulation. The smaller difference in the concentrations of IL-6 in plasma and interstitial fluid is consistent with other evidence that skeletal muscle releases IL-6 into the circulation during exercise (68, 211, 212). The time course of changes in the secretion of cytokines in this study is similar to that reported in plasma following exercise (160). The early secretion of TNF-α and IL-1β probably stimulated the sustained production of IL-6 (44, 71, 72, 125, 162). Constitutive expression of IL-10 in skeletal muscle cells is low (144), but IL-10 production may have increased in response to the early rise in TNF-α and IL-1β secretion (67).

OTHER SOURCES OF LOCAL AND SYSTEMIC CYTOKINES DURING EXERCISE

Cells within the microvasculature, namely endothelial cells and pericytes, are important regulators of angiogenesis and myogenesis, making them key players in both muscle and vascular generation following injury (2). Endothelial cells (78, 100, 111, 217, 238) and pericytes secrete various cytokines (41, 108). Fibroblasts contribute to production of the extracellular matrix of muscle connective tissue by secreting fibronectin, laminin, specific tenascins and neural cell adhesion molecules (141). In response to muscle injury, fibroblasts proliferate and begin to produce collagen-rich extracellular matrix to restore the muscle's framework (121). Fibroblasts also secrete assorted cytokines (47, 78, 118, 136, 163). Neutrophils play an important role in breaking down damaged muscle tissue in the acute phase of muscle injury (148, 174), whereas monocytes/macrophages regulate subsequent tissue regeneration (12, 218). Neutrophils (36, 58, 132, 187, 223) and monocytes/macrophages (8, 48, 54, 78, 93, 123, 131, 179) both secrete a variety of cytokines. As cytokine-producing cells, endothelial cells, pericytes, fibroblasts, neutrophils and monocytes/macrophages may all contribute to global cytokine expression in skeletal muscle.

In addition to skeletal muscle, IL-6 is also released from the brain (158) and peritendinous tissue (114) after exercise. Whereas IL-6 mRNA is expressed in adipose tissue following exercise (45, 103), IL-6 is not released from adipose tissue during exercise (126). Evidence indicates that macrophages secrete IL-1β in the brain following downhill running (35), but it is unknown whether IL-1β is released from the brain into the circulation during exercise. Exercise also increases gene expression of IL-1ra and IL-15 receptorα in the liver in fasting rats (30), but it remains unknown if the liver is a source of circulating cytokines after exercise.

Leucocytes are probably only a minor source of circulating cytokines following exercise (Table 4). Studies on cytokine production by leucocytes can be divided into those that have measured cytokine gene expression in leucocytes, intracellular cytokine production, and extracellular cytokine secretion. Leucocyte mRNA expression of IL-1β, IL-1ra, IL-8 and IL-10 increases, whereas IL-6 mRNA expression remains unchanged after exercise (1, 20, 154, 161). Monocyte intracellular cytokine production of IL-1β, IL-6, TNF-α, BDNF increases, decreases or remains unchanged following exercise (27, 185, 207, 209, 210). Extracellular cytokine secretion by mononuclear cells or in whole blood stimulated with LPS is also variable (1, 18, 19, 56, 57, 81, 87, 112, 173, 189, 202, 231). Fewer studies have investigated changes in cytokine expression or secretion by T lymphocytes following exercise. Two studies have reported that the number of IFN-γ^+ T cells decreases after exercise, while the number of IL-4^+ T cells remains unchanged (113, 213). In contrast with these findings, Zaldivar et al (239) found that the percentage of T cells that expressed IL-4, IL-6 and TNF-α increased following exercise. Kakanis et al (99) also observed that the secretion of both Th1 cytokines (IL-2 and TNF-α) and Th2 cytokines (IL-6, IL-10) by T cells stimulated with phytohemagglutinin increased after exercise. Work by La Voy et al (117) demonstrated that cytokine production during exercise may depend on changes in the numbers certain subsets of T cells.

Skeletal muscle has been proposed as the dominant source of circulating IL-6 based on the increase in the arterial-femoral venous differences in the concentration of IL-6 (215). However, this does not provide *prima facie* evidence that skeletal muscle cells are the main cell type that secretes cytokines such as IL-6 into the circulation during exercise. Skeletal muscle is composed of many other cell types such as fibroblasts, myeloid cells, pericytes which also secrete cytokines. The organs and cells that secrete other cytokines (not produced in abundance by skeletal muscle cells) into the circulation during exercise remain to be determined. The results from many of these studies on cytokine secretion by leucocytes *in vitro* highlight the importance of how we interpret such data. It is important to consider differences in stimulated versus spontaneous cytokine secretion, intracellular cytokine production versus extracellular secretion, and changes in the absolute amount of cytokines that are secreted versus cytokine secretion per cell.

REGULATION OF CYTOKINE SYNTHESIS AND SECRETION

Cytokine secretion by skeletal muscle cells involves various intracellular factors, including mitogen-activated protein

Table 4. Leucocyte cytokine mRNA expression and secretion in response to exercise.

	mRNA expression	Unstimulated production		Stimulated production			Production per cell			Change in plasma	
IL-1β	↑	↓		↑	↓		↓			↑	↔
IL-1ra	↑	↑		↑		↔				↑↑↑	
IL-6	↓	↓	↔	↑	↓	↔	↑	↓	↔	↑↑↑↑	
IL-8	↑									↑↑	↔
IL-10	↑	↓		↓				↓		↑↑↑	
TNF-α		↓		↓		↔	↑	↓	↔	↑	↔

↑ or ↓ indicate an increase/decrease in plasma cytokine concentrations (more arrows denotes a greater increase). ↔ indicates no increase in plasma cytokine concentrations. (Inclusion of two symbols indicates conflicting data.). Unstimulated production refers to assays in which whole blood or cells were incubated with no external agent. Stimulated production refers to assays in which whole blood or cells were incubated with an external agent such as lipopolysaccharide.

kinases, heat shock factor 1, histone deacetylases and transcription factors such as nuclear factor of activated T cells (NFAT), activating protein (AP)-1 and NFκB (6, 70-72, 107,128, 233, 212, 235). In addition, cellular processes such as $Ca2^{+}$ signaling and protein unfolding also stimulate muscle cells to express cytokine genes and/or secrete cytokines (92, 233). Activating transcription factor 3 is an important regulator of cytokine secretion by macrophages (76), and may also play a role in skeletal muscle cells. In comparison with our knowledge of the factors that induce cytokine synthesis, much less is known about the factors that restrict and/or inhibit cytokine expression and secretion (145). In the context of exercise, this information is important because it could account for why IL-1β and TNF-α mRNA expression in skeletal muscle increases, yet the circulating concentrations of these cytokines remains comparatively low following exercise (150-152, 212). Below, we propose some potential negative regulatory mechanisms that may govern cytokine expression and secretion by skeletal muscle cells.

RNA-binding proteins

Intracellular utilization of mRNA depends on several processes including mRNA maturation, shuttling and stability. In turn, these post-transcriptional processes are under the control of RNA-binding proteins and microRNAs. RNA-binding proteins regulate mRNA utilization by binding to adenine/uracyl-rich elements downstream of the 3' untranslated regions of transcripts (205). A small subset of RNA-binding proteins are active in skeletal muscle, including human antigen R, KH-type splicing regulatory protein, CUG binding protein 1, poly(A) binding protein, Lin-28 and tristetraprolin (9). These RNA-binding proteins are known to regulate myogenesis (9), but they may also control cytokine translation in skeletal muscle cells. Human antigen R enhances the stability of TNF-α mRNA (49, 140). However, together with CUG binding protein 1 and tristetraprolin, human antigen R may also silence TNF-α translation (33, 98, 101, 240). Tristetraprolin expression increases (221), whereas the expression of human antigen R decreases (181) in response to the anti-inflammatory cytokines IL-4 and IL-10.

Relatively little research has examined changes in the expression of RNA-binding proteins in skeletal muscle following exercise. Hubal et al. (94) reported that mRNA expression of zinc finger protein 36 (a member of the tristetraprolin family) increases in skeletal muscle following exercise. This response is greater following eccentric exercise compared with concentric exercise, and is augmented following repeated bouts of eccentric exercise (94) which suggests a role for zinc finger protein 36 in regulating skeletal muscle adaptation following injury. Another study observed that tristetraprolin mRNA expression precedes that of LPS-inducible CXC chemokine mRNA expression in C2C12 myoblasts and in skeletal muscle following freeze injury (193). Geyer et al (75) performed an elegant study in which they induced the expression of the RNA-binding protein tristetraprolin in C2C12 myotubes. This treatment suppressed mRNA expression of MCP-1, KC (IL-8) and IL-6, while it also reduced MCP-1 secretion following LPS stimulation. These findings highlight the need for further research to gain greater insights into the role of RNA-binding proteins in regulating cytokine secretion by skeletal muscle cells.

MicroRNAs

MicroRNAs also bind to the 3' untranslated regions of transcripts (222) and interact with RNA-binding proteins to regulate the fate of mRNA (59, 98, 127). MicroRNAs such as Let-7, miR-146, miR-221, miR-155 and miR-106 regulate the expression of IL-1, IL-6, IL-8, TNF-α and IL-10 by immune cells (21, 38, 59, 98, 170, 198, 224, 229). Skeletal muscle cells express a number of microRNAs, including miR-133, miR-1, miR-367, miR-135a, miR-222, miR-29a, b and c, miR-221, miR-223 and miR-206 (34, 42, 105). MicroRNA Let-7 inhibits the secretion of IL-13 in human myotubes (97). Other microRNAs such as miR-367, miR-222, and miR-29 may control cytokine secretion by skeletal muscle cells indirectly by altering the activity of endothelial nitric oxide synthase (188) and signal transducer and activator of transcription (STAT) proteins (53). As more microRNAs are identified in skeletal muscle, this may improve our knowledge of whether they regulate cytokine expression and secretion by skeletal muscle cells.

Suppressor of cytokine signaling (SOCS)

Surprisingly little research has investigated the role of SOCS proteins in regulating cytokine synthesis and signaling by skeletal muscle cells. Paradoxically, the limited evidence available indicates that overexpression of SOCS3 increases IL-6 transcription in myotubes (204). Under some conditions, the interaction between SOCS proteins and cytokines may be reciprocal. IL-6 and TNF-α induce SOCS3 mRNA expression in C2 myoblasts (5) and cardiac myoblasts (230). TNF-α infusion *in vivo* also stimulates SOCS3 mRNA in murine muscle (60). Further research is warranted to examine in greater detail the function of SOCS proteins as regulators of cytokine synthesis and signaling by skeletal muscle cells.

Soluble receptors

Soluble receptors can also restrict cytokine signaling through two main mechanisms. First, soluble receptors can act as a 'non-signaling sink' that directly competes with membrane-bound receptors for ligand binding. If the ligand-binding affinity of soluble receptors and membrane-bound receptors is similar, the capacity of soluble receptors to inhibit signaling depends on the balance between the two types of receptors (84). Soluble receptors for IL-1 and TNF-α appear to operate in this manner (3, 10). Second, soluble receptors can arise through the proteolytic cleavage of membrane-bound receptors. This process results in fewer membrane-bound receptors to bind ligands and initiate cell signaling (84). TNF-α and IL-6 can also induce shedding and/or endocytosis of their own receptors (55, 79, 88, 177). These actions may represent an autocrine negative feedback loop to prevent excess ligand stimulation. Currently, there is insufficient evidence to determine whether soluble receptors regulate cytokine signaling in skeletal muscle following exercise. Gene expression of soluble IL-6 receptor (but not gp130) is elevated in skeletal muscle between 4.5 and 9 h after exercise (104), whereas the plasma concentration of soluble IL-6 receptor does not change (104) or only increases slightly (120). The presence of circulating soluble IL-6 receptors after exercise may depend on proteolytic cleavage of IL-6 receptors (120). Gene expression of TNF-α and IL-1β in skeletal muscle is elevated for up to 24 h after exercise (124, 150-152). Although the plasma concen-

trations of soluble TNF-α receptors and IL-1ra are also elevated for several hours after exercise (152, 160, 225), it is unknown whether these receptors are derived from, and are active in skeletal muscle.

Other factors

Other factors such as IL-6 and HSP72 produced locally in skeletal muscle during exercise may also regulate cytokine synthesis. IL-6 inhibits LPS-induced synthesis of TNF-α by monocytes (196). In response to LPS treatment *in vivo*, TNF-α concentration is lower in serum and broncho-alveolar lavage fluid from IL-$6^{+/+}$ mice compared with IL-$6^{-/-}$ mice (236). An increase in plasma IL-6 concentration following IL-6 infusion or exercise inhibits the systemic release of TNF-α in response to LPS (206). Local production of IL-6 may therefore regulate the synthesis and systemic release of TNF-α during exercise.

In addition to cytokines, endurance exercise induces HSP72 mRNA expression in skeletal muscle (16). Heat exposure increases HSP72 mRNA expression and IL-6 mRNA expression and protein synthesis in C2C12 myotubes in a temperature-dependent manner (234). As further evidence for this regulatory role of HSP72, the heat shock inhibitor Knk437 attenuates HSP72 mRNA expression, and completely blocks IL-6 mRNA expression in myotubes incubated at 42°C (234). The interaction between HSP72 and IL-6 appears to be reciprocal, because IL-6 infusion induces HSP72 mRNA expression in skeletal muscle (63). Huey and Meador (95) demonstrated that IL-6 regulates the expression of HSPs in skeletal muscle in response to LPS, but not exercise. In direct contrast with IL-6, heat exposure inhibits TNF-α mRNA expression in myotubes (234) and TNF-α protein synthesis in other cell types (61, 142). These findings implicate HSP72 as a negative regulator of TNF-α mRNA expression and synthesis, but further research is required to confirm this notion. The upstream regulator of HSPs, heat shock factor -1 may play a more central role than HSPs in regulating cytokine secretion by skeletal muscle cells (233). Ohno et al (159) have also demonstrated that acute heat stress suppresses NFκB activity in C2C12 muscle cells. This response was accompanied by increased expression of HSP72 (159), but it remains to be determined if HSP72 can block NFκB activity in muscle cells.

Skeletal muscle expresses the gene for tumor necrosis factor receptor-associated factor (TRAF)-6-inhibitory zinc finger protein (TIZ) (77). By inhibiting the activation of NFκB, c-Jun N-terminal kinase and AP-1 (199), zinc finger proteins such as TIZ may restrict cytokine secretion by skeletal muscle cells. Similar to other factors described above, future research could investigate the regulatory roles of zinc finger proteins in skeletal muscle.

In another interesting study, Lee (119) demonstrated that treatment of C2C12 myotubes with IGF-1 inhibited mRNA expression of IL-6 and TNF-α. This effect was due to suppression of TLR4 signaling, which was in turn mediated by inhibition of the PI3K/Akt signaling pathway (119). These findings provide further evidence of potential autocrine loops and cross-talk between cytokines and growth factors within skeletal muscle.

Cytokine trafficking and secretion

In contrast with cells of the immune system, regulation of cytokine trafficking and secretion in muscle cells remains largely unknown. Hoier et al (90) made the first attempt to characterize the subcellular localization of VEGF in skeletal muscle. However, they did not investigate dynamic changes in trafficking of VEGF within muscle cells. Lauritzen et al (116) have provided the most detailed insights to date on the mechanisms of cytokine secretion by skeletal muscle fibers. Stow and Murray (216) have provided a comprehensive overview on the mechanisms of trafficking and secretion in immune cells, and this may be used as a guide to new research in muscle cells.

REGULATION OF CYTOKINE EXPRESSION AND SECRETION IN MYOBLASTS VERSUS MYOTUBES

As noted previously, and summarized in Table 2, the constitutive expression of cytokines varies between myoblasts and myotubes. Below we discuss some of these differences, some of the mechanisms that may govern alterations in cytokine expression and secretion by skeletal muscle cells as they differentiate, and the possible biological significance of these differences.

Gene expression of numerous chemokines and their receptors increases markedly after 16−48 h of differentiation (80). Of note, MCP-1 mRNA expression peaks at 16 h, whereas TNF-α mRNA expression increases 10-fold between 16 and 24 h and declines thereafter (80). Several studies indicate that myoblasts and myotubes constitutively express similar levels of IL-6 mRNA. However, compared with myotubes, myoblasts produce substantially more IL-6 protein upon stimulation with IL-1β, TNF-α and LPS (71, 144, 178). In response to TNF-α and LPS, myoblast production of IL-6 increases in a linear manner, whereas myotube production of IL-6 increases in a more 'bell-shaped' manner (178). The greater sensitivity of myoblasts to pro-inflammatory stimuli such as TNF-α and LPS may reflect the requirement for myoblasts to secrete factors such as IL-6 to promote myoblast proliferation (13, 110), and therefore, muscle regeneration (178). Protein expression of MCP-1 (85), IL-6 (15) and LIF (25) also increases during the first 24−48 h of differentiation. Protein expression of IL-7 and IL-15 increases more steadily between 2−7 d of differentiation (83, 180). Protein expression of IL-1β and IL-1ra (14) and MCP-1 secretion (40) increase after 12−16 d of differentiation. Gene and protein expression BDNF expression decreases after 4 d (143).

The factors responsible for changes in cytokine expression by skeletal muscle cells as they differentiate are uncertain. Some of the factors that regulate cytokine expression in skeletal muscle cells (e.g., mitogen-activated protein kinases, histone deacetylases, NFAT and NFκB) also control muscle cell differentiation (11, 15, 139, 169), and may therefore account for alterations in cytokine expression and secretion in skeletal muscle cells as they differentiate. The expression of some toll-like receptors (e.g., TLR2, TLR5) increases during muscle cell differentiation (24), which may also influence signaling path-

ways linked to cytokine expression and secretion in skeletal muscle cells (69). Changes in cytokine expression during muscle cell differentiation appear to play an important functional role. For example, increased expression of IL-6 (15) and reduced expression of BDNF (143) in skeletal muscle cells is necessary for them to differentiate. Conversely, increased expression of TNF-α during muscle cell differentiation (80) may inhibit myoblast differentiation (115, 227), although this effect is not entirely consistent (43). Using an RNAi screen, Ge et al (74) identified more than 100 cytokines that act regulate myoblast differentiation. Based on their results, they were able to classify these cytokines according to their capacity to initiate differentiation, regulate myocyte fusion and inhibit differentiation. Increased chemokine expression during differentiation may also control the migration and/or positioning of myoblasts so that they can successfully fuse with nascent myotubes (80). Alternatively, increased cytokine expression during muscle cell differentiation may promote muscle cell proliferation (122, 237), and migration of monocytes (40) and mesenchymal stem cells (176) to support muscle growth. Last, autocrine cross-talk between cytokines may also control muscle cell differentiation (4, 7, 71, 125).

CONCLUSION

Our understanding of the importance of skeletal muscle and cytokines as mediators of metabolism has increased substantially over the last decade or so. Research to date has identified more than 600 different proteins that are secreted by skeletal muscle cells (168). In this rapidly advancing age of '-omics' technologies, the muscle cell secretome will continue to grow and provide new targets on which to focus. We now know that myokines exert various endocrine effects on various metabolically active organs, including adipose tissue, the liver, the pancreas and the brain. Nevertheless, perhaps with the exception of IL-6 and LIF, our knowledge of the factors and mechanisms that regulate cytokine production and release from skeletal muscle cells during exercise remains somewhat limited. Skeletal muscle cells produce numerous cytokines in response to various agents, but not all these agents are present at similar concentrations and/or are active in the muscle microenvironment during exercise. The capacity for these individual agents to stimulate skeletal muscle cells to produce cytokines is well characterized. However, the control of cytokine production in skeletal muscle during exercise is more complex than the *in vitro* setting, and depends on interactions between a variety of local and systemic factors. Future research should aim to treat skeletal muscle cells with combinations and concentrations of agents that are present in skeletal muscle during exercise.

Cyclic strain and electromagnetic stimulation of skeletal muscle myotubes *in vitro* has generated useful insights into the signaling pathways that govern cytokine production by skeletal muscle cells during exercise. Adding other factors—such as cytokines themselves—to this experimental system could simulate the muscle microenvironment during exercise. In doing so, this approach may assist in characterizing interactions between factors that stimulate or inhibit the ability of skeletal muscle cells to produce cytokines during exercise.

More research is warranted to identify the feedback mechanisms that govern cytokine synthesis by skeletal muscle cells; in particular, which mechanisms are most important, how they interact with each other, and how they are induced and regulated. These research endeavors are important for several reasons. First, this information may help to understand the factors governing the systemic release of cytokines. Second, this information may help to understand the processes that regulate acute inflammatory responses to tissue injury. Last, this information may help to determine why some pro-inflammatory cytokines are chronically elevated in skeletal muscle of patients with idiopathic myopathies, rheumatoid arthritis and muscular dystrophy.

Finally, much has been written about the anti-inflammatory effects of exercise. Petersen et al (171) first proposed the notion that exercise-induced increases in IL-6, IL-1ra and IL-10 exert beneficial anti-inflammatory effects to counteract obesity and insulin resistance. Although this theory is appealing from a mechanistic perspective (206), cytokines may play a relatively minor role in regulating the health benefits of exercise training. Evidence in support of this notion is that brisk walking does not stimulate any discernible increase in circulating cytokines (135), yet regular walking is associated with many health benefits. Furthermore, marathon running induces high physiological stress and a large cytokine response (155), but it is doubtful the cytokines are increased for the purposes of health. Instead, exercise-induced cytokine changes may represent a more generalized response to internal and/or external stress. Factors such as oxidative or nitrosative stress, damaged or unfolded proteins, hyperthermia or energy imbalance likely induce cytokine production during exercise through catecholamines, endotoxin, alarmins, ATP and pro-inflammatory cytokines themselves (232). These issues highlight the need for further research to enhance our understanding of the biological significance of exercise-induced cytokine responses.

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Exercise and inflammation-related epigenetic modifications: focus on DNA methylation

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Abstract

Epigenetics is the study of mitotically or meiotically heritable phenotypes that occur as a result of modifications to DNA, thereby regulating gene expression independently of changes in base sequence due to manipulation of the chromatin structure. These modifications occur through a variety of mechanisms, such as DNA methylation, post-translational histone modifications, and non-coding RNAs, and can cause transcriptional suppression or activation depending on the location within the gene. Environmental stimuli, such as diet and exercise, are thought to be able to regulate these mechanisms, with inflammation as a probable contributory factor. Research into these areas is still in its infancy however.
This review will focus on DNA methylation in the context of inflammation (both pro- and anti-inflammatory processes) and exercise. The complexity and relative shortcomings of some existing techniques for studying epigenetics will be highlighted, and recommendations for future study approaches made.

Keywords: DNA methyltransferase, NLRP3, stress, glucocorticoid, physical activity.

1.0 - INTRODUCTION

Although epigenetics is an emerging area of research within sport and exercise sciences, it has been of interest to the wider scientific community for many decades. The word 'epigenetics' was first coined by Conrad Waddington in 1942, later defined as 'a branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being' (90). This broad description has since been refined and is generally accepted nowadays as meaning, 'the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in the sequence of DNA' (98).
Epigenetics seems to go against the traditional principles of genetics where early 20th century data supports the Darwinian theory that genes are the basis of phenotype, and any change in phenotype is due to alterations in DNA sequence. A competing but generally discredited hypothesis to Darwin's concept of evolution was the one proposed by Jean-Baptiste Lamark in 1809. The Lamarkian theory of heritability of acquired characteristics suggests that traits acquired during a lifetime can be passed on to future generations. This theory was generally abandoned in biology and replaced by the classical Mendelian laws of inheritance, namely, the law of segregation, the law of independent assortment, and the law of dominance. Indeed, it was widely accepted that the only way for traits to be passed on through generations was through the inheritance of genes and that the environment could not influence them. Lamark's theory, that environment plays a role in inherited phenotype, is now being recredited by the scientific community.
In light of a greater current understanding of epigenetic change, and the recent evidence indicating a role for the epigenome in inheritance and development, an appreciation that the genome and epigenome work 'in concert' is of paramount importance to future research. By acknowledging the combined influence of both genetic and epigenetic factors, significant progress is being made on the molecular understanding of the pathogenesis of many disease states and resultant therapeutic interventions. In future, due to the apparent dynamic nature of epigenetic changes, it may be possible to prescribe lifestyle interventions to prevent the accumulation of aberrant modifications to the epigenome that are associated with disease and ageing. Research into the impact of environmental stimuli, such as diet and exercise, is still in its infancy however. Thus, this area represents a worthwhile and fruitful avenue of investigation for sport and exercise science research.

The following review serves to provide a background understanding of epigenetic mechanisms and in particular the role of DNA methylation in normal functioning and in the pathogenesis of disease. Modifications to methylation in the context of inflammation and exercise will also be discussed.

2.0 - EPIGENETICS: UNDERSTANDING THE EPIGENOME

2.1 - Fundamentals of Epigenetics

DNA consists of nucleotides: a deoxyribose molecule bound to a phosphate group on one side, creating the backbone of DNA, and bound to one of four nitrogenous bases on the opposing side. The double-ringed purine bases Adenine (A) and Guanine (G) pair with the single-ringed pyrimidine bases Thymine (T) and Cytosine (C) (A with T, G with C) (figure 1). Nucleosomes, which consist of ~147 base pairs of double

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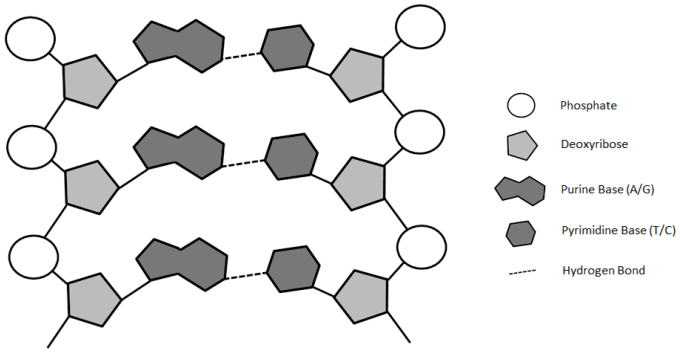

Figure 1 - Schematic of the molecules that make up a short section of DNA.

helix structured DNA wrapped around an octamer of histone proteins, are the packaging units of DNA that form chromatin fibres, and when condensed further, form chromosomes (figure 2). Post-translational modifications to histones are key moderators of gene activity, with acetylation and methylation the best characterised, although ubiquitation, phosphorylation, sumoylation, ADP-ribosylation and citrullination also occur.

Acetylation of lysine (K) residues within the N-terminal tail of the histone proteins is associated with gene activation by neutralising the positive charge of lysine, thus decreasing attraction between histones and DNA. Additionally, the attachment of an acetyl group, via histone acetyltransferase (HAT), can act as an attachment site for other proteins that are able to recruit chromatin remodelling complexes. Consequently, chromatin is less tightly bound which allows transcription factor binding, thus resulting in gene activation and protein formation.

In contrast, methylation of histones, catalysed by histone methyltransferase (HMT), can correlate with either transcription or repression, depending upon the locus of modification. For example, tri-methylation of lysine residue 4 of histone 3 (H3K4me3) causes gene transcription, whereas tri-methylation of lysine 9 or 27 (H3K9me3/H3K27me3) results in gene silencing.

Non-coding RNAs (ncRNA), RNA molecules that are not translated into a protein, can be classified into many subgroups, including, but not limited to, micro RNAs (miRNA), involved in post-transcriptional gene silencing; piwi-interacting RNAs (piRNA), which direct DNA methylation at transposable elements; and long non-coding RNAs (lncRNA), which direct epigenetic machinery such as chromatin remodelling complexes.

There is a complex interplay between histone modifiers, chromatin remodelling complexes, ncRNAs, and DNA methylation, however, for the purpose of this review, only DNA methylation will be discussed further.

2.2 - DNA Methylation

DNA methylation, characterised by the DNA methyltransferase (DNMT) regulated addition of a methyl group to the nucleotide cytosine, creating 5-methylcytosine (5mC), is the most abundantly studied of the aforementioned epigenetic modifications. This process occurs at CpG dinucleotides (cytosine and guanine separated by phosphate in the linear sequence along DNA), which contribute to less than 1% of the genome (51). Clusters of CpG dinucleotides are often located at transcription start sites of genes known as promoter regions, and although DNA methylation has also been found to occur at non-CpG sites (33), the process is more commonly reported at the former. The effect of methylation at gene promoter CpG islands is transcriptional silencing of gene expression, of which the inhibition of transcription factor binding, and the recruitment of methyl-CpG binding proteins (MBPs) which repress the chromatin structure, are key mechanisms (5).

A number of DNMTs regulate the methylation process (figure 3). DNMT1 methylates hemi-methylated DNA, and therefore, has an important role with regards to the maintenance of methylation. DNMT3A and DNMT3B, on the other hand, show preference toward unmethylated CpG dinucleotides and are both involved in *de novo* methylation during development, albeit at different stages; DNMT3B is the primary enzyme involved in the earlier embryonic stages such as implantation, whereas DNMT3A expression is greater in the latter stages of embryonic development (72), as well as during methylation of maturing gametes (35, 81). Another DNMT variant, DNMT3L, despite a lack of methyltransferase activity, assists DNMT3A and DNMT3B by increasing their ability to bind to the methyl donor, S-adenosyl-L-methionine (SAM) (46). Although the maintenance of methylation is primarily thought to be regulated by DNMT1, there is evidence to suggest that DNMT3A and DNMT3B also contribute to this process (13). All three of the aforementioned DNMTs are essential in mammalian development, as demonstrated by the death of DNMT deficient mice (52, 72). Mutation of the DNMT3B gene, and the subsequent loss of methyltransferase activity, can cause ICF (Immunodeficiency, Centromere instability and Facial anomalies) syndrome, an extremely rare recessive disease that affects serum immunoglobulin levels and leads to severe infections, often of the pulmonary or gastrointestinal tracts. Psychomotor and growth retardation are also common symptomologies of ICF patients (22). In addition, DNMT3B has been linked to the fatty acid induced non-CpG methylation of the PGC1α promoter observed in Type-2 Diabetes Mellitus (T2DM) patients (6).

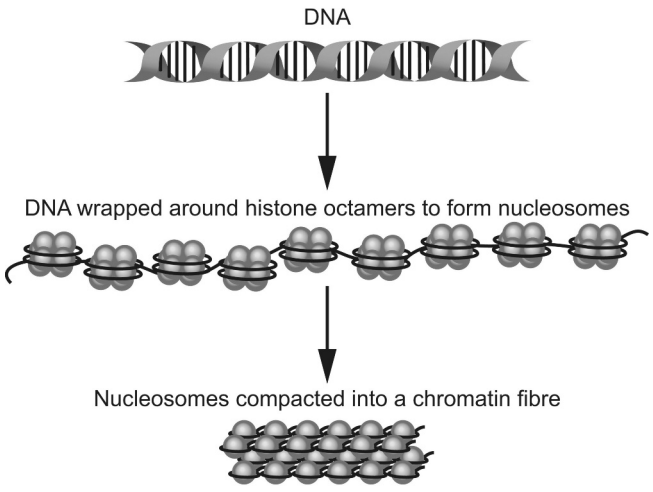

Figure 2 - Structure of a chromatin fibre (image provided courtesy of Abcam Inc. Image copyright©2014 Abcam).

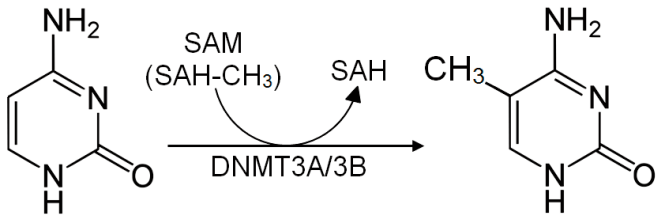

Figure 3 - DNMT regulated transfer of a methyl group (H3C) from the methyl donor S-Adenosyl methionine (SAM), converting cytosine (left) into 5-methylcytosine (right).

Despite sharing structural similarity to the other DNMTs, DNMT2 is located primarily in the cytoplasm, in contrast to DNMT1, DNMT3A and DNMT3B, which are located in the nucleus. Additionally, DNMT2 does not appear to alter genomic methylation, as demonstrated by DNMT2 deficient mouse embryonic stem cells, but rather, methylates aspartic acid transfer RNA ($tRNA^{Asp}$) (32).

As a brief example of the overlap between epigenetic mechanisms, miRNA-143 downregulates DNMT3A mRNA and protein levels in colorectal cancer cell lines (67), while both DNMT3A and DNMT3B have been shown to be direct targets of miRNA-29 in lung cancer samples (24). Similarly, DNMT1 has been verified as a target for miRNA-148a and miRNA-152 (9).

2.3 - DNA Demethylation

An abundance of research has allowed extensive characterisation of both structure and functionality of the enzymes that catalyse DNA methylation. Currently however, less is known regarding the enzymes involved in active demethylation; the removal of a methyl group from 5mC. If hypermethylation of a gene's promoter region causes suppression of activity, reversal of this process should logically result in gene transcription and protein translation. DNA glycosylases, involved in base excision repair of damaged DNA, have been considered to be involved in the demethylation process, however, the identification of 5-hydroxymethylcytosine (5hmC) via the TET1 (ten-eleven translocation enzyme) mediated oxidation of 5mC (85), was of key importance in understanding the molecular mechanisms of active demethylation. Following 5mC oxidation, a number of possible pathways for demethylation have been proposed, including passive dilution of the oxidised base, direct removal of the oxidised 5'-position substituent, and DNA repair-mediated excision of modified nucleotides (50). This section serves as a brief summary of the current understanding of the demethylation process, and the reader is referred to the recent review by Kohli and Zhang (50) for elaboration on the topic.

2.4 - Role of Methyl-CpG Binding Proteins

MBPs play an important role in transcriptional repression and heterochromatin (closed) structure formation (figure 4). Three structural families have been identified; methyl CpG-binding domain (MBD), Zinc Finger, and SET and RING finger-associated domain (SRA). MBD1, 2 and 4, which are able to bind to methylated CpG sites, are largely considered to mediate the suppressive effect of DNA methylation. Conversely, MBD3, 5 and 6 do not bind with methylated DNA. MeCP2, another MBD, is thought to interact with a Sin3 and histone deacetylase (HDAC) complex at methylated regions, which results in the repression of chromatin structure (91). There are, however, other mechanisms, including interactions between MeCP2 and histone methyltransferases (HMT) (31). Kaiso, a Zinc Finger protein, is able to differentiate between methylated and unmethylated regions, and acts as a transcriptional repressor. Other Zinc Finger protein family members include ZBTB4, ZBTB38 and ZFP57 (15). UHRF1 and UHRF2 (Ubiquitin-like, containing PHD and RING finger domains) have the ability to bind with methylated DNA through their SRA domains, with the former recruiting DNMT1, and therefore aiding the maintenance of methylation. Thus far, UHRF1 is the only MBP that has been shown to bind 5hmC as well as 5mC (11).

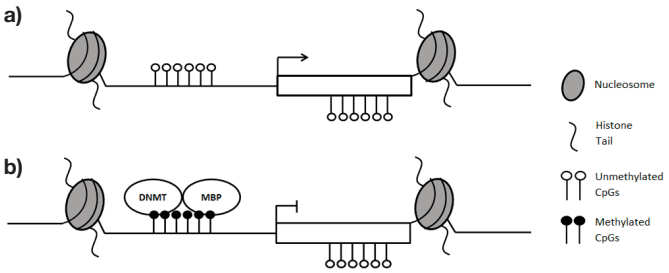

Figure 4 - a) Unmethylated CpG dinucleotides at a gene promoter region. Gene is active; b) DNMT mediated methylation of CpG dinucleotides, followed by MBP recruitment which causes chromatin remodelling and blocking of transcription factors, resulting in transcriptional suppression.

The importance of MBPs in normal developmental regulation is highlighted by Rett's syndrome, a neurodevelopmental disorder of the brain that affects 1 in 10,000 to 1 in 15,000 females. The syndrome is caused by germline mutations in MeCP2 and is commonly mistaken for autism during the early stages of onset, while common symptoms include microcephaly, chorea, ataxia, apraxia, and seizures. Given the role of MeCP2 in binding to methylated portions of DNA and subsequent recruitment of the aforementioned transcriptional repressor complex (Sin3 and HDAC), mutations, of which more than 60 have been identified, generally result in reduced affinity for methylated DNA. Consequently, improper gene suppression occurs (19). The interactions between the various enzymes and proteins discussed thus far highlights that DNA methylation does not occur in isolation, but rather, is part of a complex cascade of events that regulates epigenetic modification.

Up to now, the discussion of epigenetic modification, such as changes in DNA methylation status, have been focused on findings reporting disease or condition-specific epigenetic changes which are directly associated with causing a specific illness, such as the recent review linking immunity, cancer and epigenetics in the context of inflammatory bowel disease (17). However, literature focused on non-disease related epigenetic changes with relevance to inflammatory processes, is relatively lacking.To add complexity to the interpretation of epigenetic data, more general epigenetic changes that are seemingly unrelated to a particular disease, also occur. These

general changes may alter the cellular environment in such a manner as to predispose an individual to a number of diseases. One example of such a general change which may increase susceptibility to various chronic diseases, and one that is very relevant to the exercise arena, is inflammation, which will be discussed in this context in the next section.

3.0 - INFLAMMATION: INTERLINKED ROLES OF THE INFLAMMASOME AND GLUCOCORTICOIDS IN MODIFYING DNA METHYLATION STATUS?

Sterile inflammation is increasingly named as a secondary aetiological factor in modern lifestyle related diseases, such as cardiovascular disease (70), diabetes (73) and depression (38). Chronic stress is a further aetiological role player, since chronic activation of the glucocorticoid system, and subsequent insensitivity to glucocorticoids is known to contribute to low grade inflammation. Furthermore, obesity-related chronic low grade inflammation is also implicated on an epigenetic level in the development of some forms of cancer, such as colorectal cancer (56), further highlighting the prominence of a chronic inflammatory condition as an adverse health factor. Interestingly, for most lifestyle-related diseases such as the aforementioned, moderate exercise, a known anti-inflammatory modality, is prescribed as a preventative and/or complementary treatment. However, the plasticity of exercise-induced changes, and thus its longer-term impact on inflammation and/or glucocorticoid resistance in the context of the development of these pathologies, could be largely dependent on epigenetic modification. In this section, following a brief background on the (non-epigenetic) inflammasome and related immunology, relevant literature available on the epigenetic changes associated with inflammation and glucocorticoid function will be discussed, followed by the reported modulatory effects of exercise.

3.1 - Linking Peripheral Inflammatory Markers to the Inflammasome

3.1.1 - Sterile Inflammation and Innate Immunity

Sterile inflammation is commonly known as the response to either psychological or physical stressors that evoke an innate immune response, in the absence of pathogenic stimuli (23, 59). The exact mechanism of activation remains unclear, though several signals that trigger the immune response have been identified, such as catecholamines, glucocorticoids, intestinal microbiota, as well as molecular signals from host tissue.

Among these signals recognised by the innate immune system are danger-associated molecular patterns (DAMPs), which account for the initiation of an inflammatory response in the absence of microbial stimuli (26). In response to stressors, the host tissue releases these danger signals in response to a local and/or systemic challenge. DAMPs, like pathogen-associated molecular patterns (PAMPs), share several characteristics: they are host-derived proteins, endogenous within cells, and go undetected by the immune system. Several of these DAMPs have been identified in recent years, with many being endogenous molecules that act as alarm signals when released extracellularly, for example, high mobility group 1 (HMG1), ATP, uric acid, glucose and heat shock proteins (23, 59).

Regardless of how the inflammatory branch of the innate immune system is activated, it inevitably results in the appearance of pro-inflammatory markers that are commonly measured and reported in the exercise science literature.

3.1.2 - Importance of IL-1β and the NLRP3 Inflammasome

IL-1β is one of the most important and most potent inflammatory mediators. In its active form, this inflammatory cytokine is primarily released from myeloid cells such as monocytes, macrophages and dendritic cells, but is also readily secreted by most other tissues on stimulation. IL-1β triggers the acute phase response, characterised by the release of C-reactive protein and amyloid β from the liver, the release of inflammatory cytokines such as IL-6 and TNF-α, and secretion of adrenocorticotropic hormone. It also evokes the symptoms fever and hypotension within the host via a plethora of chemical mediators.

Upon stimulus, IL-1β is produced in its inactive 35kDa form. Proteolytic cleavage with caspase-1 results in the generation of the mature 17kDa protein. The process of activation of caspase-1 is essential for IL-1β maturation, and is tightly regulated by multi-protein complexes, known as inflammasomes. To date, several inflammasomes have been identified, namely, NLRP1, 2, 3, 6, NLRC4 and AIM-2. The NLRP3 inflammasome, activation of which regulates IL-1 β and IL-18 in obesity (89), is the most well studied and will be the focus of this section of the review.

3.1.3 - NLRP Inflammasome Structure

The NLRP3 inflammasome (also known as cryopyrin and NALP3) is a multiprotein complex expressed in myeloid cells. This structure consists of a central nucleotide binding

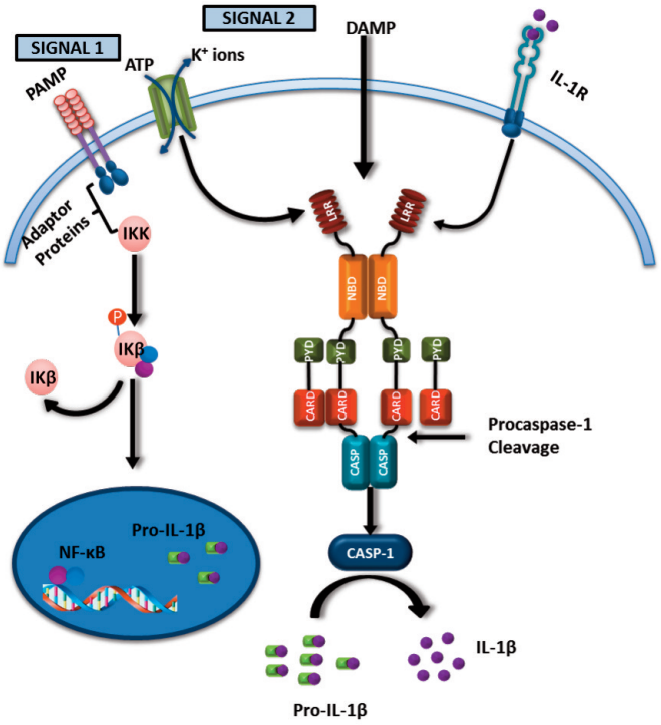

Figure 5 - Schematic illustration of NLRP3 inflammasome activation and subsequent intracellular signalling that produces a pro-inflammatory outcome.

domain (NBD/NACHT/NOD), together with a C-terminal LRR domain. This structure lacks a caspase recruitment domain (CARD), and requires an adaptor molecule (apoptosis-associated speck-like protein containing a CARD (ASC)) to recruit a procaspase-1. NLRP3 interacts with ASC via PYD homophilic dimerization. Similarly, NLRP3 interacts with CARD8 (also known as CARDINAL), to achieve activation of caspase-1. The NLRP3 activation cycle is presented schematically in figure 5.

NLRP3 activation markers are divided into two categories, namely sterile, which include host and environmental stimuli (extracellular ATP, hyaluronic acid, fibrillar amyloid β, silica, asbestos, uric acid), and non-sterile, pathogen-associated activators, which are PAMPs from bacteria, fungi, virus and protozoa.

NLRP3 activation can be triggered by direct PAMPs/DAMPs binding to pattern recognition receptors (PRR) (signal 1), or with incorporation of an addition external ATP (signal 2). The stimulation of PRRs by PAMPs leads to the activation of adaptor proteins, such as MyD88 that activate IKK complexes. These complexes phosphorylate IκB proteins, and subsequently results in ubiquitination and degradation by the proteasome. This frees the p50/p65 heterodimer of the NF-κB, which enters the nucleus and activates gene transcription. Among the cytokines release, pro-IL-1β is expressed.

External ATP acts as a second signal of NLRP3 inflammasome activation, resulting in the efflux of K^+ ions from the P2X7 receptor. Endogenous DAMPs are also able to trigger inflammasome activation. Upon NLRP3 activation, the NLRP3 oligomerizes and results in PYD domain clustering, which leads to the homotypic interaction with the PYD and CARD domains of the ASC adaptors. The CARD domains of the ASC, in turn, react with the CARD of pro-caspase-1, which allows for auto-cleaving and the construction of an active caspase-1 p10/20 tetramer. Caspase-1 is able to produce active IL-1β through its cleave of the pro-IL-1β molecules.

3.2 - Interdependency of Inflammasome and Glucocorticoids: Relative Lack of Studies on Epigenetic Involvement

In the context of non-sterile inflammation, lipopolysaccharide (LPS) infection is known to increase pro-inflammatory cytokine concentrations (IL-1β, IL-6, TNF-α) and to activate the HPA-axis. In a model of maternal infection, LPS infection of mothers during late gestation (day 17) was associated with higher stress responsiveness (higher corticosterone) and anxiety behaviour ('elevated plus maze' rodent model of anxiety) in offspring, both in adolescence (day 40) and adulthood (day 80) (23). Although the mechanisms by which these effects were facilitated were not investigated, it suggests transgenerational transfer of effects resulting from inflammation, thus potentially, epigenetic modulation.

Furthermore, in the context of severe trauma, known to result in a glucocorticoid response, acute stress in the form of 100 tail shocks in rats has been shown to activate the inflammasome to increase circulating inflammatory cytokine (IL-1β, IL-18, IL-6, IL-10 and MCP-1) and danger associated molecular pattern (DAMP – hsp72 and uric acid) levels, in a caspase-1 dependent manner (59). In this study, use of a caspase-1 inhibitor attenuated the stress-induced pro-inflammatory response (IL-1β, IL-6 and IL-18) both in the circulating and tissue compartments. Furthermore, the DAMPs assessed were implicated in the caspase-1 activation seen after stress exposure. From this study it is clear that a connection exists between the stress response and inflammation. Despite this, we could not find any studies jointly reporting on both inflammation and glucocorticoid epigenetic changes in the absence of specific pathology such as cancer. In our opinion, given the proven links between these responses reported more downstream, as shown above, this omission is an important gap in the literature that should be addressed. Therefore, while reviewing epigenetic changes impacting on inflammation here, we have included what is known about epigenetic modification in the context of the glucocorticoid response, to encourage inclusion of these parameters in future studies for a more all-encompassing approach.

3.2.1 - Information from Stress Studies

In considering stress and glucocorticoid-related epigenetic modification as an "additive factor" determining the susceptibility to inflammation-induced epigenetic changes, it is important to consider both acute and chronic changes in the glucocorticoid system. Ever since the first maternal separation rodent study by Levine in the 1950s (53), stress as an early life environmental factor has been known to have long-term deleterious effects on stress-susceptibility into adulthood. Similarly, rodents raised by non-caring mothers, who neglected to lick and groom pups during the first week after birth, resulted in decreased resistance to stress. Interestingly, this effect could be reversed by cross-fostering pups with more caring mothers directly after birth (28), suggesting that individual differences in stress reactivity were not genetically inherited, but likely occur via nongenomic transmission in the early developmental phase. Although the critical site for glucocorticoid receptor (GR) regulation remains to be identified, increased nerve growth factor-induced protein A (NGFI-A), also known as early growth response protein-1 (EGR-1), expression has been linked to up-regulation of GR in the hippocampus (63). However, since the increased NGFI-A expression seen in offspring of caring mothers does not persist into adulthood, while the beneficial effect on stress reactivity does, data again points toward epigenetic modification.

Indeed, more recent epigenetic studies confirmed this. Rodent studies showed that the 5' CpG dinucleotide of the NGFI-A consensus sequence within the exon I_7 GR promoter is always methylated in offspring of non-caring mothers, while in offspring of caring mothers, it is rarely methylated. After cross-fostering offspring to caring mothers, this methylation was reversed, suggesting site-specific DNA methylation silencing of the GR promoter is reversible by environmental factors, in this case, maternal care (93). Interestingly, in this study, modification of DNA methylation was shown to occur in cytosines at very specific sites, since, for example, the neighbouring 3' CpG dinucleotide of the AP-1 consensus sequence within the exon I_7 GR promoter was not affected. Thus, it appears likely that epigenetic modification of DNA methylation by any particular intervention is a highly specific, targeted response. Of specific interest was the fact that these patterns in methylation were not present at birth, but developed within the first 6 days of life (12), ruling out genetic inheritance of stress reactivity.

Rather, postnatal maternal conduct seems to play a huge role in the epigenetic outcome of offspring in the context of glucocorticoid receptor expression and stress reactivity. An important message here is that even a relatively acute stressor, in this case only 6 to 7 days, may result in epigenetic modification that persists chronically. Thus, relatively acute stressors may alter chronic glucocorticoid sensitivity and thus more chronically predispose an individual to pro-inflammatory epigenetic modification.

The studies in rodents as described above may give the impression that dynamic DNA methylation and demethylation occurs early in life only. However, treatment of adult rodents with the HDAC inhibitor trichostatin A (TSA) for 4 days was reported to significantly increase histone acetylation at the exon I_7 site, which increased NGFI-A protein binding and resulted in demethylation of the CpG dinucleotide of the NGFI-A consensus sequence within the exon I_7 GR promoter (93). This suggested that the DNA methylation status in fully differentiated cells can be modified, which has far-reaching therapeutic implications.

Taken together, in the context of stress, DNA methylation patterns seem to be largely dependent on environmental and maternal influence early in life. However, pharmacological intervention seems to be an option, at least theoretically, for modification of DNA methylation status in adulthood to reverse this "environmental programming". The extent to which environmental changes may influence this epigenetic programming is still uncertain.

Although the link between chronic stress and inflammation is well established in non-disease models, for example, in humans exposed to the chronic stress of maltreatment during childhood (62), literature on epigenetic links in this context seems to be lacking. Also, in disease conditions linked to chronic stress, pro-inflammatory changes have been reported; in the context of major depressive disorder, increased NLRP3 inflammasome activation was very recently found (3). In this study, increased gene expression of NLRP3 and caspase-1 was discovered in mononuclear blood cells (PBMCs), and was associated with increased serum IL-1β and IL-18 levels, both of which correlated with depression scores, according to the Beck Depression Inventory questionnaire. Similarly, chronic glucocorticoid treatment in rats was shown to increase gene expression of NLRP3, Iba-1, MHCII and NF-κBIα in the rat hippocampus (29). Thus, it is clear that chronic stress has a pro-inflammatory outcome that is associated with the NLRP3 inflammasome. Frustratingly however, direct proof of epigenetic involvement is once again, not available. This point was highlighted in a recent review (44) which stated that the epigenetic mechanisms for the upregulation of the NLRP3 inflammasome have not been elucidated. It is uncertain, therefore, whether only the NLRP3 gene is modified epigenetically, or whether glucocorticoid-associated genes, such as NGFI-A, are also implicated. The targets of stress-induced epigenetic modifications with inflammatory outcomes have huge therapeutic implications, so that the relative absence of epigenetic studies linking stress and inflammation highlights the need for a multidisciplinary approach in epigenetic studies.

Since the epigenetic mechanisms resulting in activation or inactivation of specific pro-inflammatory processes, such as the NLRP3 inflammasome, have not been elucidated, especially not in the absence of pre-existing disease, we will briefly review what is known on epigenetic modulation of these systems in the context of different diseases with an inflammatory component, trying to tease out the commonly reported parameters associated with inflammation and not with the primary disease itself, before moving on to a discussion of epigenetic modulation by physical activity (PA) and exercise.

3.3 - Inflammation and DNA Methylation in the Context of Inflammation-associated Conditions

While DNA methylation is an essential component of normal development and transcriptional regulation, aberrant patterns of DNA methylation are associated with a number of inflammatory diseases and conditions. However, in terms of epigenetic modification and inflammation, the causal directionality remains questionable. This is briefly discussed here, in the context of a variety of inflammatory models.

Obesity is well-known to contribute to a chronic low-grade inflammation via increased secretion of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β from macrophages infiltrating adipose tissue (73). In this context, high-fat diet feeding in two subsequent generations of mice has recently been reported to result in DNA hypomethylation of inflammation-associated genes in adipose tissue of third generation mice (18). One of the most notable findings in this study was an upregulation of NLRC4, a critical component in the formation of the inflammasome which plays an important role in obesity-related inflammation, in both first and second generation offspring (90). Another significant upregulation was that of the toll-like receptors (TLRs), and specifically TLR4, which facilitates obesity-induced inflammation by inhibition of PCK1. Notably, the study also reported hypomethylation of inflammation-associated promoters of the genes TLR1, TLR2 and Lat. This data is in accordance with other inflammatory models. For example, an inverse correlation between DNA methylation of TLR4 and the inflammatory response to LPS stimulation in intestinal epithelial cells has been reported (86), while DNA methylation of TLR2 in circulation was also inversely associated with the C-reactive protein, ICAM-1 and VCAM-1 responses to air pollution (8). Furthermore, in the context of cystic fibrosis (CF), a disease characterised by chronic inflammation and recurring infections of the pulmonary tract, upregulation of TLR2 in a CF bronchial epithelial cell line was due to DNA hypomethylation (83). Finally, hypomethylation of a number of gene promoters has been reported in synovial fluid of rheumatoid arthritis (RA) patients. These included CXCL12 (47), as well as CHI3L1, CASP1, STAT3, MAP3K5, MEFV, and WISP3 (66), many of which are associated with inflammation. Even in circulation this effect was evident, with hypomethylation of a single CpG motif of the IL-6 gene reported in PBMCs of RA patients (68). Augmented expression of these genes could contribute to RA due to their roles in the activation and differentiation of various immune cells and pro-inflammatory cytokines.

Thus, in lieu of epigenetic data from non-disease models of inflammation, by using obesity as a disease-free model of inflammation, and comparing results reported in this model to that of inflammatory diseases, we have been able to identify epigenetic changes associated with clinical symptoms of inflammation. The available evidence appears to indicate that aberrant DNA methylation contributes to inflammation

through hypomethylation and subsequent upregulation of inflammatory gene expression.

3.3.1 - Are Athletes at Risk of Aberrant Inflammation-induced Epigenetic Modifications?

Up to now, this section has focused on DNA methylation changes as a causal factor in the development of chronic inflammation. However, we should also address the question of whether this communication is bidirectional, i.e. can chronic inflammation in the absence of pre-existing disease also lead to other epigenetic modifications? Data now suggests that changes in the DNMT enzymes that catalyse changes to the epigenome may indeed be regulated by inflammatory mechanisms. For example, inflammatory bowel disease-associated colorectal cancer manifests with high levels of IL-6 (4), while DNMT1 is also overexpressed in this condition (27). *In vitro* studies have shown that IL-6 stimulation of human cancer cell lines (HCT116/K562) resulted in elevated expression and activity of DNMT1 (27, 42). This augmentation of DNMT1 could occur through PI3K activation of AKT, and subsequent AKT dependent phosphorylation of the nuclear localisation signal of DNMT1, allowing nuclear translocation and subsequent binding of DNA (39). IL-6 has also been implicated in the maintenance of promoter methylation in cholangiocarcinoma (94) and multiple myeloma cells (41), which is likely to be due to DNMT1, given its functional role. Furthermore, upregulation of DNMT1, but not DNMT3A or DNMT3B, was reported to be responsible for the IL-6 induced hypermethylation of tumour suppressors p53 and p21 in A549 (adenocarcinomic human alveolar basal epithelial cells) cells (55). Western blotting showed that the IL-6 mediated increase in DNMT1 expression was due to activation of the JAK2/STAT3 pathway, which is in support of previous findings that STAT3 induces DNMT1 expression, while STAT3 depletion downregulates DNMT1, in malignant T lymphocytes (104). Furthermore, IL-6 induced DNMT1 mRNA and protein expression correlated with SOCS3 promoter methylation via STAT3 signalling in human colorectal cancer cell cultures (54).

Similar to the IL-6-stimulation studies, IL-1β stimulation of fibroblast-like synoviocytes isolated from RA patients subsequently decreased expression of DNMT1, whereas both IL-1β and TNFα decreased levels of DNMT3A (65). TNFα-induced inhibition of Notch-1, a transmembrane protein, has been attributed to Ezh2 (histone methyltransferase) and DNMT3B recruitment via NF-κB, resulting in hypermethylation in mouse myoblast cells (1).

Interestingly, a recent study (79 - discussed in more detail in the next section) also provides support for the link between transient exercise-induced increases in plasma IL-6 and methylation status of several genes related to immune function, in a sample of healthy, trained men.

Given these findings, it is possible that the acute inflammatory state associated with intense exercise (25) could drive alterations in the epigenome, possibly predisposing athletes to disease. Despite the fact that many of the aforementioned studies were conducted *in vitro* using various cancer cell lines, the findings of Robson-Ansley et al (79) corroborate the notion that exercise-induced inflammation may be related to modifications to DNA methylation in a non-diseased population such as athletes.

4.0 - EXERCISE AND EPIGENETIC MODIFICATION

In contrast to the bleak picture painted above in terms of the potential aberrant epigenetic effects of excessive exercise, chronic moderate exercise is commonly accepted to decrease levels of inflammatory biomarkers (30, 84) and reduce the risk of developing many major non-communicable diseases. Data linking exercise and altered DNA methylation suggests that there may be a possible epigenetic mechanism with regards to these protective effects. The following section is a review of all known data linking PA and exercise to DNA methylation (see table 1).

4.1 – DNA Methylation in the Context of Habitual Physical Activity

A number of studies have sought to elucidate the relationship between PA history and measures of global methylation, i.e. changes in methylation across the genome. The association between PA, measured by accelerometry over four days, and global methylation in LINE-1, a marker shown to correlate well with other measures of global methylation (97), has been investigated (102). Those who performed approximately 30 minutes of PA per day, as measured by a portable accelerometer, when compared to those who performed less than 10 minutes, had a significantly greater level of global DNA methylation. However, following multivariate adjustment for age, gender, smoking status, ethnicity and body mass index (BMI), the association was no longer statistically significant, and thus, this data is more difficult to interpret.

More recently, White et al (96) retrospectively assessed childhood, adolescent, and previous 12 months PA of over 600 non-Hispanic, white women with a family history of breast cancer. The women that reported being active above the median amount for all three periods were shown to have significantly greater LINE-1 methylation than those below the median. A trend was also reported for women that performed above the median for one or two time periods. Similarly, in an elderly population, Luttropp et al (57) divided 509 individuals aged 70 years and over into four discrete groups based on the amount of light and heavy PA they performed. Global methylation, in this case assessed using the Luminometric Methylation Assay (LUMA) method, was found to be significantly correlated with self-reported activity level, even after adjustment for gender, systolic and diastolic blood pressure, LDL and HDL cholesterol, serum triglycerides, smoking status, and BMI.

However, in contrast with the above results which seem to suggest that PA is associated with hypermethylation of DNA across board, results from the Commuting Mode and Inflammatory Response Study did not support this association, as PA was not correlated with global (LINE-1) methylation. Interestingly, levels of IL-6 promoter methylation were not significantly associated with any of the study variables, which also included age, gender, ethnicity and BMI, in addition to PA and diet (103). Most recently, as part of the Cardiovascular Health Study, the association between gene-specific methylation changes in PBMCs and PA energy expenditure (PAEE) over eight years, was assessed in a sample of elderly men and women (82). Maintenance of increased PAEE of 500 kcal or more per week, resulted in significant hypermethylation of the

TNF gene, while the IL-10 gene was significantly hypomethylated in those who increased their PAEE by 500 kcal per week, compared with those who decreased their PAEE by 500 kcal per week. Given the pro-inflammatory role of TNFα, and the anti-inflammatory role of IL-10, these PAEE-induced modifications are a favourable outcome. These two studies differed drastically from a methodological standpoint; the former (104) measured PA through the use of a questionnaire that selected 26 specific activities performed over the previous year, in contrast to the latter study (82) which involved participants recalling any physical activities over the previous eight years. Furthermore, Shaw et al's (82) study utilised a much more defined elderly age group in comparison with the Zhang et al's (104) study that included individuals ranging from 18 to 78 years of age. Interestingly, in a cross-sectional comparison of experienced (defined as three or more years) and novice tai chi practitioners (78), six CpG sites showed differential methylation between the groups, with the more experienced group demonstrating a slowing of the usual age-related pattern of hypo- or demethylation change. This result, as well as the fact that age-related hypomethylation has been reported in PBMC samples (1), clearly illustrates how the lack of a properly defined study population can clearly be a large confounding variable, which may account for the lack of significant findings in Zhang et al's study.

From these studies, no firm conclusion can be made regarding the effects of habitual PA on epigenetic modification, however, it is clear that methodologies must be appropriately selected in order to truly quantify any changes that are occurring. Apart from the issues related to population selection and quantification of PA already mentioned above, the overall inconsistency in results is likely a product of the utilisation of global methylation as an outcome measure, as this does not reflect changes in DNA methylation at the gene-specific level. For example, particular genes may be differentially methylated in response to activity, however, some may be hypomethylated, and others hypermethylated, resulting in little to no global change. Furthermore, while de- or hypomethylation of particular genes is an undesired outcome, hypomethylation, and thus transcription, of tumour suppressor genes is highly desired in the context of cancer. It is thus clear that neither hyper-, nor hypomethylation is desired across the board for all genes, illustrating that interpretation of a "crude" assessment such as global methylation has limited value. This highlights the need to investigate gene and CpG sequence-specific changes. This point has already been illustrated by the gene-specific epigenetic changes reported in other disciplines, as also described in the overview of the stress-related literature in section 3.2.1.

4.2 - Disease-specific DNA Methylation in the Context of Habitual Physical Activity

In addition to gene-specific studies in the context of inflammation, several studies have attempted to elucidate the epigenetic effects of PA related to disease-specific genetic loci. For example, Coyle et al (16) utilised a cross-sectional design in order to investigate the effects of self-reported PA on promoter methylation of the tumour suppressor genes APC and RASSF1A, an epigenetic alteration commonly associated with breast cancer risk. They reported that lifetime, previous five years, and previous year levels of PA were all inversely correlated with promoter methylation of APC but not RASSF1A, although this association did not reach statistical significance. Similarly, hypermethylation of APC, but again, not RASSF1A, was inversely associated with requirement to have breast biopsies. These results appear to suggest that PA may regulate epigenetic modifications in certain tumour suppressor genes thereby reducing the risk of breast tumour growth. PA has also been shown to be inversely correlated with methylation of CACNA2D3, a tumour-suppressor gene, in gastric carcinoma patients (100), suggesting an anti-tumorigenic effect, although no significant associations were reported for the remaining five tumour-related genes (CDX2, BMP-2, p16, GATA5, ER) that were tested. The data from these two studies shows that PA may convey protective anti-oncogenic effects through modulation of tumour-suppressor methylation.

Given the relative complexity of measuring physical activity, which may differ substantially between individuals, in combination with cancer, which again, differs substantially depending on the type and location, cross-sectional studies, such as the aforementioned, are probably insufficient evidence for firmer conclusions. Thus, the role of PA in the methylation status of selected cancer related genes is far from clear and warrants further investigation of this intriguing area.

4.3 - Epigenetic Effects of an Acute Exercise Bout

With regard to an acute bout of exercise, global methylation of vastus lateralis skeletal muscle was reported to be reduced in a sample of sedentary young men and women following a VO_{2peak} test on a cycle ergometer. Further analysis demonstrated that hypomethylation occurred at promoter regions of PGC-1α, PDK4 and PPAR-δ immediately post exercise. Consequently, transcription was upregulated, and given the role of these genes in metabolism, this would be regarded to be a health-beneficial outcome. This appears to contradict the hypothesis that an intense acute bout could have deleterious epigenetic effects via inflammatory mechanisms, although in this case, the bout may have simply been too short or not intense enough to elicit a drastic inflammatory response. The fact that the methylation status of muscle-specific transcription factors MEF2A and MyoD1 remained unchanged (7) supports this notion. Another possible explanation is that IL-6 (section 3.3.1) and other pro-inflammatory proteins only regulate the epigenetic machinery involved in hypermethylation, whereas demethylation, as in the context of this study, is a consequence of other regulatory pathways.

Bisulfite sequencing, a technique used for validation of DNA methylation, demonstrated that non-CpG sites (CpA, CpT, CpC) comprised the majority of modified cytosines in this study. It has been suggested that oxidation of the cytosine's methyl group could provide a possible mechanism as to how an acute exercise bout could cause demethylation. However, due to poor specificity of the bisulfite technique with regard to distinguishing between methylated and hydroxymethylated cytosines (43) this mechanism has not yet been clarified. TET-assisted bisulfite sequencing (TAB-seq) on the other hand, is able to quantify 5mC and 5hmC independently from one

another (99), and thus, may be a more appropriate method when investigating changes in demethylation.

A recent study (21) investigated the importance of exercise intensity on epigenetic changes in terms of mitochondrial biogenesis. Healthy male subjects performed interval cycling at 73, 100 or 133% of peak power output (PPO) and post-exercise changes in gene expression of PGC-1α and its regulators were assessed in skeletal muscle biopsies. Cycling at 100% of PPO was reported to increase PGC-1α mRNA more than cycling at 73% PPO, but supramaximal exercise seemed to blunt this response, so that a lower increase in levels of PGC-1α mRNA was seen when compared to both 100% and 73% PPO. Interestingly, increases in the mRNA levels of the regulators Sirt-1, PDK4 and RIP140 occurred in a manner independent of exercise intensity and muscle activation. This upregulation of PGC-1α is regulated by HDACs, one of the ways in which adaptation to exercise is facilitated (74). Although these results aren't directly related to DNA methylation, a recent broad review on epigenetic modulation by exercise (71) pointed out that this mechanism may suggest a way by which the hypermethylated status of PGC-1α in diabetic patients (6) could be modified. A relative lack of literature dealing with inflammasome epigenetics indicates a huge area for future research focus, especially since the PGC-1α results above suggest that at least some of the adaptive epigenetic changes seen after exercise may, in fact, translate to a more permanent and prolonged beneficial outcome.

The effect of acute exercise on cells of the immune system has recently been investigated (79). A 120 minute treadmill run at 60% of vVO_{2max} interspersed with sprints at 90% of vVO_{2max} for the last 30 seconds of every 10 minutes, followed by a 5km time trial, a protocol previously shown to induce transient elevations in IL-6 (92), was utilised in order to quantify changes in the methylation of PBMCs, measured using the Infinium Human Methylation 27 microarray. Despite no significant alteration in global methylation, an interesting finding was that the exercise-induced increase in plasma IL-6 concentration immediately following the bout was significantly correlated with the methylation status of 11 genes (SLAMF1, IRAK3, LDB2, TMEM156, FCRL2, CDK9, SIT1, AER61, RAG2, C10orf89, CD40LG), a number of which are regulators of immune activities. Of particular interest was the effect on IRAK3, a key inhibitor of inflammation associated with the metabolic syndrome and obesity.

Although research into the relationship between acute exercise, inflammation and epigenetic modification is clearly still in its infancy, and the plasticity of the observed effects remains to be established, the reviewed literature appears to support the notion that inflammation associated with acute exercise is likely to be a regulatory mechanism of changes in DNA methylation. This opens up an exciting new subdiscipline in exercise immunology, which may be mined for information beneficial not only to healthy and active individuals, but also to those suffering from a variety of disease states associated with chronic inflammation.

4.4 - Impact of Exercise Training and Physical Activity Interventions on Epigenetic Modification

Experimental manipulation of mode and intensity of exercise has begun to enhance our understanding of how the epigenome responds to prolonged periods of exercise training. For example, a six month training study (64) consisting of high intensity interval walking exercise, utilising an aging sample matched to both aging and young control groups, demonstrated that methylation status of the p15 tumour suppressor gene was unaffected by exercise or age. However, methylation of the ASC gene, involved in IL-1β and IL-18 production (as described in section 3.1.3), was significantly lower within the elderly population when compared with the young controls, which potentially explains, at least in part, the commonly described age-associated inflammatory state (14), and thus, is an important finding within the context of this review. ASC methylation of DNA extracted from peripheral blood samples was found to be higher in the older group subjected to the exercise protocol compared with the aging control group, which may indicate that the known anti-inflammatory effect of longer-term moderate exercise may be facilitated via attenuation of the well-documented age-related hypomethylation (2, 30, 37). Future studies focusing on gene-specific methylation may shed more light on this possibility.

Longer-term moderate exercise has also been reported to have beneficial effects on DNA methylation when employed as remedial or complementary therapy. For example, in primarily sedentary cancer patients, a six-month clinical exercise intervention (150min/week of moderate intensity aerobic exercise on a treadmill for experimental group; control group received only usual clinical care) altered the methylation profile of 43 genes (101). Most profoundly, hypermethylation of CXCL10, involved in chemoattraction of monocytes, T cells and NK cells, and EPS15, a protein involved in the EGFR pathway, was reported. In addition, hypomethylation of ABCB1, a protein involved in cell membrane efflux, RP11-450P7.3, a gene for a kelch-like family protein, and KIAA0980, which encodes ninein-like protein which contributes to chromosome segregation and cytokinesis, was reported. Six of the 43 genes were associated with overall patient survival, with three of these hypomethylated following exercise, suggesting augmented gene expression. One gene was of particular interest in the context of cancer; L3MBTL1, a candidate tumour suppressor gene (34, 76), was found to be inversely correlated with gene expression, while there was also an association between low risk of breast cancer death and high levels of expression.

Of interest is that this study measured changes in methylation status in peripheral blood leukocyte samples. It has been reported (77) that considerable variation exists between PBMCs and granulocytes, and even within each cell population (T cells/natural killer/B cells/monocytes), a variation that is considerably more pronounced in adult blood than cord blood (45). The investigators did, however, expand on their initial observations by analysing tumour samples, and reported concordance between the two measures in terms of exercise-induced L3MBTL1 methylation, although it is unlikely that blood will be a useful surrogate for all tissues or tumour samples, given the differences in gene-specific methylation reported between muscle, colon, brain, heart, kidney and liver (49, 75).

Exactly what type of exercise is optimal to achieve these beneficial effects in the context of cancer has been only partially elucidated. Bryan et al (10) selected 45 CpG sites that are potentially associated with breast cancer, and investigated the relationship between self-reported PA, in addition to objectively measured cardiovascular fitness using a sample of

sedentary men and women. The intervention consisted of individually tailored self-help materials, designed to increase PA participation based on the participants' motivational readiness, which, after 12 months, significantly increased time spent exercising, but not VO_{2max}, when compared with the control group. At baseline, average methylation of the selected CpG sites was inversely correlated with PAR (7 Day Physical Activity Recall) minutes, which remained significant after controlling for age, but not BMI. Following the intervention, the increase in PAR score was significantly correlated with a decrease in methylation, even after controlling for age, BMI and baseline VO_{2max}, highlighting that chronic PA may convey protective effects due to inhibition of DNMT activity that may result in aberrant DNA methylation at particular sites which could promote tumorigenesis.

Turning attention now to another globally relevant disease, T2DM patients could also potentially benefit from exercise-related epigenetic modulation. A controlled study on a cohort of individuals with a family history of T2DM indicates that a six-month exercise intervention was sufficient to induce alterations in both global and gene-specific methylation, independent of family history of T2DM (69). Overall, vastus lateralis skeletal muscle biopsy showed that hypomethylation occurred in 115 genes, and hypermethylation occurred in 19 genes. Specifically, hypomethylation of RUNX1 and MEF2A, key transcription factors involved in exercise training adaptation (48, 61), THADA, associated with T2DM (60), and NDUFC2, which encodes NADH hydrogenase, the first enzyme of the oxidative phosphorylation system within the mitochondrial inner membrane (95), were reported following the intervention. Additionally, methylation of IL-7, which stimulates proliferation of lymphocytes, was decreased and associated with an increase in mRNA expression and serum concentration post-exercise. A separate analysis of the same cohort (80) demonstrated that the exercise intervention resulted in global adipose tissue hypermethylation, decreased abdominal adiposity and diastolic blood pressure, and increased VO_{2max} and HDL. In addition to the "crude" assessment of global methylation, more than likely performed to enable comparison of results with existing literature, more gene-specific analyses were also included, and thus it was confirmed that the intervention indeed facilitated differential CpG site methylation of subcutaneous adipose tissue. The majority of sites were located within gene bodies and intergenic regions of 18 obesity and 21 T2DM candidate genes, such as ITPR2, a locus associated with waist-hip ratio (36), as well as KCNQ1 and TCF712, which have both been implicated in the pathogenesis of T2DM (60, 87). An inverse relationship between methylation and mRNA expression was observed for TCF712, in addition to other candidate genes. Overall, 197 genes showed changes in both methylation level and mRNA expression, with an inverse relationship reported in 58% of these.

Not all studies show exercise training programmes to have an effect on DNA methylation (20), although this is more than likely due to a number of methodological issues that have previously been discussed within this paper. The majority of studies reviewed here indeed agree that a period of six to 12 months is sufficient to modify gene-specific methylation of a number of different genes associated with pathologies such as aging, cancer and T2DM. The significance of this on prognosis or long-term clinical outcome is an important aspect to consider for future investigation, given the potentially far-reaching implications for public health.

5.0 – CONCLUSION AND FUTURE DIRECTIONS

Although the aforementioned studies have begun to characterise the epigenetic response associated with exercise and inflammation, much of the available research has been conducted in the context of pathologies with an inflammatory component. For the scientific community to achieve a thorough understanding of the relationship between exercise, inflammation and the epigenome, we propose that a collaborative interdisciplinary approach is utilised. Research into this relationship is made more complex by the apparent interchangeable roles of inflammation and DNA methylation as the causative factor; on one hand, hypermethylation can cause upregulation of inflammation-associated genes, while on the other hand, pro-inflammatory cytokines can regulate expression of DNA methyltransferases. It remains to be clarified whether the inflammatory state associated with intense exercise causes detrimental modifications to the epigenome; *in vitro* studies suggest that this could be the case, which could potentially predispose athletes to disease. Conversely, since regular moderate exercise is known to reduce chronic inflammation, the health beneficial effects of regular exercise may be due in part to favourable epigenetic changes. This, therefore, suggests that there is potential for novel epigenetic-based preventative and therapeutic strategies through non-pharmacologic methods such as lifestyle manipulation.

From our review of the literature, a few points to consider in the technical design of an epigenetic study have also become evident. Firstly, it is clear that the original method of global methylation assessment is not sufficient in isolation, and that gene-specific analyses are now required to provide accurate information that adds to the scientific knowledge base, particularly when studying epigenetic markers that increase susceptibility to a disease. Secondly, future research should attempt to identify regions in the genome which may be particularly susceptible to epigenetic modification in response to exercise, and to investigate to what extent differences in activity type, duration or intensity may yield differential effects. Thirdly, it has been shown that variation of DNA methylation is greater between tissues (liver, heart and kidney) than between species (human and chimpanzee) (75), while another study (49) reported that CpG methylation of a single gene varies between muscle, kidney, colon, heart and brain. Even within blood, considerable variation exists between cells (45, 77). These findings highlight the need to look at tissue- and even cell-specific differences. Fourthly, it is important to keep in mind that although epigenetic modification such as DNA methylation is most probably a dynamic phenomenon, both the longevity and degree of reversibility of these adaptations are largely unknown at this stage. In the context of disease, reversibility has been demonstrated by the treatment of cutaneous T cell lymphoma with HDAC inhibitors, and myelodysplastic syndrome with DNMT inhibitors, which comprise the first generation of epigenetic drugs to be approved (58). However, in the absence of pharmacological intervention, as dis-

Study	Population	Activity	Measurement	Results
Habitual PA				
Zhang et al (2011)	131 men/women, >45 years, various ethnicities, no history of heart/kidney disease or cancer.	PA assessed over 4 days using accelerometry.	GM (LINE-1) in peripheral blood.	>30 minutes of PA per day = ↑ LINE-1 methylation compared with those who performed <10 minutes per day.
White et al (2013)	647 women, aged 35-74, non-Hispanic, sister diagnosed with BC.	PA (hours per week) retrospectively recalled for ages 5-12, 13-19 and previous 12 months.	GM (LINE-1) in peripheral blood.	PA levels above median for all 3 time periods = significantly ↑ GM vs. those below median.
Luttropp et al (2013)	509 men/women, aged 70, healthy.	Self-reported weekly PA participation assessed.	GM (LUMA) in leukocytes.	GM significantly correlated with activity level (after adjustment for gender, systolic and diastolic blood pressure, LDL and HDL cholesterol, serum triglycerides, smoking status and BMI).
Zhang et al (2012)	165 men/women, aged 18-78, college commuters.	Block adult energy expenditure survey (assesses frequency and duration of 26 activities within the past year).	GM (LINE-1) and IL6 PM in leukocytes.	No association between PA and LINE-1 methylation, or PA and IL6 PM.
Shaw et al (2014)	253 white males, 137 white females. Elderly.	Self-reported PA energy expenditure assessed over 8 years.	Quantitative methylation-specific PCR of leukocytes.	Hypermethylation of TNF in those who increased PA energy expenditure by 500 kcal or more per week. Hypomethylation of IL-10 in those who increased vs. those who decreased by 500 kcal or more per week.
Ren et al (2012)	237 female tai chi practitioners compared with 263 female beginners, aged 45-88.	Experienced practitioners defined as >3 years, while beginners had just enrolled in beginner classes.	Saliva DNA isolated from mouthwash, with methylation quantified at 60 CpG sites.	Differential methylation of 6 CpG sites in the experienced, compared with beginner, group.
Disease-specific				
Coyle et al (2007)	106 women without BC diagnosis, mean age = 43 years.	Interviewer-administered lifetime PA questionnaire.	PM of APC and RASSF1A genes in biopsied breast tissue.	Lifetime, previous 5 years, and previous year PA inversely correlated with PM of APC but not RASSF1A.
Yuasa et al (2009)	106 male/female primary gastric carcinoma patients.	Self-administered pre-cancer PA history questionnaire.	Methylation of 6 tumour-related genes; CDX2, BMP-2, p16, CACNA2D3, GATA-5, ER following tumour biopsy.	PA inversely correlated with CACNA2D methylation.
Acute Exercise				
Barrès et al (2012)	14 sedentary, healthy men/women.	Acute bout: cycle ergometer VO_{2peak} test.	GM of vastuslateralis skeletal muscle. PM of selected genes also quantified.	GM reduced following the acute exercise bout. PM decreased at PGC1α, PDK4 and PPARδ. No change in PM of MEF2A or MYoD1.
Robson-Ansley et al (2014)	8 healthy, trained men.	Acute bout: 120 minutes of treadmill running at 60% vVO_{2max}followed by 5km time trial.	HumanMethylation27 Beadchip analysis of PBMC samples.	GM and CpG site-specific methylation remained unchanged. IL-6 protein levels correlated with CpG methylation at 11 CpG sites.
Exercise Interventions				
Nakajima et al (2010)	162 controls (aged 40-87), 274 exercise group (aged 41-86), 37 young controls (aged 18-22), healthy, Japanese.	6 months of several sets of 3 minute low-intensity walking at 40% of VO_{2peak}, followed by 3 minutes of high intensity walking above 70% $VO_{2peak,}$ at least 2 days per week. Tracked by accelerometry.	Peripheral blood ASC gene methylation.	ASC methylation decreased with age, while the exercise intervention attenuated this age related decrease.
Zeng et al (2011)	12 women (6 = exercise intervention, 6 = control), BC diagnosis.	6 months of 150 minutes of moderate intensity treadmill exercise.	HumanMethylation27 Beadchip analysed 27,578 CpG sites in peripheral blood leukocyte/tumour samples.	Methylation of 43 genes were altered, 6 were associated with overall survival (IFT172, EPS15, GLUD1, PPP2R3A, MSX1, L3MBTL1). Concordance between blood and tumour samples.
Bryan et al (2013)	64 sedentary men/women, mean age = 29 years.	Psychologically tailored materials designed to increase PA participation over 12 months.	Methylation of 45 CpG sites from saliva samples.	Post-intervention, self-reported PA score inversely correlated with methylation (after controlling for age, BMI and baseline VO_{2max}).
Nitert et al (2012)	15 men with a first-degree FH of T2DM, and 13 men without.	6 months of 1 hour of spinning and 2x1 hour aerobic class per week.	Genome wide analysis (MeDIP) of vastuslateralis muscle biopsy.	Hypomethylation of 115 genes, and hypermethylation of 19 genes.
Rönn et al (2013)	15 men with a first-degree FH of T2DM, and 16 men without.	6 months of 1 hour of spinning and 2x1 hour aerobic class per week.	Genome wide analysis (HumanMethylation450 Beadchip) of subcutaneous adipose tissue of the right thigh.	Changes in methylation of 24 CpG sites in 18 candidate obesity genes, and 45 CpG sites in 21 candidate T2DM genes.
Duggan et al (2014)	Postmenopausal, healthy, overweight women aged 50-75 (70 = exercise intervention, 59 = control).	12 months of 3 supervised aerobic sessions (treadmill walking, cycling) per week, with encouragement to complete at least 2 more sessions at home.	GM (LINE-1) of leukocytes.	No sig. change in GM.

PA = Physical Activity; BC = Breast Cancer; GM = Global Methylation; PM = Promoter Methylation; LINE-1 = Long Interspersed Nuclear Element 1; LUMA = Luminometric Methylation Assay; PCR = Polymerase Chain Reaction; PBMC = Peripheral Blood Mononuclear Cells; BMI = Body Mass Index; FH = Family History; T2DM = Type 2 Diabetes Mellitus; MeDIP = Methylated DNA Immunoprecipitation.

cussed elsewhere in this review in the context of glucocorticoid sensitivity, epigenetic changes appeared to persist relatively longer. The notion that exercise may potentially be able to reverse epigenetic-induced aberrations in gene expression associated with disease pathogenesis, thereby suppressing the disease state, is an exciting new avenue to pursue in exercise science. Finally, in terms of exercise, the available, published epigenetic studies have focused on therapeutic training interventions and anti-inflammatory outcomes, employing moderate intensity exercise training protocols. However, in contrast, the effect of strenuous or excessive exercise on epigenetic modulation has received scant attention leaving a potentially fruitful avenue for future researchers to investigate, and would allow more extensive characterisation of the precise relationship between exercise-induced inflammation and epigenetic regulation.

We have identified three important burning issues or questions that still need to be addressed within in this domain. Firstly, the optimal intensity, duration and mode of exercise that would elicit beneficial changes to the methylome needs to be established. Protocols used in training studies that have shown peripheral benefits in terms of inflammation are probably a good starting point. Secondly, to inform on potential target sites susceptible to epigenetic modification, the exact molecular mechanisms by which these changes are regulated needs to be elucidated. Thirdly, other factors contributing to the complexity of the exercise-inflammation relationship have not received much attention. For example, there is a close relationship between inflammation and oxidative stress, and thus, the possibility of this as another causative factor within the context of exercise, should be investigated. Similarly, the high-carbohydrate diet traditionally consumed by athletes is now associated with inflammation in the context of heart disease, but this potential role player has not been the focus of exercise studies with an epigenetic focus.

In conclusion, the need to further understand the effects of both unaccustomed and more moderate, habitual exercise on inflammation in the context of epigenetic mediators and signalling pathways is essential if we are to fully understand the way in which changes occur. With the application of considered, standardised techniques and study design, inclusion of an epigenetic approach to exercise-related research may add vital information that would otherwise have remained elusive.

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Exercise, skeletal muscle and inflammation: ARE-binding proteins as key regulators in inflammatory and adaptive networks

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ABSTRACT

The role of inflammation in skeletal muscle adaptation to exercise is complex and has hardly been elucidated so far. While the acute inflammatory response to exercise seems to promote skeletal muscle training adaptation and regeneration, persistent, low-grade inflammation, as seen in a multitude of chronic diseases, is obviously detrimental. The regulation of cytokine production in skeletal muscle cells has been relatively well studied, yet little is known about the compensatory and anti-inflammatory mechanisms that resolve inflammation and restore tissue homeostasis. One important strategy to ensure sequential, timely and controlled resolution of inflammation relies on the regulated stability of mRNAs encoding pro-inflammatory mediators. Many key transcripts in early immune responses are characterized by the presence of AU-rich elements (AREs) in the 3'-untranslated regions of their mRNAs, allowing efficient fine-tuning of gene expression patterns at the post-transcriptional level. AREs exert their function by recruiting particular RNA-binding proteins, resulting, in most cases, in de-stabilization of the target transcripts. The best-characterized ARE-binding proteins are HuR, CUGBP1, KSRP, AUF1, and the three ZFP36 proteins, especially TTP/ZFP36. Here, we give a general introduction into the role of inflammation in the adaptation of skeletal muscle to exercise. Subsequently, we focus on potential roles of ARE-binding proteins in skeletal muscle tissue in general and specifically exercise-induced skeletal muscle remodeling. Finally, we present novel data suggesting a specific function of TTP/ZFP36 in exercise-induced skeletal muscle plasticity.

Keywords: skeletal muscle, exercise, regeneration, inflammation, resolution of inflammation, cachexia, mRNA stability, AU-rich element binding protein (ABP), ZFP36/TTP, HuR

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1. INFLAMMATION AND SKELETAL MUSCLE PLASTICITY

Skeletal muscle shows an enormous plasticity to regulate its functional, structural and metabolic properties in order to adapt to varying physiological demands. Specifically, skeletal muscle maintenance, remodeling, growth, and repair depend on sequential gene expression programs that orchestrate complex protein synthesis and degradation pathways to determine fiber type composition and metabolic profiles, as well as satellite cell activation status and myogenic differentiation (22, 67). Prolonged disturbance of metabolic and immune homeostasis inevitably results in loss of skeletal muscle mass, power and/or functional capacity, as evidenced during aging (sarcopenia), inactivity (atrophy), or disease (cachexia). In particular, chronic diseases with features of persistent inflammation and immune dysregulation profoundly impair skeletal muscle strength, endurance capacity, and regeneration potential. Examples of clinical conditions associated with excessive loss of muscle mass and strength include atherosclerosis and cardiovascular conditions, rheumatoid arthritis, chronic obstructive pulmonary disease, obesity, and type-2 diabetes, as well as several malignant diseases (3, 92, 106, 109).

Physical activity is well recognized as an important strategy not only to prevent, but also to improve and—in some cases—even cure chronic inflammatory disease states which threaten to become the worldwide scourge of the new millennium (142). In recent years, an intricate crosstalk between skeletal muscle tissue and multiple levels of host immunity has become apparent. Specifically, myocytes are capable of producing a variety of immune-relevant receptors, mediators, attractants, and immunomodulatory cytokines. In this way, insulted, mechanically stretched, as well as contracting muscle fibers have a remarkable capacity to specifically alter the local inflammatory milieu, and thus, to attract distinct subsets of leukocytes that exert essential supportive functions in skeletal muscle adaptation, remodeling, and repair processes (56, 60, 91, 118). This inflammatory response is both an inevitable consequence of myofiber damage by eccentric overload, but also an indispensable prerequisite for subsequent structural remodeling and functional adaptation of skeletal muscle tissue (for review, see 95, 108). How this response is fine-tuned to meet the specific demands of different exercise regimens is only poorly understood.

Quite evidently, a short-lived inflammatory response, initiated or promoted by the exercising muscle itself, is necessary to initiate its adaptation to exercise. By contrast, chronic systemic inflammation, as well as a disturbed metabolism, have a profound negative impact on skeletal muscle homeostasis, resulting in unbalanced proteolytic activity and impaired regenerative capacity (for review see 8, 9, 23, 70, 81, 87). Conversely, the exercising muscle seems well-equipped with signaling devices to actively resolve inflammatory responses, and thus, to prevent and even counteract chronic inflammation.

Surprisingly, while the mediators and signaling pathways that initiate and promote the inflammatory response are relatively well known, little is known on the question how acute inflammation resolves to prevent chronic inflammation. We have to consider the fact that inflammation generally does not passively subside, not even when the initial trigger has ceased. By contrast, resolution of inflammation is an active process that involves activation of multiple well-timed counter-regulatory mechanisms that promote a sequential, timely and controlled decline of the inflammatory response (for review, see 28, 93, 97, 123). With regard to skeletal muscle, there are still large knowledge gaps concerning the molecular pathways that are involved in disrupting the physiological inflammatory phase during repair and remodeling, thereby allowing proper return to homeostasis.

It has long been thought that the magnitude and duration of an inflammatory response is mainly controlled by the transcriptional up-regulation of anti-inflammatory as well as by the repression of pro-inflammatory gene activity. However, in recent years, a plethora of post-transcriptional regulatory mechanisms have emerged that are involved in immediate and effective fine-tuning of gene expression programs at multiple levels. There is increasing evidence suggesting that proper regulation of inflammatory gene expression, besides transcriptional regulation, involves a variety of post-transcriptional checkpoints that function at the levels of mRNA splicing, mRNA polyadenylation, mRNA stability, and protein translation (for review, 36). Among these, particularly regulated decay of mRNAs encoding inflammatory mediators emerges as an important inflammatory control mechanism.

Many inflammatory key transcripts are equipped with adjustable "sell by date" labels, located within their 3'-untranslated regions (3'-UTRs). Specific RNA-binding proteins recognize these labels and decide whether to postpone or extinguish the 'expiration date', thereby allowing immediate control of inflammatory transcript levels. Remarkably, similar control mechanisms can orchestrate gene expression in the stepwise differentiation of tissue-specific stem cells, such as satellite cells, in skeletal muscle tissue. In the following review, we focus on the most important mRNA de-stabilizing motif, the adenylate/uridylate (AU)-rich element (ARE), which is present in a broad variety of mRNAs encoding inflammatory mediators and cell cycle regulators. We will summarize accumulating knowledge on ARE-mediated gene expression control during myogenic differentiation, and discuss its potential relevance in orchestrating the inflammatory response of skeletal muscle tissue to physical exercise. Specifically, we discuss the effects of different ARE-binding proteins in skeletal muscle regeneration, remodeling and repair processes.

2. REGULATION OF mRNA STABILITY BY AREs

AU-rich elements (AREs), located within the 3'-UTRs of 8-10% of all human transcripts, are the most common recruiting motifs (*cis*-acting factors) for RNA-binding proteins (*trans*-acting factors) (14). They all contain one or more core pentamers (AUUUA), often arranged in tandem repeats, and integrated in a U-rich region (158). Generally, AREs are characteristic for mRNAs encoding short-lived proteins, such as proto-oncogenes, cell cycle regulators, and—most importantly—pro-inflammatory cytokines (73). Upon binding to their target sequences, ARE-binding proteins (ABPs) exert specific effects on the respective transcripts. These effects include regulation of their translation, intracellular localization/transport, interaction with miRNAs, as well as their stabilization/destabilization. In general, destabilizing ABPs stimulate poly(A) shortening and decapping of their target transcripts, and subsequent 5'-3' or 3'-5' exonuclease degradation (Fig.1, for review, see 98).

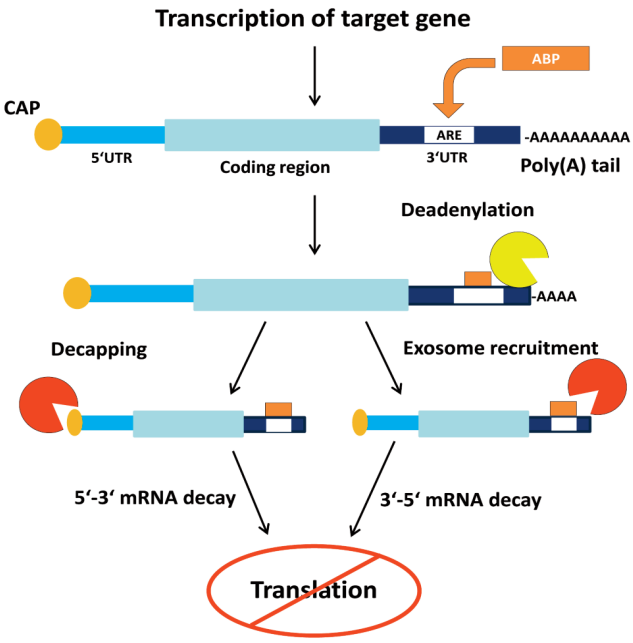

Fig.1. General mechanism of ARE-mediated mRNA decay. Upon binding to the AU-rich element (ARE) in the 3' untranslated region (3'UTR) of the target transcript, ABPs (ARE-binding proteins) activate degradation of the poly(A) tail (deadenylation) and removal of the 5' cap structure (decapping), resulting in subsequent recruitment of the exosome, and eventual 3'-5'- or 5'-3' exonucleolytic decay.

Within the last few years, specific algorithms, like AREScore (126), as well as databases, such as ARED (14) or AREsite (58), have been established to gather and connect information on AREs and ABPs. Using these devices, it is now possible to identify (potential) AREs, to investigate selected AREs in more detail, and to gain information on experimentally validated targets for specific AREs. .

Relatively well-characterized ABPs include the human antigen R (HuR), the CUG-binding protein 1 (CUGBP1), the KH-type splicing regulatory protein (KSRP), the AU-rich element

RNA-binding protein 1 (AUF1), and members of the tristetraprolin (TTP) family of tandem CCCH zinc finger proteins (ZFPs). Whereas HuR binding generally (but not exclusively) stabilizes the respective target transcripts, binding of CUGBP1, KSRP, AUF1, or TTP proteins commonly promotes destabilization of mRNAs and initiates their rapid decay. This can lead to temporal and spatial competition of antagonistic ABPs for the same mRNA binding sites. In turn, this allows translational control of the respective targets in a refined manner (for review, see 1). For a summary of these five ABPs and their characteristics, see Table 1.

Tab.1. Overview of all ABPs discussed in this review.

	HuR (human antigen R)	**CUGBP1** (CUG-binding protein 1)	**KSRP** (KH-type splicing regulatory protein)	**AUF1** (AU-rich element RNA-binding protein-1)	**ZFP36** (zinc finger protein 36)	**ZFP36L1** (zinc finger protein 36 like 1)
Alternative names	ELAV-like protein 1	-	KHSRP	hnRNP D	Tristetraprolin (TTP), TIS11, Nup34	TIS11B, ERF1, BRF1, berg36
Main effect on ARE-containing transcript	Stabilization	Destabilization	Destabilization	Destabilization	Destabilization	Destabilization
Other effects	Regulation of translation	Regulation of mRNA splicing and translation	mRNA splicing, miRNA processing	mRNA stabilization, regulation of translation	Regulation of transcription, translation, mRNA transport, interaction with miRNA pathways	
Induction in muscle during exercise	Chronic electrical stimulation (rat) (78)	Treadmill exercise (mouse) (88)		Chronic electrical stimulation (rat) (78) Treadmill exercise (mouse) (88)	Eccentric exercise (human) (68) Treadmill exercise (mouse) (this study) Cycling exercise (human) (this study)	

3. ABPs IN SKELETAL MUSCLE DEVELOPMENT, GROWTH AND REGENERATION

Thanks to a small population of dormant myogenic stem cells, termed satellite cells, adult skeletal muscle tissue has a remarkable capacity to regenerate and restore its structure and function after acute injury (for review, see 146). Upon activation, satellite cells partly recapitulate some myogenic pathways of embryonic muscle development by use of analogous, but not necessarily identical, mechanisms. Similar to their embryonic counterparts, the so-called myoblasts, satellite cells are capable of self-renewal and new fiber formation. When activated, they undergo a staged and controlled process of proliferation, fusion and differentiation. Each step is dependent on a timely sequence of activation and resolution of stage-specific differential gene expression patterns (150).

It appears that ABPs are essential gatekeepers and caretakers to ensure controlled progression through the myogenic differentiation program (for review, see 7). In particular, ABPs are involved in regulating the transition from myoblasts to myotubes. The latter is a complex process, requiring the well-coordinated crosstalk of a multitude of external and internal stimuli. These stimuli include growth factors and proteins of the extracellular matrix, signal transduction pathways, transcription factors, and cell cycle regulatory proteins. Of particular importance are the myogenic transcription factors of the MRF (myogenic regulatory factor) and MEF2 (myocyte enhancer factor-2) families, as well as diverse cell cycle stimulators (e.g., cyclin D1) and inhibitors (e.g., p21) (reviewed in 89).

In the following section, the specific roles of the five ARE-binding proteins mentioned above (i.e., HuR, CUGBP1, KSRP, AUF1, and ZFP36/TTP) in skeletal muscle cells will be discussed in more detail.

HuR (human antigen R)

HuR is a member of the ELAV-1 (embryonic lethal abnormal vision in *Drosophila*) protein family. The *HuR* gene is believed to be ubiquitously expressed in all tissues and cell types. HuR is involved in the regulation of a multitude of processes, such as proliferation, cell death, and inflammation. In contrast to most other ABPs that promote mRNA degradation, HuR generally stabilizes its target mRNAs and promotes enhanced translation (for review, see (143)). Thus, it appears that differential occupation of 3'UTR-binding sites by HuR versus other ABPs can antagonistically dictate the posttranscriptional fate of target transcripts.

During myogenic differentiation, the nuclear import of HuR is blocked by a caspase-dependent mechanism, resulting in cytoplasmic accumulation of the protein (16). Cytoplasmic HuR has been shown to stabilize several transcripts that are

essential for myogenesis. These include, for example, the myogenic transcription factors MyoD and myogenin, as well as the p21 transcript whose translation product enhances myoblast exit from the cell cycle (51, 125). In the early phase of myogenesis, HuR appears to stabilize the mRNA encoding the cell cycle regulator cyclin D1, thereby allowing myoblast proliferation and subsequent expansion of the pool of myogenic cells. At later stages, HuR dissociates from the cyclin D1 mRNA, which, as a consequence, becomes less stable, thus paving the way for the cells to exit the cell cycle and enter the differentiation program (for review, see 6). Moreover, at the early stages of myogenesis, HuR seems to 'collaborate' with its counterpart KSRP (see below), a known destabilizer of the p21 and myogenin transcripts, by promoting the rapid decay of the mRNA encoding the cell cycle promoter nucleophosmin (31).

HuR also appears to play a role in the development of the neuromuscular junction. Increased levels of mRNA encoding acetylcholinesterase in differentiated myotubes versus undifferentiated myoblasts have in part been attributed to the stabilizing effect of ARE-mediated HuR binding (45, 46). HuR has further been shown to promote the translation of the endogenous danger signaling molecule HMGB1 (high-mobility-group box 1), which promotes inflammatory as well as tissue repair processes upon its release during infection, injury, or strenuous exercise (17, 32). In skeletal muscle, HMGB1 expression is up-regulated during regeneration, and the HMGB1 protein has been shown to promote the commitment of myoblasts to myogenesis (112). Obviously, this process has to be tightly controlled. Here, HuR exerts a key regulatory function by antagonizing miRNA-mediated translational repression of HMGB1 (49).

Finally, HuR is a good example for the frequent observation that the same ABP may exert divergent and even opposing functions on the same target transcript. These functions depend on the respective external trigger, the ABP's intracellular localization, its binding partner, or its phosphorylation status. For instance, in cachectic muscle, HuR does not seem to be involved in the induction, but rather in the repression of myogenic differentiation. Here, HuR, being predominantly confined to the nucleus, appears to be a driving force towards inflammation-associated degradation of the MyoD transcript. In particular, HuR stabilizes and promotes nuclear export of the *iNOS* gene transcript, encoding the inducible nitric oxide synthase, thereby enhancing production of NO as well as subsequent formation of peroxynitrite, which has been shown to decrease mRNA levels of MyoD (47).

CUGBP1 (CUG-binding protein 1)

CUGBP1 belongs to a protein family termed CELF (CUGBP- and ETR-3-like factors). The protein not only binds to AREs, but, as its name indicates, also to GC- and GU-rich elements, and, as a consequence, regulates not only mRNA stability, but also other features, such as translation or mRNA splicing (for review, see 143).

Particularly interesting is the binding of CUGBP1 to the tumor necrosis factor (TNF)-α transcript, since the role of this cytokine in myogenesis is complex and not completely understood. On the one hand, prolonged exposure to elevated levels of TNF-α inhibits myogenesis, and promotes muscle wasting and cachexia (for review, see (96)). On the other hand, a certain amount of this cytokine appears to be necessary to allow progression of early myogenesis, characterized by expansion of the pool of myogenic cells and early differentiation steps (for review, see 138). Thus, CUGBP1 might be an important player here, regulating TNF-α mRNA stability in a well-coordinated manner and, as a consequence, restricting TNF-α autocrine effects on the muscle cells themselves (155).

Finally, CUGBP1 is involved in the pathogenesis of myotonic dystrophies, multisystemic disorders associated with myotonia, muscle atrophy, and muscle weakness. Here, expanded (C)CTG repeats within the non-coding regions of specific mRNAs appear to be targeted for enforced degradation by CUGBP1 and MBNL1 (muscleblind-like 1), another RNA-binding protein (for review, see 139).

KSRP (KH-type splicing regulatory protein)

KSRP is a member of the FUSE (far upstream element binding) protein family. The protein appears to be involved in the regulation of RNA splicing and miRNA processing, however, its role as an ARE-dependent mRNA decay factor has been most extensively studied. The binding of KSRP and HuR to a specific ARE is mutually exclusive. Thus, these two factors are considered as competitors with regard to binding to a particular transcript, especially since they exert opposite effects (destabilization versus stabilization) on their targets (for review, see 24).

Interestingly, the MyoD, myogenin, and p21 transcripts, which, as mentioned, are important HuR targets, can also be bound by KSRP. Early in myogenesis, when cells still proliferate, the predominant binding partner for these transcripts is KSRP, which induces their destabilization and rapid decay. Upon the initiation of differentiation, activation of p38 mitogen-activated protein kinase (MAPK) leads to phosphorylation of KSRP, which reduces its affinity for the AREs in the 3'-UTR of the three mRNAs (7, 24, 25, 75). By contrast, these mRNAs are stabilized by increasing cytosolic HuR levels, as mentioned above.

The physiological relevance of KSRP in skeletal muscle is further underscored by the phenotype of *KSRP*-deficient mice, which show defects with respect to skeletal muscle regeneration, suggesting a crucial role for KSRP in this process (26).

AUF1 (AU-rich Element RNA-binding)

The AUF1 protein family consists of four members (p37, p40, p42, and p45), which are generated by alternative splicing of the same transcript. AUF1 proteins predominantly promote mRNA decay; nevertheless, they have also been described to engage in other activities, such as RNA stabilization and translation. Their target transcripts mainly encode mediators of the inflammatory response, such as cytokines, but also proto-oncogenes, such as c-myc and c-fos, and cell cycle regulators (for review, see 148). Because these factors are central players in myogenesis (48), it is likely that AUF1 proteins regulate this process in a similar manner to HuR, CUGBP1, and KSRP, even though this has not been studied in detail.

Furthermore, it is interesting that AUF1 can also influence the impact of transcripts encoding regulators of myogenesis through mechanisms other than transcript (de)stabilization. Specifically, binding of AUF1 to the 3'-UTR of the MEF2C transcript promotes translation of the latter, without affecting mRNA stability (99). The respective mechanism awaits further investigation.

ZFP36 (Tristetraprolin, TTP)

The ZFP36 family of zinc finger proteins was discovered in 1989, when cultured fibroblasts were stimulated with tetradecanoylphorbol 13-acetate (TPA), in search of rapidly induced target genes (141). The acronym "TIS" in the alternative name "TIS11" thus stands for "TPA-induced sequence".

Tristetraprolin (ZFP36/TTP, TIS11, Nup34) is the founding member of the ZFP36 family of ABPs. In humans, this family comprises two other proteins, namely ZFP36L1 (TIS11B, ERF1, BRF1, berg36) and ZFP36L2 (TIS11D, BRF2), while in rodent placenta, a fourth ZFP36 protein family member (ZFP36L3) has been identified (20).

All ZFP36 proteins share a tandem zinc finger motif, which mediates their binding to AREs (53). Otherwise, ZFP36L1 and ZFP36L2 are more similar to each other than each one of them to ZFP36. Known ZFP36 targets are mainly transcripts encoding pro-inflammatory cytokines, such as the TNF-α transcript, but also mRNAs coding for growth factors, such as VEGF, or cell cycle regulators (e.g., cyclin D1 or p21). For a summary of the most important ZFP36 targets, see Table 2. Expression of the *ZFP36* genes themselves is particularly and immediately induced by pro-inflammatory cytokines. Thus, it appears that these ABPs function as 'rapid reaction force' to efficiently attenuate inflammatory gene expression at the post-transcriptional level by immediately destabilizing transcripts encoding pro-inflammatory cytokines (for review, see 15, 40, 116).

Besides their function as mRNA-destabilizing agents, ZFP36 proteins act as regulators of transcription, translation, and RNA transport. In addition, a complex crosstalk with miRNA pathways has been described (71). These diverse, and occasionally opposing, functions seem to be determined by multisite post-translational modifications. Specifically for ZFP36/TTP, a complex pattern of phosphorylation, which regulates the protein's mRNA binding activity, as well as its interaction with other proteins and its nucleocytoplasmic shuttling, has been demonstrated (for review, see 15, 40, 116). Moreover, ZFP36 has been shown to bind to the ARE of its own gene transcript, suggesting the existence of an auto-regulatory feedback loop (137). In addition, interestingly, transcription of the *HuR* gene is regulated by ZFP36/TTP, indicating intimate regulatory connections between these two pathways (4).

Furthermore, in addition to regulating the stability of mRNAs encoding pro-inflammatory cytokines, ZFP36/TTP can also influence inflammation through an alternative mechanism. The protein has been shown to directly interact with the p65 subunit of NF-κB (nuclear factor kappa B), a transcription factor which plays a central role in inflammatory signaling pathways. Binding of ZFP36/TTP to p65 attenuates NF-κB nuclear translocation, and thus prevents the transcriptional activation of NF-κB target genes, including those encoding pro-inflammatory cytokines (59, 82, 120). In addition, ZFP36/TTP appears to recruit specific histone deacetylases to NF-κB target promoters, thereby repressing NF-κB-dependent transcription (82). Taken together, ZFP36/TTP appears to regulate inflammation in a highly complex manner at multiple levels (Fig.2).

Despite a significant overlap with regard to their mRNA targets, the functions of the three ZFP36 proteins are probably mostly non-redundant. *ZFP36/TTP* knockout mice are viable but exhibit a hyperinflammatory phenotype with features of cachexia, conjunctivitis, and dermatitis (136). Deletion of the *ZFP36L1* gene is lethal in embryogenesis, and *ZFP36L2*-deficient mice die during the early postnatal period, mainly as a result of defective hematopoiesis (Table 2) (19, 133, 134).

Tab.2. Characteristics of the ZFP36 protein family.

	ZFP36	ZFP36L1	ZFP36L2
Aliases	TIS11, TTP, Nup475	TIS11B, BRF1, ERF1, BERG36	TIS11D, BRF2, ERF2
Mechanism of action	mRNA destabilization	mRNA destabilization, regulation of translation (VEGF transcript) (19)	mRNA destabilization
Targets	TNF-α (34), IL-6 (130), IL-3 (129), IL-10 (131), p21 (100), cyclin D1 (86), GM-CSF(35), ZFP36 (27), c-myc (86)	IL-3 (128), VEGF (39), TNF-α (80), GM-CSF(79)	TNF-α (80), GM-CSF(79), IL-3 (79)
Mouse knockout phenotype	Hyperinflammation, treatable with anti-TNF-α antibody (136)	Lethal at embryonic stage E10-E12 (19, 134)	Die of internal bleeding shortly after birth (133)

To date, only very few data are available on potential regulatory functions of ZFP36 proteins in the skeletal muscle context. However, interestingly, findings by Geyer et al. indicate that nicotinergic stimulation of skeletal myotubes, mimicking activation of the motor endplate by neuronal signals, leads to increased ZFP36/TTP levels. This results in reduced inflammation, as reflected by decreased production of pro-inflammatory cytokines (54). Thus, it appears that ZFP36/TTP, besides functioning in inflammation and repair, might also be involved in multiple signaling cascades of skeletal muscle homeostasis.

Finally, specific ZFP36 proteins might also play an important role in skeletal muscle regeneration, which might be an important feature in the regulation of skeletal muscle adaptation to exercise. As early as in 2002, Sachidanandan and colleagues demonstrated induction of *ZFP36/TTP* expression after skeletal muscle injury, presumably in satellite cells, suggesting that ZFP36/TTP might play a role in the regulation of skeletal muscle regeneration (114). Similarly, in 2008, we demonstrated induction of *ZFP36L1/TIS11B* gene expression in skeletal myoblast differentiation. Most importantly, we also

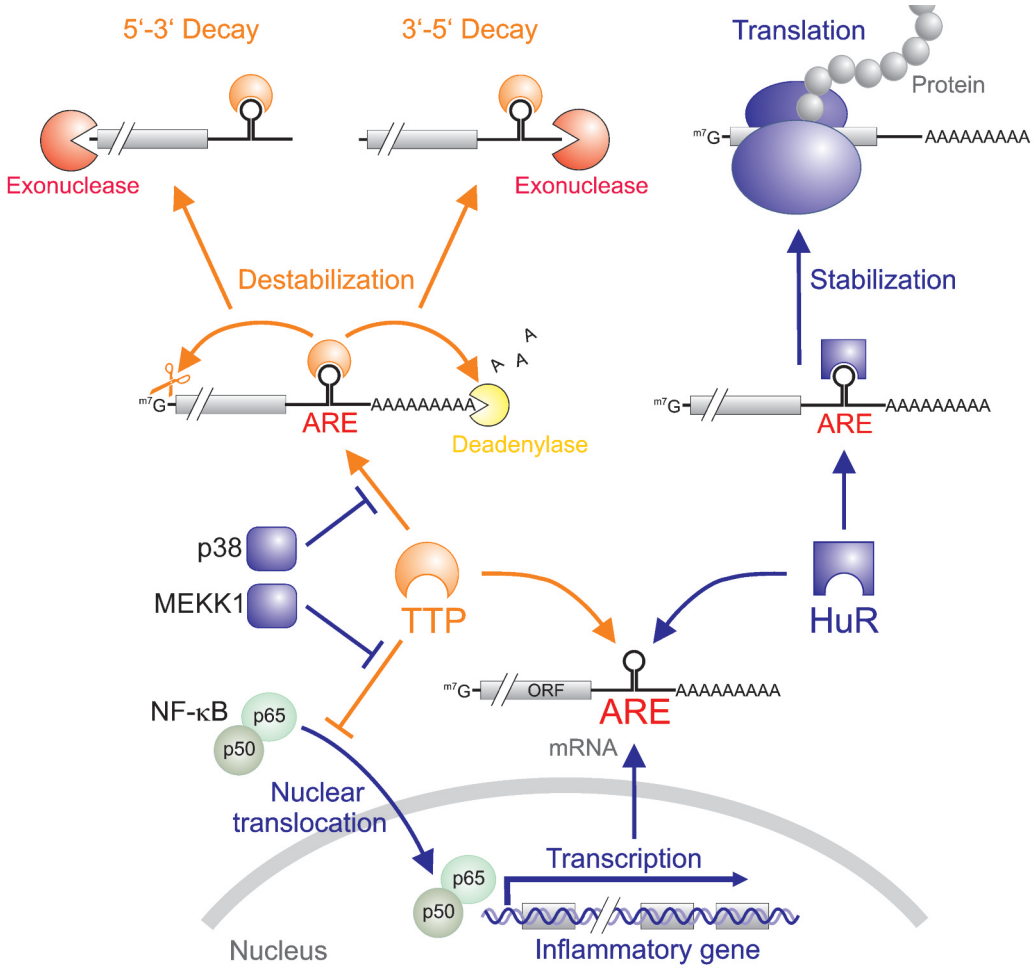

Fig.2. Reciprocal regulation of mRNA stability by ZFP36/TTP and HuR. Multiple inflammatory pathways involve activation and nuclear translocation of the transcription factor NF-κB to drive inflammatory gene expression. In the course of transcription, the nascent messenger RNA (mRNA) is spliced, 5'-capped, and 3'-polyadenylated to protect the transcript from exonucleolytic decay. The processed, mature mRNA is exported to the cytoplasm where the open reading frame (ORF) is translated into functional pro-inflammatory mediators. Many pro-inflammatory transcripts contain AU-rich elements (AREs) in their 3' untranslated regions (3'UTR) that can be recognized by specific ARE-binding proteins (ABPs). The destabilizing ABP ZFP36/TTP initiates mRNA degradation by recruiting components of the cellular mRNA decay machinery resulting in shortening of the 3' poly(A) tail, removal of the 5' 7-methylguanosine cap (^{m7}G), and subsequent degradation by 5' and 3' exonucleases. Moreover, ZFP36/TTP can interact with the p65 subunit of NF-κB to hinder its nuclear translocation, thus preventing the transcriptional activation of NF-κB target genes. In contrast, binding of the stabilizing ABP HuR to the ARE motif competes with the degradation machinery, fosters the recruitment to ribosomes, and thus ensures prolonged and enhanced translation. Likewise, specific phosphorylation of ZFP36/TTP by activation of the p38 and MEKK1-JNK pathways can promote inflammatory activity by preventing the recruitment of the RNA decay machinery to pro-inflammatory transcripts, as well as by preventing repressive interaction of ZFP36/TTP with NF-κB. Blue lines indicate pro-inflammatory pathways, anti-inflammatory pathways are marked in orange.

reported differential expression of this gene in skeletal muscle tissue from dystrophic *mdx* mice when compared to normal, healthy mice. Because *mdx* mice undergo continuous cycles of skeletal muscle degeneration and regeneration, this finding further supports a role of ZFP36 proteins in skeletal muscle regeneration (30).

4. ADAPTATION OF SKELETAL MUSCLE TO EXERCISE, INFLAMMATION, AND ABPs

Adult skeletal muscles are composed of heterogeneous fiber types that differ in the molecular organization and structure of the contractile apparatus and with respect to metabolic characteristics. Dependent upon mode (endurance/resistance), contraction type (concentric/eccentric), duration and intensity, physical exercise provokes dynamic alterations in skeletal muscle size and fiber type composition, eventually resulting in long-term adaptations with improved force production, contraction time, and/or fatigue resistance (for a comprehensive review, see 119).

As outlined in section 1, acute exercise provokes a transient pro-inflammatory state, which affects whole-body homeostasis and metabolism, and specifically targets local inflammatory circuits in the skeletal muscle microenvironment (13, 106, 145). It appears that a well-controlled inflammatory response is important for subsequent adaptive and reparative processes, including satellite cell activation and differentiation (13, 106). However, the specific contribution of the contracting muscle itself, either by passive or active release of inflammatory mediators, is still a matter of controversy. Moreover, hardly anything is known about the refined mechanisms that are

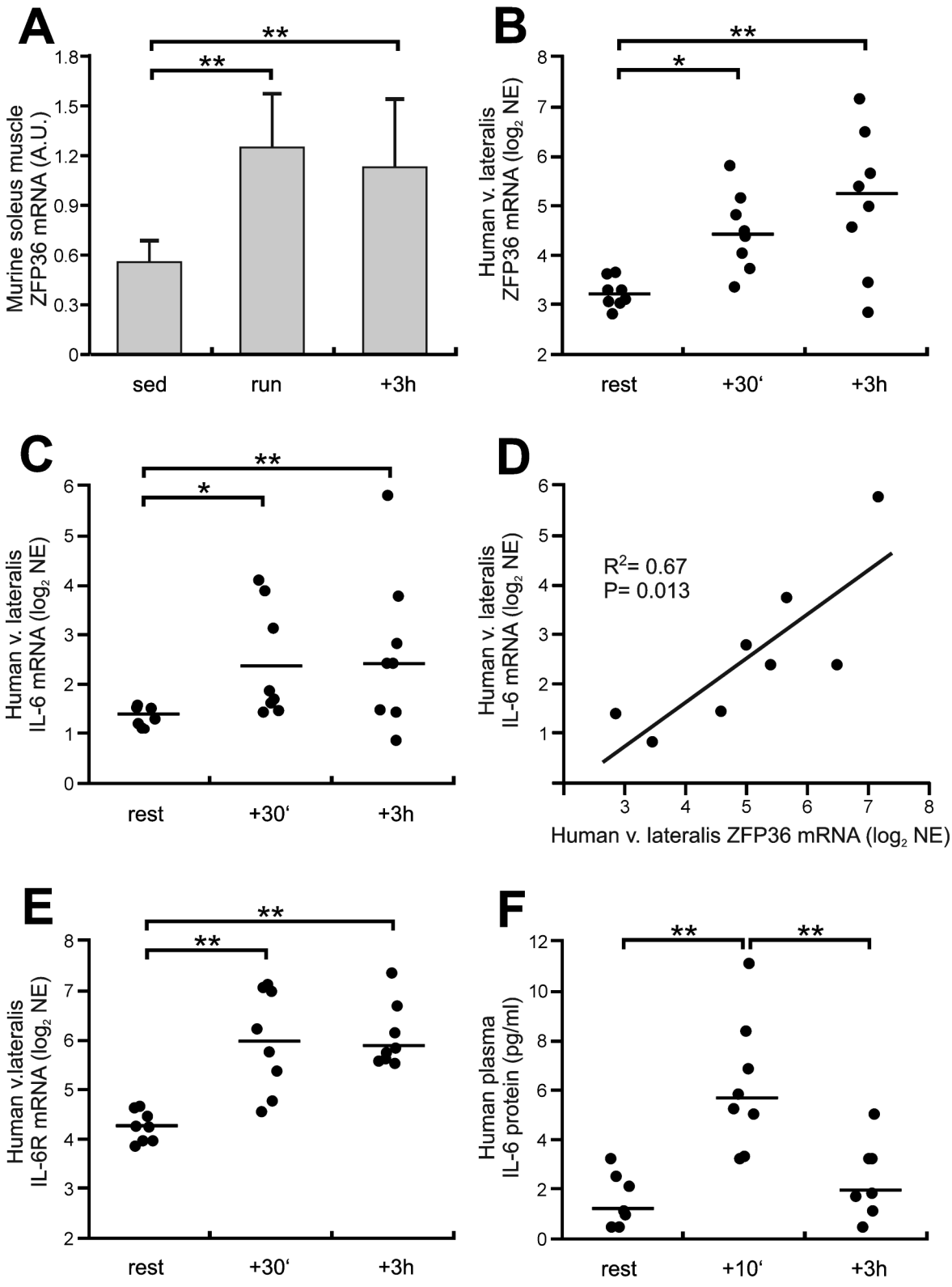

Fig.3. Induction of *ZFP36/TTP* expression in exercising skeletal muscle. (A): Expression of *ZFP36* in mouse *soleus* muscle in the sedentary state (sed), immediately after an acute bout of exercise (run), and after a recovery period of 3 hours (+3h). Male C57BL/6 mice were exercised for one hour at moderately intense conditions on an electric treadmill and were either killed immediately after the run or placed back in their cages for 3 h of recovery. mRNA was isolated from *soleus* muscles and quantified by real-time PCR. The detailed experimental procedure has been published elsewhere (64). Shown are the arithmetic means and standard deviations from n=8 mice. (B-F): Effect of one hour of cycling exercise at 80% VO_2max (262 ± 58 Watt) on gene expression levels of *ZFP36* (B), *IL-6* (C), and *IL6R* (E) in the *vastus lateralis* muscle of 8 endurance-trained male individuals (VO_2max, 67.2 ± 8.9 ml/min/kg). Values were retrieved from a microarray dataset (Affymetrix U219) of microbiopsies taken at rest, as well as 30 min (+30') and 3 hours (+3h) after the cycling protocol (unpublished data set). The bar represents the arithmetic mean of normalized log_2-transformed expression levels (*P < 0.05, **P < 0.01; paired t-test). In addition, circulating plasma levels of IL-6 were determined from blood samples taken at rest, as well as 10 min (+10') and 3 hours (+3h) after the cycling protocol (F). A significant positive association between individual gene expression levels of *ZFP36* and *IL-6* could be detected in *vastus lateralis* at 3 hours after cessation of exercise (D). Data derive from a multicenter trial performed at the facilities of Sports Medicine at the Universities of Gießen, Ulm, and Tuebingen (see supplementary methods section for details).

responsible for both induction and resolution of inflammatory circuits, and, particularly, regulation of pro- and anti-inflammatory mediators, in response to physical exercise.

Myofibrillar disruption in the course of strenuous and eccentric exercise is commonly considered a driving force in exercised-induce inflammatory responses to promote subsequent muscular adaptations in the course of damage-repair processes (101). Experimental muscle damage, as any disruption of tissue integrity, is known to provoke a transient, and locally confined inflammatory stage, initially featured by active recruitment of neutrophils, CD8 T cells, and pro-inflammatory M1-type macrophages (10, 94, 154). Within days, the leukocyte infiltrate shifts from a pro- to an anti-inflammatory phenotype, then characterized by predominantly anti-inflammatory M2 macrophage subtypes, and, most remarkably, accumulation of regulatory T cells (10, 29, 74, 110). As outlined above, tailored and short-term inflammatory activity, as well as active resolution of inflammation, are both indispensable requirements for the promotion of regeneration, repair and adaptation of skeletal muscle. These processes are intimately linked by a well-balanced pattern of pro- and anti-inflammatory mechanisms to facilitate return to tissue homeostasis (13, 74). In particular, pro-inflammatory M1 macrophages are necessarily involved in the removal of muscle debris, but have further been shown to activate satellite cell proliferation by NO-mediated mechanisms, and through the release of pro-inflammatory mediators, including interleukin (IL)-6, IL-1β, TNF-α, and granulocyte colony-stimulating factor (G-CSF) (10, 13, 110, 111). However, if initial pro-inflammatory signaling is not adequately controlled, M1 macrophages harbor destructive potential to amplify tissue damage and to block repair (13, 57, 63). Currently, there is no clear understanding as to what extent invading phagocytes may initially accelerate subtle myofiber damages caused by strenuous exercise. Moreover, there is no consensus as to whether exercise-induced mechanical damage of myofibers is an irrevocable necessity to promote skeletal muscle adaptation to specific exercise regimens, and whether contracting muscle fibers are capable of actively recruiting inflammatory cells to set the scene for subsequent remodeling processes, irrespective of a muscle-damaging insult. Indisputably, efficient regeneration and remodeling of damaged muscle fibers depends on a precise coordination of multiple staged processes, highlighted by the macrophage skewing from M1 toward M2 phenotypes at the time of resolution of inflammation (10, 74). M2 macrophages promote angiogenesis and matrix remodeling, while actively counteracting destructive immunity through the release of anti-inflammatory cytokines like IL-10. Moreover, M2 macrophages have recently been shown to contribute to the recruitment, differentiation and growth of myogenic precursor cells by production of insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) (138, 152).

Against this background, it appears obvious that inflammation and satellite cell dynamics in response to exercise and muscle injury are intertwined, and have to be tightly controlled with respect to timing and resolution. If initial pro-inflammatory signals persist, excessive tissue damage can occur while the differentiation capacity of satellite cells is impaired. Conversely, premature initiation of the anti-inflammatory program can also disrupt efficient tissue healing. Similar to the sequel of inflammatory circuits, satellite cell activation, proliferation, differentiation, and fusion follow a tight schedule. In particular, whereas the cell cycle regulator cyclin D1 enhances satellite cell proliferation within the first few hours after activation, the gene encoding the MRF MyoD is upregulated when the cells exit the cell cycle, in parallel with the cell cycle inhibitor p21. By contrast, myogenin is a rather 'late' MRF, being upregulated during differentiation and fusion (for review, see 138, 152). At present, we are far from a clear understanding of how contracting myofibers, invading inflammatory cells and resident stem cells orchestrate their activities to promote skeletal muscle regeneration and remodeling, but also to control and restore tissue homeostasis.

This is where ABPs come into the picture, as they regulate, restrict, or fine-tune the production of inflammatory mediators. Reciprocally, expression of the genes encoding ABPs is controlled and commonly up-regulated by cytokine- and growth factor-mediated pathways (1, 2, 143). In addition, as discussed in detail in section 3, several ABPs control the expression of genes encoding regulators of satellite cell proliferation, differentiation, and fusion. These include the cell cycle regulators cyclin D1 and p21, and the MRFs MyoD and myogenin. Therefore, ABPs might control skeletal muscle adaptation to exercise at multiple levels.

Against this background, it is surprising that a potential role for ABPs in skeletal muscle adaptation to exercise has hardly been analyzed so far. However, a few studies could demonstrate differential expression of genes encoding ARE-binding proteins in response to skeletal muscle contraction and/or physical exercise. Lai et al. showed enhanced expression of both *HuR* and *AUF1* in rat muscle after electrically stimulated, chronic contractile activity (78). Matravadia et al. demonstrated long-term induction of the *CUGBP1* and *AUF1* genes in the *quadriceps* muscle of mice after treadmill training, but found no effect of acute treadmill exercise on the expression levels of *HuR, CUGBP1*, or *AUF1* (88). By comparing short bout effects of eccentric versus concentric exercise in human *vastus lateralis* muscle, Chen at al. described a more pronounced expression of *ZFP36/TTP* following eccentric contractions (38). The same group reported additive effects on post-exercise *ZFP36/TTP* expression levels after two repeated bouts of eccentric exercise, separated by four weeks (68). As illustrated in Figure 3, our own unpublished data indicate that endurance exercise is also characterized by a significant up-regulation of *ZFP36/TTP* gene expression in the murine *soleus* muscle after an acute bout of running exercise (Figure 3A), and in the human *vastus lateralis* muscle in response to one hour of intense cycling exercise (supplementary methods; Figure 3B). These novel data demonstrate immediate up-regulation of *ZFP36* gene expression in skeletal muscle in response to acute endurance exercise.

In particular, simultaneous induction of *ZFP36/TTP* gene expression along with ZFP36 target transcripts in skeletal muscle after exercise suggests the existence of concerted gene activation programs that ensure immediate modulation of inflammatory gene expression by the concomitant supply of balancing ABPs. As exemplified in Figure 3, exercise-induced levels of the IL-6 transcript are significantly correlated with the individual magnitude of *ZFP36/TTP* gene expression in *vastus lateralis* muscle after intense cycling exercise (Figure 3D). Likewise, in post-exercise samples, ZFP36/TTP mRNA levels correlate with the levels of various well-known ARE-

containing transcripts, including those encoding chemokine ligand CXCL2, urokinase plasminogen activator PLAU, thrombomodulin THBD, serum/glucocorticoid regulated kinase SGK1, and cell cycle regulators like FOS, FOSL1, and MYC (unpublished data; see supplementary methods section for details). Interestingly, as outlined by Piccirillo et al., simultaneous induction of pro- as well as counter-regulatory gene expression networks at the transcriptional level, and subsequent establishment of post-transcriptional regulatory checkpoints is emerging as a rather general mechanism in a broad variety of settings, allowing rapid changes and adaptations in the proteome in response to inflammatory or metabolic stimuli (104).

An analogous, but even more complex pattern of transcriptional and post-transcriptional feedback control is found when looking at the tight control mechanisms that determine the outcome and duration of MAPK signaling cascades. Activation of MAPK pathways is known to trigger the transcription of multiple immediately early genes, including enhanced expression of ABP-encoding genes, as well as genes encoding dual-specificity phosphatases (DUSPs) which are reciprocally destined to extinguish MAPK signaling by dephosphorylation (69). DUSP transcripts contain ARE-binding motifs and are targeted by ABPs for post-transcriptional regulation, including stabilization by HuR (77), or enhanced degradation by ZFP36/TTP (83), respectively. In addition, as outlined above, activity, localization, and binding capacity of ABPs is determined by specific phosphorylation patterns, which in turn are again controlled by the activity of MAPK signaling cascades (11).

In this way, inflammatory pathways are intrinsically coupled to multiple self-regulatory feedback mechanisms, allowing precise control whether to shut-off, resolve, perpetuate, or amplify the inflammatory response. It is thus tempting to suggest that ABPs might constitute a highly flexible toolbox in skeletal muscle to set the balance between pro- and anti-inflammatory pathways at multiple levels, allowing fine-tuning of inflammatory and functional networks in response to the demands of different exercise regimens and insults.

The importance of precise and flexible post-transcriptional coordination of inflammatory responses can be exemplified by the most extensively studied (but probably least understood) exercise-induced inflammatory mediator, namely IL-6. Circulating IL-6 functions as a ubiquitous warning signal to indicate disturbed tissue homeostasis in case of emergent events. Thus, it is commonly acknowledged that IL-6 serum levels parallel the onset, progression, and remission of the exercise-provoked acute inflammatory response (Figure 3F) (102). Apparently, the contracting muscle itself is triggered to produce and release IL-6 (127) and to up-regulate expression of the gene encoding the IL-6 receptor (IL-6R) (Figures 3C,E) (72). Exogenous IL-6 has further been shown to up-regulate its own production in skeletal muscle by stabilizing the transcribed IL-6 mRNA, thus fostering a self-promoting feedback loop of IL-6 signaling in the exercising muscle (147). Although the true contribution of skeletal muscle to exercise-provoked IL-6 serum levels is unknown, there is clear evidence that IL-6 seems to be involved in driving metabolic homeostasis and insulin sensitization (21, 66, 90). Moreover, IL-6 signaling is beneficially involved in driving muscle regeneration, satellite cell proliferation and the formation of myotubes (65, 124, 153). Conversely, persistent elevation of IL-6 has been associated with muscle wasting in chronic inflammatory conditions, including cachexia, insulin resistance, and diabetes, as well as in the progression of sarcopenia (12, 132, 149). Likewise, persistent up-regulation of *IL6R* expression, along with other genes involved in inflammatory pathways, has been observed in skeletal muscle tissue of women with metabolic syndrome and insulin resistance (107). Thus, the need for efficient strategies to control the production of this cytokine in response to exercise is obvious. Indeed, recent studies reveal complex and interweaving controlling circuits that regulate *IL-6* expression at the post-transcriptional level, governed by specific MEKK1-dependent phosphorylation patterns of the ABP ZFP36/TTP (120, 121, 156, 157).

Similar to IL-6 signaling, dichotomous functionality in inflammatory and metabolic responses is characteristic of several other inflammatory mediators that are released upon exercise, and are known to be targeted by ABPs. For instance, CXCL1 and CXCL2, two chemokines involved in neutrophil and macrophage recruitment in response to exercise and muscle injury (85, 138), have recently been shown to improve fat oxidation in skeletal muscle (103). However, they are also implicated in insulin resistance and chronic muscle inflammation in obesity (105). The transcripts of both chemokines contain ARE elements within their 3'UTRs, and are under reciprocal control of HuR and ZFP36/TTP at the post-transcriptional level (43, 50, 54, 61). Similarly, both HuR and ZFP36/TTP have been implicated in the regulation of prostaglandin production in inflamed muscle tissue by reciprocally targeting COX-2 mRNA (33), thereby shaping M1 and M2 macrophage polarization (62, 84, 151). It appears that systemic chronic inflammation with dysregulated cytokine signaling interferes with skewing from M1 towards M2 phenotypes in skeletal muscle, thereby promoting insulin resistance, fibrosis, fat deposition, exhaustion of the satellite cell pool, and muscle wasting (44, 52, 111, 113). Accumulating evidence suggests that prostaglandins can promote muscular pain, chronic inflammation and atrophy, but are also indispensable for driving myogenesis and muscle repair (76). As such, administration of COX inhibitors has the potential to block skeletal muscle inflammation, as well as to promote but also to interfere with the resolution of inflammation (122, 140). Similarly, administration of glucocorticoids in chronic inflammatory conditions may aggravate disease by triggering expression of atrophy-related genes *(FOXO1, atrogin-1, MuRF1)*, and by interfering with mammalian target of rapamycin (mTOR) signaling in skeletal muscle, thereby promoting muscle wasting (41, 115, 117). In fact, no effective drug is currently available that sufficiently attenuates chronic inflammatory symptoms without imposing an increased risk of disturbed skeletal muscle homeostasis and metabolism. Consequently, a better understanding of pro-resolving, in contrast to anti-inflammatory, mechanisms is necessary to open novel avenues for the treatment of chronic inflammatory conditions, as has recently been emphasized by Buckley et al. (28). We should also consider the fact that we already have the probably most effective 'pro-resolving drug' at hand, namely physical exercise. This further emphasizes the need for a better understanding of regulatory pathways involved in skeletal muscle adaptation to exercise.

Besides mRNAs encoding inflammatory mediators, other ABP targets encode proteins known to have diverse functions in skeletal muscle adaptation to exercise. Specifically, given the fact that ABPs target mRNAs encoding cell cycle regulators and MRFs, such as cyclin D1, p21, MyoD, or myogenin, which are all involved in the regulation of satellite cell dynamics, it is very likely that ABPs are major players in this process.

In addition, ABPs might be involved in the regulation of fiber type specification in response to exercise. For example, Chakkalakal et al. demonstrated that the stability of the mRNA encoding the skeletal muscle protein utrophin A, a structural protein located at the neuromuscular junction, is controlled by a conserved ARE within the utrophin transcript (37). This is particularly interesting, since the utrophin A transcript is more stable in slow versus fast muscle fibers (37, 55). The upstream regulator appears to depend on calcineurin signaling, which is activated in response to endurance exercise, and is generally considered to have important roles in maintaining the slow-twitch, oxidative myofiber program (135). The underlying mechanism might be less efficient binding of the mRNA de-stabilizing protein KSRP to this specific ARE, since it has been demonstrated that the p38-dependent stabilization of the utrophin A transcript is mediated by KSRP (5, 6). Thus, it is likely that both the phosphatase calcineurin and the kinase p38 initiate a signaling cascade eventually leading to decreased de-stabilizing activity of KSRP. Utrophin A is a good candidate for novel therapeutic approaches in DMD (Duchenne muscular dystrophy)—it might partially replace the dystrophin protein, which is missing in DMD patients. Thus, these findings might have important clinical implications. Furthermore, D'souza et al. presented data indicating that the stability of transcripts encoding regulators of oxidative metabolic pathways, such as PGC-1α (peroxisome proliferator-activated receptor-gamma coactivator 1α), was different in red slow and white fast skeletal muscle fibers. This finding indicates that regulation of muscle fiber type composition is distinctively controlled at the level of mRNA stability (42). Consistently, the authors found that levels of several ABPs, specifically AUF1, HuR, KSRP, and CUGBP1, were different in red compared with white white muscle cells.

Finally, ABPs might also be important players in the regulation of exercise-associated angiogenesis. Because the VEGF transcript is an important target of the ZFP36 proteins in different cell types, the latter might be central in the control of the enhanced vascularization that is regularly observed in response to endurance exercise (144).

5. CONCLUDING REMARKS

Regulation of mRNA stability by ABPs might be a powerful strategy to control the response of skeletal muscle tissue to exercise. In particular, ABPs are involved in the regulation of genes encoding pro-inflammatory cytokines and chemokines, thus temporally and spatially restricting the inflammatory response to a training stimulus. This is of specific relevance, because ordered and timely resolution of inflammation is crucial for restoration of tissue homeostasis in response to exercise. Whether inflammation acts as a 'good guy', enhancing skeletal muscle training adaptation, or as a 'bad guy', inducing skeletal muscle decay in chronic disease, depends on an intricate network of cellular and molecular signaling circuits that are poorly understood at present. In addition, ABPs control the expression of a plethora of other, non inflammation-associated genes involved in the adaptation of skeletal muscle to exercise, such as the MRF genes or genes encoding cell cycle regulators, which are involved in the control of satellite cell proliferation and differentiation. Thus, in the future, it will be crucial to systematically identify and functionally characterize ABPs and ABP targets in the exercising skeletal muscle, via, for example transgenic mouse models. A better understanding of the multiple layers of post-transcriptional regulatory control mechanisms might eventually pave the way for the development of novel therapeutic approaches for disorders associated with chronic inflammation and skeletal muscle degeneration.

SUPPLEMENTARY METHODS SECTION

Microarray data from *musculus vastus lateralis* at rest and in response to exercise were kindly provided by Frank C. Mooren, Jürgen Steinacker, and Andreas M. Niess. Expression values of selected genes were extracted from an unpublished data set that derives from an ongoing multicenter trial performed at the facilities of Sports Medicine at the Universities of Gießen, Ulm, and Tübingen, Germany. All experimental procedures were approved by the Research Ethics Committees of the Justus-Liebig-University Gießen, the University Hospital of Tübingen, and the University of Ulm, Germany.

Briefly, expression data were derived from healthy male endurance-trained individuals (n=8; age, 25.4 ±4 years; VO_2max, 67.2 ±9 ml/min/kg) who completed 60 min of high intensity cycling on a bicycle ergometer at a power requiring 80% of the VO_2max (262 ± 58 Watt); including 10 min of warm-up cycling at 60% VO_2max. A more detailed description of the exercise protocol is given in Beiter et al. (18). Percutaneous muscle biopsies from the *musculus vastus lateralis* were taken at rest, as well as 30 min (+30'), and 3 hours (+3h) after the cycling protocol. All muscle biopsies were performed on the right leg of the participants using a biopsy gun and a fine biopsy needle (Plus Speed; Peter Pflugbeil, Zorneding, Germany) under local anesthesia (1% Meaverin). Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C.

Muscle tissue was homogenized with a TissueRuptor homogenizer (Qiagen, Hilden, Germany), and total RNA was extracted using RNeasy Fibrous Tissue Mini kit (Qiagen) according to the manufacturer's recommendations. RNA integrity was assessed using the Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Pico Kit (Agilent Technologies, Waldbronn, Germany). Microarray analysis was performed by the Microarray Facility Tübingen (MFT Services, Germany). Briefly, biotin-labeled cRNA synthesis and cRNA fragmentation were performed using the Affymetrix GeneChip Kit reagents, according to the procedure described in the Affymetrix GeneAtlas 3'IVT Express Kit technical manual (Affymetrix, Santa Clara, CA, USA). Samples were hybridized using Human Genome U219 microarray platform (Affymetrix). Gene expression data were analyzed using Affymetrix Expression Console software (Affymetrix) and

Partek Genomics Suite 6.5 software (Partek Incorporated, St Louis, MO, USA). Data were normalized and filtered for transcripts which were differentially expressed between sampling points. Significance was calculated using paired t-test without multiple testing corrections, selecting all transcripts with a minimum change in expression level of 1.5-fold together with $P < 0.05$. Pearson correlation and linear regression analysis was used to estimate the correlation between expression level of *ZFP36/TTP* and other exercise-affected transcripts from the list of differentially expressed probe sets. The expression changes of three selected genes *(LDHA, LDHB, PPARGC1A)* were validated by SYBR green quantitative real-time PCR.

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CONFLICTS OF INTEREST

None of the authors has any conflicts of interest to declare.

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Metabolic signals and innate immune activation in obesity and exercise

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ABSTRACT

The combination of a sedentary lifestyle and excess energy intake has led to an increased prevalence of obesity which constitutes a major risk factor for several co-morbidities including type 2 diabetes and cardiovascular diseases. Intensive research during the last two decades has revealed that a characteristic feature of obesity linking it to insulin resistance is the presence of chronic low-grade inflammation being indicative of activation of the innate immune system. Recent evidence suggests that activation of the innate immune system in the course of obesity is mediated by metabolic signals, such as free fatty acids (FFAs), being elevated in many obese subjects, through activation of pattern recognition receptors thereby leading to stimulation of critical inflammatory signaling cascades, like IκBα kinase/nuclear factor-κB (IKK/NF-κB), endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) and NOD-like receptor P3 (NLRP3) inflammasome pathway, that interfere with insulin signaling. Exercise is one of the main prescribed interventions in obesity management improving insulin sensitivity and reducing obesity-induced chronic inflammation. This review summarizes current knowledge of the cellular recognition mechanisms for FFAs, the inflammatory signaling pathways triggered by excess FFAs in obesity and the counteractive effects of both acute and chronic exercise on obesity-induced activation of inflammatory signaling pathways. A deeper understanding of the effects of exercise on inflammatory signaling pathways in obesity is useful to optimize preventive and therapeutic strategies to combat the increasing incidence of obesity and its co-morbidities.

Key words: exercise, immune system, obesity, fatty acids, inflammation, adipose tissue

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1. INTRODUCTION

Excess energy intake and reduced energy expenditure have been recognized as the most important aetiologic factors for obesity indicating that obesity is largely preventable by an appropriate lifestyle. Despite this knowledge, obesity is one of the major public health concerns with prevalence rates dramatically rising worldwide. According to prospective estimations, about 2.3 billion adults in the world will be overweight or obese by the year 2015 (104). This fact poses significant problems for healthcare systems, since obesity constitutes a risk factor for a large number of health problems, ranging from merely the physical burden of the excess adipose tissue itself (e.g. joint pain, back pain, dyspnoea) to serious endocrine and metabolic disturbances, such as type 2 diabetes (T2D), cardiovascular disease, hepatic steatosis, airway disease, biliary diseases and certain cancers (104). Aggravating this situation is the fact that the obesity-related disturbances are linked to reduced life expectancy and premature death. Thus, understanding the biological basis for the development of obesity-related disturbances is an important need.

Obesity is characterized by a pathologic expansion of adipose tissue (AT) that is caused mainly by an enlargement of pre-existing fully differentiated adipocytes due to the storage of excess energy as fat (87). AT expansion through adipocyte enlargement is critical, because it leads to insulin resistance (IR) (54, 91, 103); a condition of reduced glucose utilization by insulin-sensitive tissues due to an impaired insulin action. IR is thought to be a key driver for developing obesity-related endocrine and metabolic disturbances (8, 39, 45, 76). Intensive research during the last two decades has revealed that a characteristic feature of obesity linking it to IR is the presence of chronic low-grade inflammation, which develops locally in the expanding AT, but becomes systemic through the release of numerous pro-inflammatory mediators including cytokines into the blood stream (24-26, 97). As the metabolic surplus (excess nutrients and energy) is thought to be the initial signal for the inflammatory response, the chronic inflammation associated with obesity is referred also to as metaflammation (metabolically triggered inflammation) (28). The AT-derived pro-inflammatory mediators are initially secreted by the enlarged adipocytes, but with increasing AT expansion also by macrophages infiltrating the AT (29, 100). Interestingly, diet induced obesity was found to cause a phenotypic shift of AT macrophages from the M2 polarization state, which exhibits secretion of anti-inflammatory cytokines, to the M1 polarization state, which produces large amounts of pro-inflammatory cytokines, (53). Apart from

macrophages, other immune cells such as mast cells and natural killer T cells are known to increase in AT in obesity and contribute to the pro-inflammatory milieu (51, 69). Moreover, the ratio of $CD8^+$ to $CD4^+$ T cells has been found to increase in the obese AT, whereas the number of immunosuppressive $CD4^+$ regulatory T cells, which are known to secrete anti-inflammatory cytokines that inhibit macrophage migration, in obese AT decreases (17, 66, 105). This clearly indicates that obesity is associated with the activation of the innate immune system, a system that is important to respond to microbial stimuli, such as bacterial, viral and fungal infections, by coordinating an inflammatory reaction and subsequent tissue repair. Detection of microbial stimuli by the innate immune system occurs by a set of pattern recognition receptors (PRRs), which have evolved in mammals to sense and trigger a response to common microbial structures. Considerable evidence exists that many PRRs act also as sensors of metabolic signals, such as free fatty acids (FFAs), and upon activation critical inflammatory signaling cascades, such as the IκBα kinase/nuclear factor-κB (IKK/NF-κB) pathway, endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) pathway and the NOD-like receptor P3 (NLRP3) inflammasome pathway are stimulated providing a plausible explanation that an inflammatory response is induced during metabolic surplus.

This review summarizes current knowledge of the cellular recognition mechanisms for FFAs, which are chronically elevated in the circulation of obese subjects (35), and critical intracellular inflammatory signaling pathways triggered by excess FFAs in obesity. The review also examines the beneficial effects of exercise, which is one of the main prescribed interventions in obesity management improving insulin sensitivity and reducing obesity-induced chronic inflammation.

2. RECOGNITION MECHANISMS FOR FFAs

FFAs are known to interact with several receptors, such as PRRs, like Toll-like receptors (TLRs) and nucleotide-binding, oligomerization domain containing receptors [NOD-like receptors (NLRs)], and FFA receptors (FFARs). Interestingly, despite being essential components of the innate immune system, PRRs are present in both immune cells and metabolically active tissue cells including hepatocytes, myofibrils, and adipocytes to initiate inflammatory signaling cascades (64).

2.1 PATTERN RECOGNITION RECEPTORS (PRRs)

The PRRs have been initially described to be involved in the response to microbial attack through sensing/detecting unique pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, microbial peptides and proteins (muramyl dipeptides, flagellin), and double-stranded ribonucleic acids (dsRNA), and triggering a subsequent immune response. More recently, it has become apparent that PRRs not only recognize microbial signals but also mediate immune responses to endogenous "danger" signals [danger-associated molecular patterns (DAMPs)], including those arising from metabolic disturbances, like saturated fatty acids, ceramides, cholesterol crystals, monosodium urate crystals, amyloid beta, and extracellular ATP (64, 81).

TOLL-LIKE RECEPTORS (TLRs)

One class of PRRs are the TLRs, which are mammalian homologues to the Toll gene in drosophila (the fruit fly), where it encodes a receptor for host defence against microbial infections. The TLRs, of which more than 10 members exist in humans and mice, sense PAMPs via their extracellular leucine-rich repeat domain leading to TLR dimerization through adaptor protein recruitment to their cytoplasmic Toll/IL-1 (TIR) domain, which activates downstream signaling adaptor proteins including MyD88, IRAKs and TRAF6. At least two TLRs, namely TLR4, which is best-known to be activated by the fatty acid residue in the lipid A component of LPS, and TLR2 have been shown to be activated by saturated FFAs (16, 84), and to mediate FFA-induced impairment of insulin sensitivity through negatively interfering with insulin signaling (84). In addition, FFAs were found to exacerbate pro-inflammatory cytokine secretion from M1 macrophages through activation of TLR4, which is highly expressed by pro-inflammatory M1 macrophages and thus used as a marker for M1 macrophages (29), in genetically and diet-induced obese mice thereby promoting AT inflammation (65). A key role of TLR4 for developing IR was evidenced from the observation that TLR4 knockout mice are protected from IR when fed a high-fat diet (HFD) (74, 96). Likewise, knockout of TLR2 was found to provide protection from HFD-induced IR and to reduce AT macrophage accumulation and AT inflammation (10, 14, 21).

NOD-LIKE RECEPTORS (NLRs)

The cytoplasmic NLRs, of which 22 members are known in humans (81) are classified into five subfamilies: NLRA, NLRB, NLRC, NLRP and NLRX. All NLRs contain a central NACHT domain, which is responsible for oligomerization, and a C-terminal leucine-rich repeat domain, which facilitates sensing of PAMPs and DAMPs. The NLR subfamilies differ in their N-terminal effector domains, which ascribe unique functional properties to the NLRs. Members of the NLRP family, which contain a pyrin domain (PYD) as effector domain for downstream signaling, are best known for their role in inducing the formation of the multi-protein inflammatory complex, called "inflammasome", in response to stress signals (56). The NLRP3 inflammasome is the most commonly studied and has attracted great attention as the protagonist in obesity-associated inflammation and IR (63) due to its ability to be activated by saturated fatty acids, ceramide and reactive oxygen species (ROS) and to negatively regulate insulin receptor signaling (52, 101). Upon activation by PAMPs and DAMPs, the NLRP3 inflammasome is formed through recruiting the apoptosis-associated speck-like protein (ASC), containing an N-terminal PYD and a C-terminal caspase activation and recruitment domain (CARD), and the zymogen pro-caspase-1 to NLRP3, which is a prerequisite to induce caspase-1 activation. The activated caspase-1 subsequently cleaves the inactive cytokine precursors pro-IL-1β and pro-IL-18 into their biologically active forms, thereby inducing pro-inflammatory cell death, called pyroptosis. The mature IL-1β, in particular, is critical with regard to the induction of IR because it causes cell death of pancreatic β-cells, inhibits AKT phosphorylation coincident with serine phosphorylation of IRS-1, and, interestingly, mediates inter-organ cross-talk between adipocytes and the liver, promoting systemic inflam-

mation and lipotoxicity (30, 47, 67). The critical role of NLRP3 in obesity-induced AT inflammation and IR is shown by the fact that NLRP3 knockout mice are resistant to IR and exhibit reduced AT inflammation when kept on a HFD (92, 98, 101). In humans, weight loss in obese T2D subjects leads to reduced AT expression of NLRP3 and AT inflammation and improved insulin sensitivity (98).

2.2 FREE FATTY ACID RECEPTORS (FFARs)

The FFARs are G-protein coupled receptors (GPRs) that are widely expressed in tissues and activated by either medium and long-chain fatty acids (FFA1 and FFA4 also known as GPR40 and GPR120, respectively) or short chain fatty acids (FFA2, FFA3). FFA1 in particular is considered to be an important key that links chronically elevated FFAs in obesity to IR and T2D. FFA1 was found to mediate the lipotoxic effects of saturated FFA in pancreatic β-cells through disturbing cytosolic calcium ion (Ca^{2+}) homeostasis, amplifying glucose-stimulated insulin secretion, thereby causing β-cell dysfunction and β-cell apoptosis (1, 83). In contrast, inhibition of GPR40 protected MIN6 β-cells from palmitate-induced apoptosis (108). In line with this, loss of FFA1/GPR40 was found to protect mice from obesity-induced hyperinsulinaemia, hepatic steatosis, and glucose intolerance (89).

3. INFLAMMATORY SIGNALING PATHWAYS ACTIVATED BY FFAs IN OBESITY

3.1 IKK/NF-KB PATHWAY

The IKK/NF-κB pathway is a well-known inflammatory signaling pathway that is activated upon stimulation of the cell by various agents, including cytokines, growth factors, ROS and microbial components like LPS. All these agents trigger different upstream signaling cascades that converge in the activation of the IKK enzyme complex leading to phosphorylation of the NF-κB inhibitor IκBα and its subsequent degradation via the proteasome. This causes the release of the nuclear localization sequence of NF-κB and its translocation into the nucleus where it stimulates transcription of genes involved in inflammation including pro-inflammatory cytokines, chemokines, adhesion molecules and many others. In addition, IKK activation impairs insulin signaling, thereby inducing IR.

Like LPS, saturated FFAs, such as palmitic acid, are reported to cause IKK activation and IκBα phosphorylation through the activation of TLR4 in different cell types including adipocytes, vascular cells, and macrophages (75, 84). Conversely, NF-κB target genes are not induced by FFAs in TLR4 knockdown adipocytes and obese mice lacking TLR4 are partially protected from HFD-induced inflammatory gene expression and IR (84). Likewise, mice with a spontaneous loss-of-function mutation in TLR4 are protected from IR, weight gain and adiposity when kept on a HFD (96).

3.2 ENDOPLASMIC RETICULUM STRESS-INDUCED UNFOLDED PROTEIN RESPONSE

The ER is a dynamic continuous membrane-enclosed organelle with distribution throughout the cytoplasm and has important functions in protein biosynthesis, folding and trafficking and Ca^{2+} storage and signaling. Consequently, when the protein synthetic and folding capacity and Ca^{2+} homeostasis of the ER are perturbed - a condition referred as ER stress - a complex cascade of cytoplasmic and nuclear signaling pathways is activated, which is collectively called the UPR and which is comprised of the inositol requiring 1 (IRE1), the PKR-like ER kinase (PERK) and the activating factor 6 (ATF6) pathway. Initially, the UPR aims to reestablish ER homeostasis through inhibition of protein translation to decrease ER load, transcriptional activation of chaperone genes to increase the ER folding capacity, and activation of the ER-associated degradation (ERAD) machinery to clear misfolded proteins (7). In addition, the UPR pathway enhances inflammation through PERK-eIF2α-dependent translational suppression of IκB leading to nuclear translocation of NF-κB. Moreover, sinceIRE1 and PERK cause activation of JNK and IKKβ signaling pathways, ER stress-induced UPR also mediates IR through impairing insulin signaling. Ultimately, the ER stress-induced UPR can trigger cell death by the induction of apoptosis, if ER stress cannot be resolved (3, 80).

Convincing evidence demonstrates that ER stress is present in tissues including AT, liver, and skeletal muscle, of obese subjects and obese animals (19, 27, 38, 72) and it has been proposed that ER stress links obesity, IR and T2D (72). Conversely, weight loss decreases ER stress coincident with improved insulin signaling in tissues of obese subjects (19). In addition, several studies have shown that saturated FFAs, which are present at elevated levels in obesity, cause ER stress in different cell types including liver cells, pancreatic β-cells and adipocytes (13, 31, 40, 99) indicating that FFAs are potential mediators for the induction of ER stress in obesity. Evidence has been provided that ER stress can be induced by FFAs via both FFARs and TLRs; for example, while inhibition of FFA1/GPR40 was found to protect MIN6 cells from palmitate-induced ER stress (108), TLR4 deficiency was reported to prevent HFD-induced ER stress and IR in the main organs for glucose and lipid metabolism (skeletal muscle, liver, and AT) in mice (74).

3.3 NLRP3 INFLAMMASOME PATHWAY

Formation of the cytosolic NLRP3 inflammasome complex, consisting of NLRP3, caspase-1 and ASC, involves a two-step process: 1) In the priming step, a first hit (signal 1) causes transcriptional induction of inflammasome components, including NLRP3 and pro-IL-1β and pro-IL-18, via TLR-mediated activation of NF-κB. This induction is necessary because basal expression of NLRP3 in resting cells is insufficient for effective inflammasome activation, with the exception of human blood monocytes and dendritic cells which have constitutive NLRP3 inflammasome activity (20, 86). 2) In the activation step, a second hit (signal 2) promotes the NLRPs to undergo homotypic oligomerization and assemble the active inflammasome capable of converting the cytokine precursors into active IL-1β and IL-18. Although the precise molecular mechanisms involved in the activation of the NLRP3 inflammasome in response to PAMPs including bacterial pore-forming toxins (nigericin, listeriolysin O, pneumolysin, α-haemolysin) and DAMPs remain to be elucidated, it has been suggested that potassium ion (K^+) efflux is one common cellular response to diverse stimuli triggering

inflammasome activation (81). For instance, crystals (e.g., urate) and particulate DAMPs, entering the cell via endocytosis, and pore-forming toxins activate the NLRP3 inflammasome via facilitating K^+ efflux (23, 55, 58, 61). Extracellular ATP released from dying or damaged cells activates the NLRP3 inflammasome through binding the purinergic receptor P2X7, which induces opening of pannexin-1 channels thus resulting in K^+ efflux and influx of any DAMPs and PAMPs present in the extracellular space (2, 18, 34). Besides triggering K^+ efflux, recent studies suggested that Ca^{2+} signaling and mitochondrial destabilization plays a critical role for NLRP3 inflammasome activation (22, 48, 79). For instance, different crystals (e.g., urate, silica, cholesterol) trigger Ca^{2+} influx through opening TRPM2 channels (62, 111). As a consequence, Ca^{2+} accumulates in the cytosol causing mitochondrial destabilization or dysfunction and release of mitochondrion(mt)-associated ligands, like mtDNA and cardiolipin, and mtROS, all of which activate the NLRP3 inflammasome. Apart from stimulating extracellular Ca^{2+} influx, elevated Ca^{2+} transfer from the ER to mitochondria at the ER-mitochondria contact sites, the mitochondrial-associated ER membranes,has been shown also to trigger the cascade of mitochondrial destabilization, release of mitochondrion-associated ligands and NLRP3 inflammasome activation (60).

Saturated FFAs can act as both primers and activators in the activation of the NLRP3 inflammasome. Mechanistic studies have demonstrated that saturated FFAs are particularly potent signals to induce the priming step of NLPR3 inflammasome activation through TLR2/4-dependent activation of NF-κB (65, 102). L´Homme et al. (50) demonstrated that both palmitic acid and stearic acid increase IL-1β release through NLRP3 inflammasome activation in LPS-primed human monocytes, in human monocyte-derived macrophages and in THP-1 macrophages. Recently, it was also shown that the saturated FFA palmitic acid induces NLRP3 inflammasome activation via induction of ER stress, which primes cells for pro-IL-1β production via NF-κB and promotes IL-1β secretion (44). In addition, Kim et al. (44) found that ER stress-induced ROS production activates the NLRP3 inflammasome through binding of the ROS-sensitive NLRP3 ligand thioredoxin-interacting protein (TXNIP), resulting in IL-1β cleavage and secretion. These findings indicate that FFAs through inducing ER stress act as primers and activators of the NLRP3 inflammasome.

4. EFFECT OF EXERCISE ON OBESITY-INDUCED ACTIVATION OF INFLAMMATORY SIGNALING PATHWAYS

Exercise is a reasonable approach to attenuate diet-induced weight gain by increasing energy expenditure and counteracting a positive energy balance (57, 73), thereby, providing several benefits for skeletal muscle function including increased insulin sensitivity, stimulated utilization of metabolic substrates, and even improved protection against oxidative insults. In the following sections evidence is provided that these beneficial effects of both acute and chronic exercise are mediated on the molecular level by an inhibition on obesity-induced activation of inflammatory signaling pathways being responsible for attenuating obesity-induced metaflammation.

4.1 EFFECTS OF EXERCISE ON THE IKK/NF-KB PATHWAY

It is well established that the IKK/NF-κB pathway is activated in tissues of obese subjects and obese animals (70, 93), and that activation of this pro-inflammatory signaling pathway is a key pathogenic mechanism responsible for inhibition of insulin signaling and induction of IR. Since it is also well known that exercise reduces obesity-induced IR (77, 94), it is likely that exercise prevents the development of obesity-induced IR through inhibiting the NF-κB pathway in insulin-dependent tissues. Indeed, several studies have documented the potential of exercise training to attenuate obesity-induced activation of the IKK/NF-κB pathway. For instance, Medeiros et al. (59) demonstrated that 12 weeks of endurance exercise training (swimming at 32°C water temperature, 1h/day with 5 % overload of the body weight, 5 days/week) inhibits the NF-κB pathway and increases the activity of the mTOR/p70S6k pathway of insulin-dependent protein synthesis thereby reducing IR in the cardiac tissue of diet-induced obese rats. In addition, Da Luz et al. (9) demonstrated that 8 weeks of endurance exercise training (swimming at 32°C water temperature, 1 h/day with 5 % overload of the body weight, 5 days/week) reduces activation of the NF-κB pathway in AT and liver via ameliorating ER stress along with improving insulin sensitivity in diet-induced obese rats. Moreover, Oliveira et al. (70) reported that both acute (two 3 h swimming sessions at 34°C water temperature, separated by a 45 min rest period, with 5 % overload of the body weight) and chronic exercise (same conditions as for acute exercise, 1 h/day, 5 days/week, 8 weeks) inhibits the IKKβ/NF-κB pathway and, in parallel, improves insulin signaling in tissues (AT, skeletal muscle, liver) in diet-induced obese rats. Also in humans, an inhibitory effect of 8 weeks of aerobic exercise training on a stationary bicycle (four times/week, exercise intensity, duration, and frequency were progressively increased to 70 % of VO_2max for 45 min during the 8-week exercise program) on NF-κB pathway in vastus lateralis muscle was found in T2DM subjects (88).

With regard to the mechanism of inhibition of obesity-induced NF-κB activation by exercise, Oliveira et al. (70) demonstrated in their study that both acute and chronic swimming exercise reverses obesity-induced activation of TLR4, which is an activator of both IKKβ and JNK in tissues of diet-induced obese rats. This suggests that the effect of exercise involves suppression of FFA-induced activation of TLR4 signaling, because TLR4 is activated by FFAs whose levels are typically elevated in obese subjects. Reduced FFA-mediated activation of TLR4 signaling by chronic exercise is probably also the result of decreasing the expression of TLR4 in AT. This was observed in HFD-induced obese mice in response to a 16-weeks endurance exercise protocol on a motorized treadmill (60 min/day, 5 times/week) (37). The reduced TLR4 expression in AT is likely the consequence of exercise-induced suppression of M1 macrophage infiltration, which express high levels of TLR4 (29), and/or phenotypic switching from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages (36, 37). TLR4 plays a key role for activating the pro-inflammatory NF-κB pathway and develop-

ing IR during obesity as evidenced from the finding that TLR4 knockout mice are protected from IR and induction of pro-inflammatory gene expression when fed a HFD (74, 96). In addition, activation of NF-κB by FFAs is prevented in TLR4 knockdown adipocytes (84). Given that long-term exercise training on a stationary bicycle in humans (exercise intensity of 50-70 % of the target heart rate, 30 min/day, 5 times/week for 12 weeks) causes a reduction of body fat along with a decrease of resting plasma FFA levels (41), the reduced activation of NF-κB in tissues might be simply explained by a reduced TLR4 activation and expression due to decreased plasma FFA levels (70, 71, 110). In contrast, it is well-established that a prolonged acute exercise bout increases circulating FFA levels due to increasing mobilization from AT in order to provide energy substrates for contracting skeletal muscle. In line with this, an acute exhaustive exercise bout (treadmill running at 70% VO_2max for 50 min and then running at an elevated rate that increased by 1 m/min until exhaustion, mean exhaustion time: 61.98 ± 2.24min) was found to activate TLR4 signaling and the NF-κB pathway in AT of healthy, non-obese rats (78). However, despite their well known role in impairment of insulin action, it is expected that TLR activation during acute exercise does not impair insulin sensitivity. This assumption is supported by a study using TLR2 and TLR4 knockout mice, in which the increase of circulating FFAs during acute exercise was stronger than in wild-type mice. These data indicated a role of these proteins in metabolic regulation or repartition of energy substrates during acute exercise (109).

Noteworthy, it was also found that suppression of TLR4 signaling in tissues of HFD-induced obese rats in response to the abovementioned chronic swimming exercise protocol is accompanied by a decrease of serum levels of LPS (70), which is a known activator of TLR4 signaling and, through this, of the IKK/NF-κB pathway. Elevated serum LPS levels have been observed following the intake of a HFD and are probably the result of an increased intestinal permeability for LPS (5). These findings indicate that the inhibitory effect of exercise on NF-κB activation in HFD-induced obese rats might be also due to attenuating LPS-induced TLR4 activation. In this context it is also interesting that a strenuous endurance exercise bout (marathon run) was followed by a slight LPS IgG activity indicating a mild endotoxaemia (4), which has been suggested to be the result of an increased intestinal permeability after exercise.

4.2 EFFECT OF EXERCISE ON THE ENDOPLASMIC RETICULUM STRESS-INDUCED UNFOLDED PROTEIN RESPONSE

It is well documented that exercise (acute and chronic) causes ER stress-induced UPR in skeletal muscle of lean, metabolically healthy humans and animals (42, 43, 68, 107). One important stimulus for ER stress-induced UPR is considered to be the elevated production of ROS during acute muscle contraction (43). In addition, altered Ca^{2+} dynamics and elevated protein synthesis in response to resistance exercise could also provoke the UPR (12). Moreover, mechanical stress and/or local metabolic changes in the muscle that are directly involved in exercise are thought to play a role in UPR activation (107). In line with these divergent ER stimuli, the stimulatory effect of exercise on ER stress-induced UPR pathway was evident in response to different kinds of exercise: A single bout of exhaustive exercise in muscles heavily activated during treadmill exercise (starting at a warm up speed of 5 m/min for 5 min, every subsequent 5 min, the speed increased by 5 m/min until mice were exhausted or a maximal speed of 25 m/min was reached) in mice (M. quadriceps and M. gastrocnemius but not in non-weight bearing back muscle M. erector spinae) (107); a long lasting running exercise (200 km run, 28 ± 2 h) in M. vastus lateralis in humans (42); a single unaccustomed resistance-exercise bout (leg press and knee-extension exercise) in M. vastus lateralis in humans (68). This indicates that both endurance and resistance exercise causes ER stress-induced UPR in skeletal muscle. Interestingly, acute exercise was found to activate specifically the ATF6/IRE1α pathway of the UPR, but not the PERK/eIF2α pathway, that attenuates protein synthesis, (43, 68), thereby promoting the production of certain chaperone proteins, likely as a consequence of the increased production of various proteins during the post-exercise period (68). Activation of the UPR in skeletal muscle by chronic exercise was found to be an important mechanism in the adaptation of skeletal muscle to exercise training (107), with the transcriptional co-activator PGC1α, which regulates several exercise-induced adaptations of skeletal muscle function (mitochondrial biogenesis and function, oxidative metabolism, fibre type switching, angiogenesis), being the mediator of the UPR in skeletal muscle in response to exercise (107). Regulation of the UPR by PGC1α during exercise was shown to involve direct co-activation of ATF6, which is one of the three proximal sensors of the UPR preferentially activating UPR target genes involved in the adaptation of cells to chronic ER stress (106). The essential role of PGC1α and the UPR sensor ATF6 for the adaptation to exercise was evident from the observations that 1) muscle-specific PGC1α knockout mice are defective in up-regulating ER chaperones and experience exacerbated ER stress after repeated exercise training, and 2) ATF6a knockout mice do not recover from muscle damage after exercise (107).

Only very few studies are available in the literature investigating the potential of exercise training to attenuate obesity-induced ER stress. For instance, Da Luz et al. (9) demonstrated that 8 weeks of endurance exercise training (swimming at 32°C water temperature, 1 h/day with 5 % overload of the body weight, 5 days/week) following a 2-month HFD feeding period reduced PERK and eIF2α phosphorylation, inhibited pro-inflammatory signaling pathways (JNK, IκB and NF-κB), and reversed IR in AT and liver of obese rats suggesting that endurance exercise training reduces obesity-induced ER stress in tissues. In contrast, Deldicque et al. (11) reported the opposite finding, namely, that 6 weeks of endurance exercise training (treadmill running at 75 % VO_2max, 30-60 min/day, 5 days/week) during HFD feeding even promoted the UPR induced by HFD feeding alone in two different muscles (M. soleus, M. tibialis), liver and pancreas, despite attenuating the pro-inflammatory state induced by HFD feeding (11). Based on these findings Deldicque et al. (11) postulated that exacerbation of the HFD-induced UPR by endurance exercise might be an adaptive protective mechanism to restore ER homeostasis and to protect against obesity-induced inflammation. The opposing outcomes of endurance exercise on obesity-induced ER stress may have different reasons: 1) The availability of FFAs, which are likely mediators for the induction of ER

stress in obesity via activating both FFARs and TLRs, may be influenced by the different exercise interventions. While exercise intensities of about 75 % VO_2max as applicable to the treadmill protocol increases mobilization of FFAs from AT, glucose is preferentially utilized during the swimming protocol. This is evidenced by the observation that blood concentrations of epinephrine and lactate of mice are markedly higher following a swimming test (at 22°C water temperature until exhaustion; mean swimming time: 36.7 ± 3.7 min) compared to a running test (at 80 % VO_2max until exhaustion; mean running time: 39.5 ± 5.0 min) (46). Thus, it is likely that endurance exercise training during parallel HFD feeding amplified the obesity-induced ER stress due to the great availability of FFAs, whereas the obesity-induced ER stress could be attenuated by swimming exercise with only less FFA mobilization. 2) The authors of the treadmill study proposed that the obese rats were accustomed to the HFD feeding and that a new homeostasis level was reached before the swimming exercise stress was applied, so that the beneficial effects of exercise could be exerted. This was not the case in the treadmill study as both HFD feeding and treadmill exercise started simultaneously, each stress probably exacerbating the other. Interestingly, Deldicque et al. (11) also found muscle type-specific differences in the UPR activation by exercise in obese rats as activation of the UPR was very strong in the tonically active M. soleus and less pronounced in the phasically active M. tibialis anterior. This has been explained by a greater susceptibility of oxidative fibres to HFD feeding, an additive effect of contractile activity and HFD feeding on ER stress, or increased utilization of FFAs in M. soleus due to its higher metabolic requirements (11).

4.3 EFFECT OF EXERCISE ON THE NLRP3 INFLAMMASOME PATHWAY

To date, no published studies are available exploring the direct effect of exercise on obesity-induced activation of the NLRP3 inflammasome pathway. Unpublished data of our own group demonstrate that both endurance (treadmill running at 80 % VO_2max, 30 min/day, 5 times/week for 10 weeks) and resistance exercise (holding on a metal mesh placed in a vertical position, three 3 min bouts, with 1 min break between each bout, 5 times/week for 10 weeks) in HFD-induced obese mice decreases the mRNA level of NLRP3 in AT, which provides the first direct evidence for inhibition of obesity-induced NLRP3 activation by exercise. Interestingly, our data show that the decreased mRNA level of NLRP3 in AT of trained obese mice is accompanied by reduced plasma levels of IL-18, to which AT is considered a major contributor (15, 82, 85) and whose maturation and secretion is mediated by the NLRP3 inflammasome. Thus,

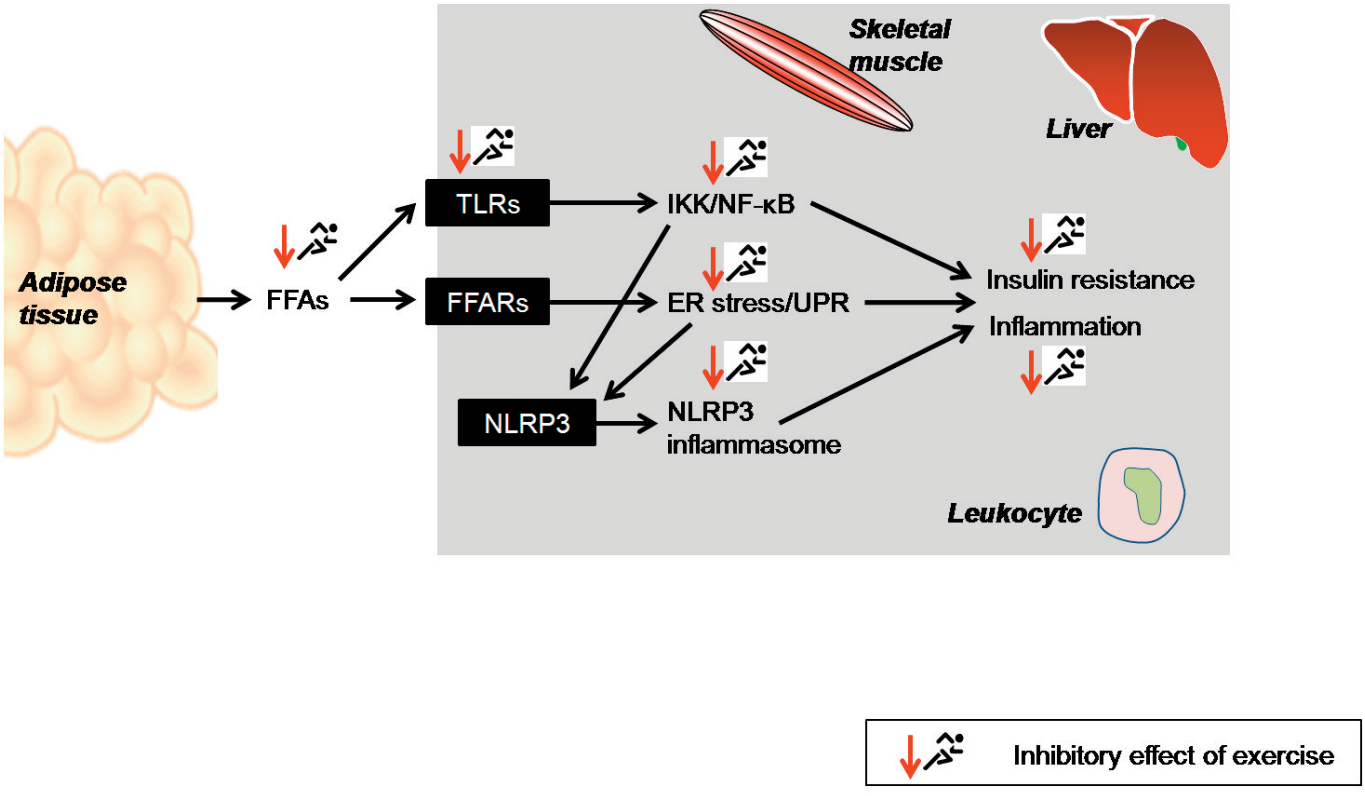

Figure 1
Schematic summary of the effects of exercise on obesity-induced activation of inflammatory signaling pathways. In the course of obesity, elevated levels of free fatty acids (FFA) led to the activation of critical inflammatory signaling pathways, like IκBα kinase/nuclear factor-κB (IKK/NF-κB) and endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) via activating pattern recognition receptors such as Toll-like receptors (TLRs) and free fatty acid receptors (FFARs). In addition, FFAs are potent signals to induce the priming step of NOD-like receptor P3 (NLRP3) inflammasome activation through TLR-dependent activation of NF-κB and via induction of ER stress. Through activation of these criticial inflammatory signaling pathways inflammation and insulin resistance is induced in metabolically active and immune cells. Exercise reduces chronic low-grade inflammation and insulin resistance through increasing utilization of FFAs, lowering expression of TLRs and attenuating activation of IKK/NF-κB, ER stress-induced UPR and NLRP3 inflammasome.

alterations in the plasma IL-18 level can be used to evaluate indirectly changes in the activity of the NLRP3 inflammasome pathway. Interestingly, a large number of human studies have reported that exercise reduces the plasma levels of IL-18 in obese subjects providing at least indirect evidence for inhibition of the NLRP3 pathway by exercise. For instance, Stensvold et al. (90) showed that the serum level of IL-18 was reduced by 43 % in response to 12 weeks of aerobic interval training (three times/week) in men and women with metabolic syndrome. Likewise, in overweight individuals with T2DM a 6-month aerobic exercise training program (four times/week, 45-60 min/session, 50-85 % VO_2max) *per se* (without affecting body weight) resulted in a significant reduction of the plasma IL-18 level (32, 33). Troseid et al. (95) found that the serum level of IL-18 was reduced by 12 weeks of exercise training in subjects with metabolic syndrome. Furthermore, 8 weeks of high-intensity exercise training on a rowing ergometer (three times/week, 30 min/session, $\geq$ 70 % VO_2max) decreased IL-18 mRNA level in abdominal AT and numerically lowered plasma IL-18 concentration in obese men and women (49). Only in one study by Christiansen et al. (6), a 12-week aerobic exercise training program (three times/week, 60-75 min/session), failed to reduce the plasma level of IL-18 in obese men and women. The lack of an exercise effect in this study may be explained by the relatively moderate exercise intensity.

Currently, it is not known whether exercise exerts its predominantly inhibitory action on NLRP3 activation in obese subjects in the priming step, which involves transcriptional induction of inflammasome components via TLR-mediated activation of NF-κB, in the activation step or in both steps of the NLRP3 activation process. Given that exercise was found to strongly reverse the activation of TLR4 signaling along with reducing IKKβ phosphorylation in tissues (AT, skeletal muscle, and liver) of diet-induced obese rats (70), suggests that exercise inhibits the NLRP3 inflammasome pathway in the priming step. Important primers of the NLRP3 activation process are saturated fatty acids and ceramide species (52, 98, 101), whose circulating levels are reduced in obese animals and subjects in response to exercise. Interestingly, our above mentioned study (unpublished) revealed that the plasma levels of ceramides in mice were increased by feeding a HFD, an effect that was significantly attenuated by both endurance and resistance exercise. Exercise might also inhibit the priming step of NLRP3 activation in obesity through its ability to reduce ER stress (9), because ER stress is known to induce the priming step of NLRP3 activation via NF-κB activation (44). Considering that ROS, which are produced in response to ER stress, act as second hit signals leading to the assembly of the NLRP3 components into the active NLRP3 inflammasome and subsequent conversion of cytokine precursors into the active cytokines, exercise might also inhibit the NLRP3 inflammasome pathway in the activation step.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Convincing evidence exists that activation of the innate immune system in the course of obesity is mediated by metabolic signals, such as FFAs, through activation of PPRs like TLR4 thereby leading to stimulation of critical inflammatory signaling cascades that interfere with insulin signaling. The present review provides evidence from the literature showing that exercise is a successful strategy to inhibit obesity- and FFA-induced activation of inflammatory signaling pathways being responsible for attenuating obesity-induced metaflammation **(Fig. 1)**. One important mechanism of exercise on inhibition of obesity-induced activation of inflammatory signaling pathways is suppression of FFA-induced expression and activation of TLR4, a receptor that is critically involved in the activation of the innate immune system by FFAs in obesity. As a consequence of this, it was consistently found in several studies that both acute and chronic exercise and different forms of exercise (running, swimming) inhibit the IKKβ/NF-κB pathway and improve insulin signaling in metabolic tissues of obese animals and humans. In contrast, only two studies are available in the literature investigating the potential of exercise training to attenuate obesity-induced ER stress, with however opposing outcomes; whereas one study reported an inhibition of obesity-induced ER stress by swimming training, the other study revealed an exacerbation of obesity-induced ER stress by running training. Thus, future studies are necessary to clarify the different mechanisms of swimming and running exercise in the regulation of ER stress in obesity. With regard to the NLRP3 inflammasome pathway several human studies reported a decrease in the plasma levels of IL-18 in obese subjects in response to chronic exercise representing at least indirect evidence for inhibition of obesity-induced NLRP3 inflammasome pathway. However, regarding that no published studies are available exploring the direct effect of exercise on obesity-induced activation of the NLRP3 inflammasome pathway future studies are required to close this gap of knowledge. Thus, despite evidence from the majority of published studies that exercise has counteractive effects on obesity-induced activation of inflammatory signaling pathways further studies are necessary to establish the most successful exercise protocol (type, intensity, duration) for preventing and treating obesity and its co-morbidities.

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The microbiota: An exercise immunology perspective

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ABSTRACT

The gut microbiota consists of a cluster of microorganisms that produces several signaling molecules of a hormonal nature which are released into the blood stream and act at distal sites. There is a growing body of evidence indicating that microbiota may be modulated by several environmental conditions, including different exercise stimulus, as well some pathologies. Enriched bacterial diversity has also been associated with improved health status and alterations in immune system, making multiple connections between host and microbiota. Experimental evidence has shown that reduced levels and variations in the bacterial community are associated with health impairments, while increased microbiota diversity improves metabolic profile and immunological responses. So far, very few controlled studies have focused on the interactions between acute or chronic exercise and the gut microbiota. However, some preliminary experimental data obtained from animal studies or probiotics studies show some interesting results at the immune level, indicating that the microbiota also acts like an endocrine organ and is sensitive to the homeostatic and physiological changes associated with exercise. Thus, our review intends to shed some light on the interaction between gut microbiota, exercise and immunomodulation.

Key Words: exercise, gut, immunity, microbiota

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1. The microbiota

Human beings have clusters of bacteria (the microbiota) in different parts of the body, such as in the surface or deep layers of skin, the mouth, gut, lungs, vagina, and all surfaces exposed to the external world. With regards to quantitative aspects, it is emerging that we are made of ten times more microbial than mammalian cells. The adult gut microbiota contains up to 100 trillion micro-organisms, including at least 1,000 different species of known bacteria, with more than 3 million genes (150 times more than human genes). Microbiota can, in total, weigh up to 2 kg. One third of our gut microbiota is common to most people, while two thirds are specific to each one of us. In other words, the single individual gut microbiota is like an individual identity card (67). This largely enhances the genetic variation among individuals that is provided by the human genome (48,57,67). The use of new molecular biology techniques using the conserved 16S rRNA gene for phylogenetic analyses that can also detect unculturable bacteria has significantly advanced our understanding of the gut microbiome (the bacteria and their genome) (81).

2. Establishment and changes of the microbiota

While the 'healthy' gut microbiota is seen to be a stable community, there are stages within the life cycle of humans during which there can be alterations in the structure and function of this population. The infant gut microbiota undergoes dynamic changes during development, resulting in an adult-like microbiome at about 3 years of age (90). This process is influenced by genetic, epigenetic and environmental factors such as country of origin, delivery mode, antibiotics and breastfeeding (2). Indeed, the delivery mode at childbirth has an impact on early microbiota composition (22). Vaginally delivered children display a microbiota that shares characteristics with the vaginal microbiota, and includes *Lactobacillus, Prevotella, Atopobium, or Sneathia spp.* On the other hand, babies delivered by caesarean section have more skin-associated microbiota including *Staphylococcus spp.* (22). This suggests that the microbiota derives at least in part from the mother during the delivery. Hence, interpersonal variations are higher

among children than among adults. In adults, individuals coming from different geographic areas also display different microbiota (90).

3. The microbiota acts like an endocrine organ

The microbiota produces numerous compounds of a hormonal nature which are released into the blood stream and act at distal sites. Among the targets for these substances are many other organs including the brain. The microbiota releases its hormonal products into interstitial tissue, to be picked up by blood and lymph capillaries. These secretions are usually effective in low concentrations on target organs or tissues remote from the enteric milieu. Thus, considering the ability to influence the function of distal organs and systems, in many respects the gut microbiota resembles an endocrine organ. It is more biochemically heterogeneous than any other endocrine organ because it has the potential to produce hundreds of chemicals with hormonal properties. For example GABA, the most important inhibitory transmitter in the brain is produced by several strains of *lactobacilli* (7), while monoamines such as noradrenaline, dopamine and serotonin are also produced by many other strains of bacteria (17). The gut microbiome has been reported to regulate psychiatric health and influence etiopathology of autism. For example, Bravo et al. (10) reported that chronic administration of *Lactobacillus rhamnosus* induced anxiolytic and antidepressant effects by modulating the expression of GABA receptors in the brain, and Lyte et al. (50) observed that infection with *Citrobacter rodentiumin* induced anxiety-like behaviours through vagal sensory regulation.

Short chain fatty acids (SCFAs) are the major products of the bacterial fermentation of carbohydrates and proteins in the gut. Bacteria that produce SCFA include, but are not limited to, *Bacteroides, Bifidobacterium, Propionibacterium, Eubacterium, Lactobacillus, Clostridium, Roseburia* and *Prevotella* (52). SCFA are produced in high amounts when poorly digestible polysaccharides from plant origin are used as a carbohydrate source. Acetate, butyrate and propionate are then secreted into the gut lumen, transported across the epithelial barrier and transported to the effector organs. SCFAs actively

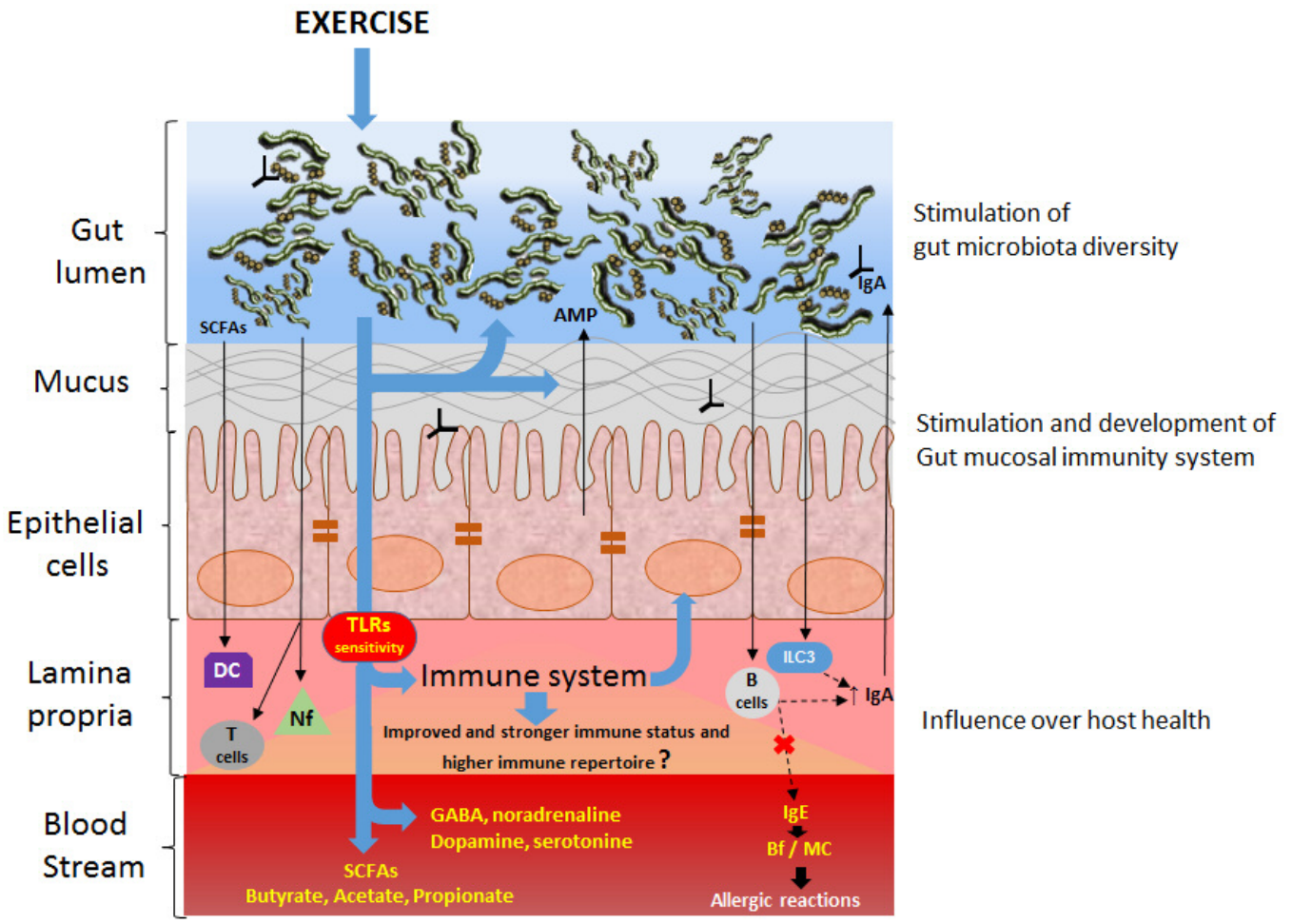

Figure 1: Exercise and the "cross-talk" between the gut microbiota and immune system.
Exercise may enhance sensitivity of toll like receptors (TLRs). Short chain fatty acids (SCAFs) then stimulate dendritic cells (DC) which are associated with inflammation protection. Microbiota also stimulates T cells and neutrophils (Nf), inducing a pathogen spreading control and B cells, where IgA production enhanced to produce IgE. IgE production and its release in serum, activates basophils (Bf) and mast cells (MC) leading to allergic reactions. Stimulation of ILC3 cells also enhances IL-22 production and IgA, and protects epithelial cells and antimicrobial peptide (AMP) synthesis and its release in gut lumen. The microbiota also produces hormonal-neurotransmitter agents (GABA, noradrenaline, dopamine and serotonin) and metabolic products (SCFAs: butyrate, acetate and propionate) which are related to neurotransmitters synthesis and food satiety control.

participate in the gut-brain axis, for instance by modulating entero-endocrine 5 hydroxy tryptophan secretion (25) and neuropeptide YY release (36). Butyrate and propionate, when in the blood stream, can be carried by monocarboxylate transporters which are abundantly expressed at the blood-brain-barrier and enter the central nervous system. They are a major energy source for neurons, but can potentially influence neurotransmitter synthesis through regulation of tyrosine hydroxylase gene expression (21).

4. The microbiota interacts with our immune system

The microbiota consists of symbiotic innocuous bacteria and potential pathogens also called pathobionts (16). The first role identified for the microbiota was the degradation of complex food macromolecules. However, there is growing evidence showing that the microbiota plays important roles in the maturation of the immune system and protection against some infectious agents (37,44,69,78). This is particularly true in the early phases of life when the microbiota 'teaches' our immune system how to deal with both innocuous and harmful bacteria, which in turn keep the microbiota under control. As far as the gut is concerned, this early programming is of utmost importance because it leads to the concept of a 'healthy' gut. When dysregulation (or dysbiosis) among these bacterial communities occurs, it can lead to inflammatory disorders, including inflammatory bowel disease, obesity, diabetes and autism (see below).

All the immune system components are directly or indirectly regulated by the microbiota. For instance, the microbiota and their metabolic by-products influence dendritic cells and macrophages either directly or through the intervention of epithelial cells. This cellular activity can be regulated by microbiota-driven epigenetic mechanisms. Similarly, T regulatory cells can be induced by metabolic products of the microbiota. The gut microbiota can induce B cell maturation as well as switching their immunoglobulin isotype. A preference for IgE rather than IgA can drive the activation of basophils and mast cells, which in turn results in a modified microbiota. The cross-talk between the gut flora and the immune system stimulates the development of the gut mucosal immune system, which is one of the mechanisms to prevent exogen pathogen intrusion (14). The pattern recognition receptors, among them, the toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain receptors are known mechanisms by which the innate immune system recognizes molecules with opposing characteristics. In turn, this leads to the recognition of, and distinction between, pathogens and non-harming elements (40). Considering that the expression of TLRs is modulated by the microbiota through the microbe-associated molecular pattern (MAMP), a complex molecular cascade is triggered, which includes the activation of the nuclear factor-kappa B pathway, followed by cytokine production and activation of T cells (40). The major components in the interaction of the microbiota with the host immune system are summarized in a review article (70) and presented in Figure 1.

SCFAs produced by the anaerobic bacterial fermentation at the gut level also act as signaling molecules. Indeed, propionate and acetate are ligands for two G protein coupled receptors, Gpr41 and Gpr43, which are broadly expressed in the distal small intestine, colon and adipocytes (11,89). SCFA linkage with Gpr43 decreases inflammatory responses, as it was shown that Gpr43 is largely expressed in neutrophils and eosinophils. This suggests that SCFA-Gpr43 signaling is one of the molecular pathways whereby commensal bacteria regulate immune and inflammatory responses (54). Moreover, SCFAs can modulate intracellular calcium levels in neutrophils suggesting another way of cell signaling (58-60). SCFAs present multiple effects in different cells involved in the inflammatory and immune responses. These fatty acids not only affect the production of inflammatory mediators, and ability of leukocytes to migrate. They can also induce apoptosis in lymphocytes, macrophages and neutrophils (82). In general, SCFAs, such as propionate and butyrate, inhibit stimuli-induced expression of adhesion molecules, chemokine production and consequently suppress monocyte/macrophage and neutrophil recruitment, suggesting an anti-inflammatory action of the microbiota byproducts.

5. Dysbiosis and immune-mediated diseases

The epidemic rise in allergic disease over the last decades has coincided with progressive Westernization (increased hygiene, smaller family sizes, dietary change and excessive antibiotic use). To explain this rise, Strachan (75) introduced the hygiene hypothesis suggesting that microbial exposures in childhood are critical for normal immune development. This hypothesis was later revised by the 'gut microbial deprivation hypothesis', which proposed that the observed changes in early intestinal colonisation patterns over the last decades in Western countries have resulted in failure to induce and maintain tolerance (12). There is emerging evidence that early gut microbiota establishment during critical periods of development has the potential to influence the risk of developing environmentally influenced disease, including allergic disease. Studies have reported that infants born by caesarean section are at greater risk for developing asthma and atopy (45,53,79), mainly because they show gut microbiota patterns with lower abundance of *Bacteroidetes* and lower diversity within the Bacteroidetes phylum (38,39,63,80). More generally, lower overall microbiota diversity and diversity within *Bacteroidetes* in early infancy have also been observed to precede development of allergic manifestations (1,9,22,86).

In recent years, considerable evidence has accumulated supporting the notion that the gut microbiota induces mucosal regulatory T cells which then play a vital role in maintaining gut homeostasis under normal conditions or in controlling inflammatory responses that would lead to disease. A breakdown in mucosal unresponsiveness to gut commensal organisms as well as a gut dysbiosis are now known to be associated with inflammatory bowel diseases such as Crohn's disease (CD) and ulcerative colitis (76). The gut microbiota may cause or aggravate CD by defective induction of regulatory T cells, or by infection of the mucosa and the induction of inflammatory cytokines. Indeed, studies of mice and humans with gastrointestinal inflammation have led to the identification of at least two kinds of bacteria that may cause or aggravate the inflammation of CD: *Faecalibacterium prausnitzii (Firmicutes),* and adherent–invasive *Escherichia coli.*

Assuming that obesity is at least partly an immune-mediated disease, it has been shown that the gut microbiota plays an important role in weight control, in addition to diet, lifestyle, genetics, and the environment. Obese mice and humans show higher proportion of *Firmicutes* and lower proportion of *Bacteroidetes* than their lean counterparts. One postulated explanation for this finding is that *Firmicutes* produce more complete metabolism of a given energy source than do *Bacteroidetes*, thus promoting more efficient absorption of calories and subsequent weight gain. Ridaura et al. (71) confirmed these findings in their pivotal study on colonization of the intestine of germ-free mice with the microbiome obtained from either obese or lean individuals. Other possible mechanisms by which the intestinal microbiome affects host obesity include induction of low-grade inflammation with lipopolysaccharide, regulation of host genes responsible for energy expenditure and storage, and hormonal communication between the intestinal microbiome and the host (42).

Together, these pieces of evidence indicate that the composition of the gut microbiota during early life, as well as a possible dysregulation, influence the way in which energy from dietary compounds is extracted, stored and expended in the host. This in turn influences the development of obesity and metabolic disorders. Diet has also been shown to rapidly alter the composition of gut microbiota independently of the obesity phenotype (26,35). In fact, it is suggested that the microbiota of lean and obese subjects responds in a different manner to alterations in caloric content of diet (41). In this sense, diet and an obese host-environment may also contribute to the modification of the gut microbiota consortia. However, the order in which these events occur is still unknown, and it is likely that both events progress in parallel. In a similar idea, up to now, it has not been established if exercise shifts the gut microbiota by promoting weight loss or if the weight loss promoted by exercise influences the regulation of the microbiota itself. Although weight loss has been shown to modulate the ratio of *Firmicutes* to *Bacteroidetes*, a role of exercise *per se* independently of a weight loss is still to be confirmed.

In this field, nutritional supplementation therapy (e.g. glutamine, prebiotics and probiotics) together with fecal microbiota transplantation have been used to manipulate and re-establish gut microbiota status (31,62,74). Glutamine is well known to be important in gut function and is avidly used by rapidly dividing cells such as enterocytes, colonocytes and gut lymphocytes. Moreover, glutamine alone or in combination with other gut-trophic nutrients improves the intestinal barrier function in children (49).

6. Exercise and the gut microbiota

As discussed above, the contribution of gut microbiota to the pathogenesis of obesity occurs through the alteration of host energy homeostasis. The ability of gut microbiota to process indigestible polysaccharides increases the viability of short chain fatty acids including butyrate, acetate and propionate (4). It has been demonstrated that butyrate is used as an energy source for colonic epithelial cells, whereas acetate and propionate are used by the liver for the lipogenesis process (73). Moreover, it is proposed that pro-inflammatory dietary compounds, such as saturated fat, together with genetic predisposition may shape the gut microbiota and increase caloric load. The inability to restore healthy gut microbiota status may lead to inflammation and bacterial metabolites leaking out to the mesenteric fat. This process is associated with the activation of pro-inflammatory gene expression, cytokine production, and macrophage infiltration. It has been proposed by Lam et al. (46) that the enhancement of adipose-derived cytokines and fatty acids promotes inflammation, steatosis and insulin resistance in the liver, which may lead to a metabolic systemic dysfunction. As exercise is known to exert a beneficial role in energy homeostasis and regulation, it might also modulate and help to restore the gut microbiota when altered by a high fat diet.

6.1. General effects of exercise on gut physiology.

There are several well-known effects of exercise on gut physiology. Exercise volume and intensity have been shown to exert an influence on gastrointestinal health status (64). For example, exercise reduces the transient stool time in the gastrointestinal tract, reducing the prolonged contact of pathogens with the gastrointestinal mucus layer and circulatory system. Moreover, moderate exercise is associated with reduced levels of cecum cancer, while exhaustive endurance exercise has been associated with a disturbance in the gastrointestinal tract due to toxicity effects induced by reduced local blood flow and bacterial translocation to blood stream (64).

6.2. The effects of voluntary exercise on the gut microbiota

To date, very few studies have investigated the role of exercise on the gut microbiota. However, exercise is a potential external agent with the capacity to change gut microbiota diversity in quantitative and qualitative ways. This was initially observed by Matsumoto et al. (55), who reported an alteration in the microbiota content and an increase of n-butyrate concentrations in rats submitted to voluntary running exercise. These authors also reported an increase in the cecum diameter in the trained rats. In addition, exercise alters the gut microbiota in mice on both a low and high fat diet, and normalizes major phylum-level changes for mice on the high fat diet. Furthermore, the total distance run by these animals inversely correlates with the *Bacteroidetes-Firmicutes* ratio (24). However, exercise, when associated with food restrictions (mimicking anorexia in a rat model), seems to have a potential negative impact on the quantity of health-promoting bacteria. In addition, it can enhance the growth of bacteria which may be related to the disruption of the gut mucosal barrier and the optimal exploitation of the very low caloric diet (68). These authors also reported that serum leptin was positively correlated with the quantity of *Bifidobacterium* and *Lactobacillus*, and negatively correlated with the quantity of Clostridium, *Bacteroides* and *Prevotella*. Conversely, serum ghrelin levels were negatively correlated with the quantity of *Bifidobacterium, Lactobacillus* and *B. coccoides–Eubacterium rectale* group, and were positively correlated with the number of *Bacteroides* and *Prevotella.* These findings highlight the associations between gut microbiota and appetite-regulating hormones. Moreover, voluntary exercise also appeared to attenuate the microbiome changes induced by polychlorinated biophenyls (PCB) (15). In this study, mice exposed to two days of PCB mixture, presented an alteration

in the abundance of 1,223 bacterial taxa, with an overall abundance reduction (2.2%) whereas the biodiversity of the gut microbiota was not altered. Interestingly, predicted analysis for micro arrays identified seven phila *(Firmicutes)* with significantly higher abundance when comparing exercising to sedentary mice.

6.3. Controlled training and gut microbiota modification in animals

Again, very few studies have investigated the alteration of gut microbiota following controlled exercise. As observed in voluntary exercise regimens, recent studies have shown that controlled training also exerted some beneficial effect on the gut microbiome of obese and hypertensive rats (65) and in obese mice with a phenotype induced by high fat diet (HFD) (43). Moderate treadmill training (around the maximal lactate steady state, 12.5 $m.min^{-1}$ for obese $Zucker^{fa/fa}$ rats and 20 $m.min^{-1}$ for non-obese and hypertensive rats (SHR), 30 min/day, 5 days/weeks, during 4 weeks) altered the composition and the diversity of the gut bacterial at genus level in non-obese, obese animals, and SHR. Exercise promoted *Allobaculum* in SHR and *Pseudomonas* and *Lactobacillus* genus in the obese rats. Moreover, the abundance of operation taxonomic units from two bacteria families (*Clostridiaceae* and *Bacteroidaceae*) and genera (*Oscillospira* and *Ruminocossus*) was significantly correlated with blood lactate concentration. These findings indicate that training status may be linked to these bacterial proliferations (65). The effect of 16 weeks of training (running wheels, 1 h, 7 m/min, 5 days/week) on rats submitted to HFD was also used to study anxiety and cognitive dysfunction which are associated with the development of obesity (65). HFD and exercise alone caused massive but opposite changes in the gut microbiome. However, exercise failed to reverse the changes induced by the HFD at the microbiome level.

6.4 Effect of training on the human gut microbiota

A study involving elite rugby players also reported that exercise increases gut microbiota richness and diversity (18). Moreover, this pioneering work in humans showed that the indices of the gut microbiota diversity positively correlated with protein intake and creatine kinase concentration, suggesting that diet and exercise are drivers of biodiversity in the gut. This work highlighted that exercise is another important factor in the complex relationship among the host, host immunity and the microbiota in elite athletes.

6.5 Gut Permeability and Ischemia –Reperfusion

One of the essential functions of the intestine is to maintain a barrier which prevents the entry of potentially harmful microorganisms to adjacent and distant sterile organs. This mechanical barrier can be disrupted through splanchnic hypoxia and subsequent reperfusion. This often results in bacterial translocation, with most of the translocating bacteria originating from the colon. Strenuous and prolonged exercise such as endurance competitions and training are associated with various levels of splanchnic hypoperfusion and ischemia and subsequent reperfusion (64). In a murine model, Gutekunst et al. (32) recently reported increased apoptosis and altered permeability following exhaustive and acute endurance exercise. Although relevant to the topic of this article, this pathophysiological phenomenon and its potential consequences on the gut microbiota have not been addressed in exercising humans. Using an ischemia-reperfusion model in rats, Wang et al. (85) reported that the damage-repair of the epithelium preceded dysbiosis and subsequent tendency to recovery of the colonic microbiota. While the epithelial barrier started repairing after 3 hours and gained full recovery at 24 hours of reperfusion, a normal microbiome was not fully recovered after 72 hours of reperfusion. Colonic flora started to change as early as 1 hour into reperfusion. At 6 hours, *Escherichia coli* (a pro-inflammatory strain of bacteria with high translocation potential) reached a construction peak. Speeding-up the gut microbiota recovery process by consuming a probiotic containing *Lactobacilli* strains prior to a lasting endurance event is a hypothesis which deserves further investigation.

6.6. The Hypothalamic-Pituitary-Adrenal (HPA) axis and the Microbiota

Cross-talk between the gut microbiota and the HPA axis has recently been described. It is now clearly established that the gut microbiota is involved in the development of the HPA axis in rodents (20,77). Animals raised in the absence of microorganisms show exaggerated release of corticosterone and ACTH after mild stress exposure, when compared with specific pathogen free controls. These results demonstrate that the early life microbial colonization of the gut is critical to the development of an appropriate stress response later in life. It has also been proven that stress and the HPA can influence the composition of the gut microbiome. The functional consequences of such changes are probably relevant to the field of exercise immunology. In animal experiments, maternal separation, an early life stressor, or exposure to social stressors (6,61) result in long-term HPA changes, and also has long term effects on the microbiome (5). These stressed animals showed decreased relative abundance of the *Bacteroidetes* genus and increased relative abundance of the *Clostridium* genus in their cecum, as well as increased circulating levels of IL-6 and MCP-1 (6). In a recent randomized controlled study, healthy humans supplemented with *Lactobacillus helveticus* R0052 and *Bacteroidetes longum* R0175 for 30 days showed reduced urinary free cortisol output (56).

An elevation in the plasma concentration of noradrenaline, associated with physical exercise or mental stress, stimulates the growth of non-pathogenic commensal *E.Coli* (27), as well as other gram-negative bacteria (50). These preliminary findings in humans attest to hormonally-driven changes in the composition and distribution of the intestinal microbiota, which in turn might modify host behavior. This topic is of particular interest in the field of exercise immunology, and deserves future studies in exercising humans or elite athletes.

7. Prebiotics and Probiotics in Exercise Immunology

Probiotics may improve health, either by the immunomodulation of local immunity by maintaining gut wall integrity or by acting on systemic immunity; enhancing non-specific and specific arms of the immune system (33). As far as the innate immune function is concerned, probiotics have been shown to enhance phagocytic capacity of peripheral blood polymor-

phonuclear cells and monocytes as well as NK cells cytotoxic activity. Acquired immunity also seems to be improved following supplementation with probiotics, with significantly higher specific IgG, IgA and IgM immunoglobulins. Local immunity is modified with an enhanced gut barrier function and an improved local immune response. One of the main clinical outcomes from these *in vitro* results is a reduced rate of upper respiratory tract illness (URTI) in children and adults when given specific strains of probiotics (33). Although there is a scarcity of supplementation studies with athletes, it seems that this particular population may also benefit from a regular probiotics use (66). This is of particular interest since athletes engaging in prolonged intense exercise may be more susceptible to URTI (84). This benefit is believed to be strain specific: the most common strains used to promote immune function are lactic acid bacteria; *Lactobacillus* and *Bifidobacterium* species. Cox et al. (19) reported that oral administration of *Lactobacillus fermentum* was associated with a substantial reduction in the number of days and severity of URTI in twenty highly trained distance runners. These beneficial effects occurred without any significant changes in salivary IgA or interleukin-4 or -12 levels. The same type of results, after using probiotics, were reported for lower respiratory illness use of cold and flu medication, and severity of gastrointestinal symptoms at higher training loads, in male (but not female) competitive cyclists (88). The authors observed a reduction in exercise-induced immune perturbations, interestingly in both anti-and pro-inflammatory cytokines, which could have mediated these effects. Two other studies performed on physically active subjects and elite rugby union players confirmed a positive effect of a probiotic supplementation on the incidence, but not severity or duration of URTI (28,34). The study using physically active subjects also observed a positive effect on salivary IgA.

Prebiotics are non-digestible polysaccharides and other substances that selectively stimulate the growth or activities of one or more species of bacteria in the gut microbiota: this confers a health benefit on the host (72). For instance, high amylose maize starch supplementation showed some beneficial effects on markers of bowel health in healthy physically active adults (87). In general, prebiotics favor the growth of *Bifidobacteria* and *Lactobacilli* over potentially harmful proteolytic and putrefactive bacteria. Prebiotics have been classified mainly into two groups, the inulin type fructans (ITF) and the galacto oligosaccharides (GOS), based on their chemical structures. So far, no studies focusing on the purported beneficial effects of prebiotics on athletic performance have been conducted. However, a study examining the effects of various GOS supplementation protocols in a large cohort of 427 students showed that supplementation with GOS was associated with lower GI illness symptom scores. Moreover, supplementation with 2.5 g of GOS was associated with reduced cold and flu severity scores (38). Another study of GOS supplementation providing a dose of 5.5 g/day, showed a significant reduction of in the incidence and duration of diarrhoea in healthy volunteers undertaking international travel (23).

Regular consumption of 16 g per day of FOS has been found to influence host metabolism favorably, increasing plasma gut peptide concentration and reducing appetite (13). Reduced levels of C-reactive protein have also been reported with FOS supplementation (82). Whether these findings could be relevant and useful to elite athletes as prophylaxis and recovery means should be further investigated.

8. Perspectives in Exercise Immunology

There are interesting questions to be discussed, such as the effect of exercise on the gut, its microbiota and the brain-gut interactions. For example, does exercise enhance the sensitivity of TLRs and the recognition of the MAMPs, leading to a stronger innate immune system? If so, does this occur through modification of the communication between the immune system and the flora? It is known that TLRs have been associated with a sedentary lifestyle and inflammation status, and that exercise reduces the expression of these receptors in the monocyte cell-surface, contributing to a post-exercise immunodepression status (29). However, the link between gut microbiota, mucosal immunity and exercise stimulation has not yet been explored, leading to several possibilities in the exercise-immunology research field.

Advances in sequencing technologies have made it possible to identify the presence of bacterial strains in the airways. The most prevalent phyla identified in the airways are *Proteobacteria, Firmicutes,* and *Bacteroidetes*. Although there is no direct evidence that the airway microbiota has, like the gut, a function in developing and maintaining the steady-state immune phenotype of the lung, several recent studies showed an association between the airway microbiota and a variety of chronic lung diseases such as asthma, chronic obstructive pulmonary diseases, and cystic fibrosis (30). It is, however, difficult to understand whether the observed differences between the compositions of the airway microbiota between healthy and diseased subjects is driven by changes at the gut level. It is also difficult to estimate the causative roles of treatments such as glucocorticosteroïds or antibiotics. As some athletes are prone to respiratory illnesses from inflammatory or viral origins (8), characterization and comparison of the airway microbiota in this population would certainly be of clinical interest.

10. Conclusion

Experimental evidence has shown that alterations in the bacterial community are associated with health impairments, while increased microbiota diversity improves metabolic profile and immunological responses, and may provide a possible biomarker for health improvement. Therefore, it is of vital importance to have a better understanding of the effects of exercise on the interaction of the microbiota and innate immune system, as well as further outcomes in relation to host health. In obesity and diabetes, the immunological system plays a key role in the development of the pathological conditions influenced by microbiota alterations. Although exercise may induce positive restorative effects on the microbiota, it is definitely too soon to define exercise as a therapeutic element for the treatment of diseases associated with a disturbance of the gut microbiota. Moreover, few groups have embraced this particular field in terms of linking exercise physiology and the possible outcomes of disturbed gut microbiota treatment. In contrast to other new treatments, such as microbiota trans-

plantation or nutritional supplements, exercise is still an effective and non-pharmacological treatment for a number of pathologies. Exercise may hopefully contribute to positive manipulations within gut microbiota and its close relationship with the immunological system. It is hypothesized that the key to this process is linked to the effects of exercise on the cross talk between the immune system and the microbiota. These effects remain largely unknown and should be a research focus in the near future.

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Understanding graft-versus-host disease. Preliminary findings regarding the effects of exercise in affected patients

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ABSTRACT

Advances in this century regarding allogeneic hematopoietic stem cell transplantation (allo-HSCT) have led to an expanding population of long-term survivors, many of whom suffer severe side effects, particularly those related to graft-versus-host disease (GVHD), a potentially multi-systemic disorder caused by immunoeffector donor lymphocytes that destroy host tissues. The GVHD, especially in its chronic form (cGVHD), generates considerable morbidity and compromises the physical capacity of patients. We have reviewed the main pathophysiological aspects of the disease as well as the data available on the effects of exercise in GVHD, based on animal and human patient research. Although exercise training as an adjunct therapy to improve health outcomes after allo-HSCT shows promise (particularly, this lifestyle intervention can improve physical fitness and possibly immune function while attenuating fatigue), there is a need for more randomized control trials that focus specifically on GVHD.

INTRODUCTION

Allogeneic hematopoietic stem cell transplant (allo-HSCT) is the only curative option for many patients with leukemia, primary or acquired marrow failure, primary immunodeficiency or inborn genetic diseases (378). Graft-versus-host disease (GVHD) is a frequent complication of allo-HSCT (288), and consists of the destruction of host tissues by donor effector lymphocytes. The incidence of the acute form of GVHD (aGVHD) has been estimated at 10%-80%, with symptoms usually developing 2-3 weeks post allo-HSCT, and 30-70% for chronic GVHD (cGVHD) in allo-HSCT recipients surviving beyond 100 days, with a median onset of 4-6 months following transplant (127) (see below for a definition of aGVHD versus cGVHD). Reasons for the wide variability in the incidence of both of these diseases might include individual differences in a variety of modifiable and nonmodifiable risk factors. These include type of conditioning regimen and impact of regimen intensity, graft source, degree of human leukocyte antigen (HLA) mismatch, previous donor alloimmunization, use of total body irradiation, GVHD prophylaxis, severity of individual organ sites, female donor–male recipient, parity of female donors, or recipient age (150, 169, 176, 244, 258, 270, 393). Mortality rates of 15-40% have been reported for patients with aGVHD and 30-50% for those with cGVHD (37). Reasons for the usually higher mortality rates found in cGVHD compared with aGVHD likely include a lower magnitude of medical advances in treatment, and the more aggressive, multi-systemic nature of the chronic disease form (151, 225). In addition, GVHD causes severe morbidity, and allo-HSCT survivors with GVHD show impaired physical and social behavior, and undergo a worse physical and psychosocial recovery than survivors without this complication. Quality of life (QoL) is thus severely compromised (119, 196, 218, 367-369).

The first-line option for the treatment of GVHD, steroid therapy, has a failure rate of 30-40% (90). In effect, GVHD refractory from steroids is an unresolved clinical challenge with a high impact on both the survival and QoL of patients (3). It is therefore imperative that researchers pursue other effective therapies for the prevention and treatment of GVHD.

The multi-system benefits of regular exercise have been linked to a lower risk of numerous chronic diseases (see (114) for an in-depth review). There is indeed strong epidemiological evidence that this simple lifestyle intervention leads to lower rates of all-cause mortality, cardiovascular disease, hypertension, stroke, metabolic syndrome, type 2 diabetes, breast cancer, colon cancer, depression, and falls (216). However, the impact exercise may have to prevent GVHD, or to influence the course of the disease in affected patients, is largely unknown. This paper reviews the main features of this life-threatening disease and discusses the rationale and preliminary findings supporting the effects of exercise training in GVHD. To our knowledge, no data are available on the possible association between previous exercise habits, and the risk or severity of GVHD.

Allo-HSCT and GVHD

Allo-HSCT was first introduced to treat patients with end-stage leukemia (381) or aplastic anemia after conventional

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treatment failure, as well as to offset the toxic effects of irradiation and chemotherapy against both of these diseases (377). The process consists of the intravenous transfer of hematopoietic stem cells from a healthy donor to an immunosuppressed recipient, to regenerate normal hematopoiesis in patients in whom it is impaired or non-existent (379). The immunosuppression caused by the transplant conditioning regimen enables the grafting of donor cells, while donor T lymphocytes provide anti-tumor therapy against the host's residual malignant cells (graft-versus-tumor (or leukemia) (GVT) effect) (212). However, several complications may arise during the process. The grafted stem cells may be rejected by the recipient (host-versus-graft (HVG) effect) or, conversely, the donor immune system may act against the recipient (graft-versus-host (GVH) effect). The latter is clinically known as GVHD. Such effects were discovered in studies conducted in the mid-20th century, in which an anti-tumor cell effect of the transplanted graft (GVT effect) was observed after allo-HSCT (23, 24). However, in these studies, transplanted mice later died from a second degenerative, or wasting, disease, which caused diarrhea and weight loss, skin inflammation and liver failure/lesions. This was the first clinical description of aGVHD (25).

In parallel, bone marrow transplants were conducted in patients with malignant tumors with the objective of inducing GVT activity without developing GVHD. This strategy was, however, unsuccessful due to failure of the transplanted hematopoietic stem cells (381) and was soon followed by the technique of total body irradiation plus allogeneic bone marrow cell transplant. This new approach led to the first cure of leukemia by the group of the Nobel prize winner E. Donnall Thomas (380). Early experience was followed by further anecdotal cases, but it was not until the mid 1970s that the first epidemiological data of long-term survival were reported for patients with acute leukemia subjected to allo-HSCT (377). Since then, allo-HSCT has been widely adopted worldwide (81) following developments made in tools designed to assess donor-recipient synergistic and competitive interactions, the selection of donors according to similarities in the human leukocyte antigen (HLA), anti-microbial therapies, cell transplant conditioning regimens and patient care (26, 137, 148). Allo-HSCT is also currently used as potentially curative treatment for many different diseases (91, 107, 140, 148, 229, 339, 374). Nevertheless, today, almost 50 years after initial studies, the challenge continues to be to maintain the GVT effect while also facilitating the grafting of donor stem cells, thus avoiding graft rejection and the complications of treatment, among which GVHD is the most frequent and life-threatening (199).

Definition and classification of GVHD

GVHD is the outcome of donor immune system cells attacking the recipient's organs (347). Donor T lymphocytes play a major role in the pathophysiology of GVHD (109). After their implant, donor T cells undergo activation upon alloantigen presentation by antigen presenting cells (APCs) and then clonally expand. Donor T cells induce damage to target organs either directly through cytolytic attack, or indirectly through the release of inflammatory mediators. As early as 1966, Billingham identified the necessary conditions for the onset of GVHD (35): (i) the graft should contain immunocompetent cells; (ii) the host should express tissue antigens not present in the donor; and (iii) the host should be incapable of organizing an effective destruction or inactivation response against the transplanted cells. A fourth postulate was added later (324): donor lymphocytes need to reach their target organs in the host.

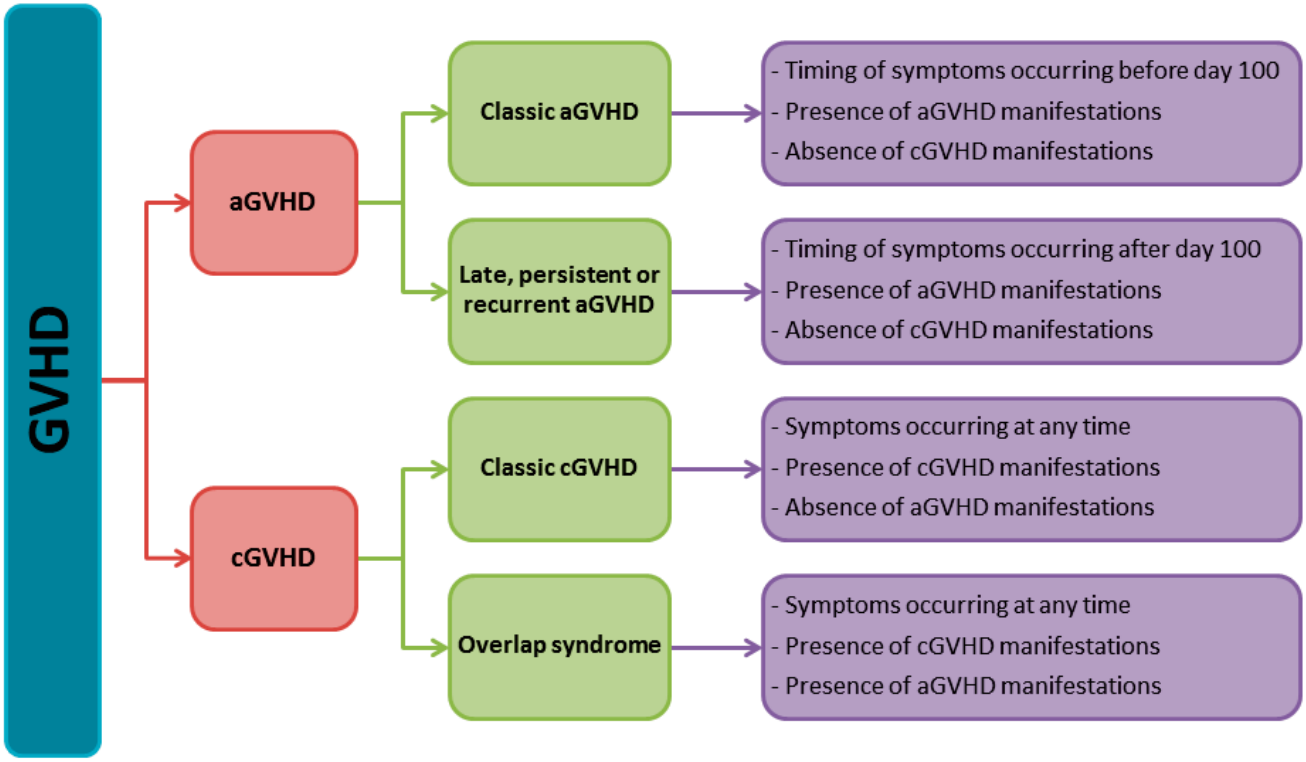

Figure 1. Clinical classification/differentiation of acute (aGVHD) and chronic graft-versus-host disease (cGVHD) according the US National Institutes of Health (NIH) (110).

In 1974, Glucksberg and co-workers classified GVHD as acute or chronic depending on its appearance before or after day 100 after transplant, respectively. However, signs of aGVHD may persist beyond 100 days post allo-HSCT and those of cGVHD may commence before the 100-day time point (397). Since then, there have been many attempts to classify this disease and, today, the accepted system is that created in 2005 by the US National Institutes of Health (NIH), based on the different clinical manifestations of GVHD (63, 110, 248, 335, 349) (see also **Figure 1**):

1) aGVHD (lack of findings of cGVHD)
   a) classic aGVHD, diagnosed before day 100 following allo-HSCT or donor lymphocyte infusion showing characteristics of aGVHD;
   b) persistent, recurrent or late-acute GVHD, showing characteristic features aGVHD diagnosed after day 100 following allo-HSCT or donor lymphocyte infusion, often after suspending immunosuppressive treatment, lacking characteristics of cGVHD;
2) cGVHD
   a) classic cGVHD, showing symptoms of cGVHD yet lacking symptoms of aGVHD;
   b) overlap GVHD syndrome, showing characteristics of both aGVHD and cGVHD.

Pathophysiology of aGVHD

The physiopathology of aGVHD is summarized in **Figure 2**. Classically, aGVHD occurs in three phases (156):

Phase I. **Transplant conditioning regimen effects and APCs activation**

Host tissues may be damaged by the underlying disease and/or infection before HSCT. In addition, the transplant conditioning regimen induces damaged cells to secrete pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6] (108, 156), endogenous non-infectious molecules known as 'damage-associated molecular patterns' (DAMPs) [e.g., adenosine triphosphate (ATP), heat shock proteins or mitochondria, extracellular matrix proteins such as biglycan] (237, 416, 435, 437) and chemokines (77, 413). These molecules serve as "danger signals" and are responsible for the activation of APCs, especially dendritic cells, via toll-like receptors (TLRs) and non-TLRs, enhancing GVHD (61, 156, 435). In the gastrointestinal tract, inflammatory stimuli are translocated to the bloodstream. These signals include microbial products (lipopolysaccharides and unmethylated cytosine-phosphate-guanine) or other "pathogen-associated molecular patterns" (PAMPs), furthering the cytokine cascade (61, 155, 156). Most innate immune cells express pattern recognition receptors (PRRs) and recognize PAMPs through PRRs, such as TLRs and nucleotide-binding oligomerization domain-like receptors. The binding of PAMPs by PRRs on APCs activates the innate immune response, which induces the upregulation of cytokines and MHC class II costimulatory molecules, and promotes dendritic cell migration to the T-cell compartment of lymph nodes so that antigens are presented to other immune cells (77, 81, 92, 155, 156, 237, 430).

Phase II. **T lymphocyte activation**

Donor T cells are recognized and activated by APCs in secondary lymph nodes (11, 81, 267) and then migrate to their target organs where they can cause tissue damage (73, 241).

II.1. Antigen presentation and T cell activation

In the setting of an HLA-identical donor allo-HSCT, the host's APCs activate donor T lymphocytes through the presentation of minor histocompatibility antigens (miHAs) by HLA proteins to T cell receptors (14, 20, 38, 108, 139, 211, 226, 342, 348). This is the first activation signal, but costimulatory molecules (second signal) are needed for a full immune response. In an HLA-non-identical allo-HSCT, aGVHD may be induced both by $CD4^+$ and $CD8^+$ (class II and I major histocompatibility antigen (MHC) coreceptors, respectively) due to the miHAs disparity (108, 424). In mouse models in which donor/recipient genetic differences are controlled for, if the disparity between T lymphocytes and APCs affects class I antigens, cytotoxic/suppressor $CD8^+$ lymphocytes are activated. By contrast, if this disparity affects class II antigens, the cells activated are cooperator $CD4^+$ T cells (202). When T cells are exposed to antigens in the presence of adjuvants such as lipopolysaccharide, their migration and survival are enhanced (104).

II.2. T lymphocyte proliferation and differentiation

T lymphocyte activation leads to their differentiation into various T cell phenotypes such as effector, memory, regulatory or helper (Th1, Th2, Th17), among other subsets (78, 409). Differentiation into T helper cells is determined by the cytokines present in the environment during the activation process (third signal): (i) interferon (IFN)-γ (228) and IL-12 (165) promote the development of Th1 cells, which express IFN-γ, lymphotoxin, IL-2 and TNF-α (266); (ii) IL-4 and IL-2 promote the development of Th2 cells (164, 215, 338, 366), which express IL-4, IL-5, IL-9, IL-10, IL-13 and TNF-α (266); and (iii) transforming growth factor (TGF)-β and IL-6 promote the Th17 cell phenotype (34, 201, 240, 396) expressing IL-17A, IL-17F, IL-22 (149, 286, 396) or IL-21 (278, 438). Th1 cytokines (IFN-γ, IL-2, TNF-α) have been correlated with aGVHD (103, 106, 311). The balance between Th1/Th2 subsets as well as other subsets such as Th17 and the production of cytokines affects the manifestations of GVHD (432). Although there is some controversy as how Th1/Th2 balance might affect GVHD and various contributions of each of these elements are still under investigation, some explanations have been postulated, as briefly summarized below. aGVHD has been proposed to be mediated by Th1 cells (102), whereas Th2 cells have been reported to suppress aGVHD (208). Yet Th2-biased donor cells deficient in signal transducer and activator of transcription 4 gene ($STAT4^{-/-}$) can induce lethal GVHD (276). On the other hand, although the absence of Th17 cells can exacerbate aGVHD (433), Th17 cells have been shown to augment GVHD in some circumstances (76, 185), with in vitro-generated Th17 cells mediating lung and skin GVHD (57). Yi et al., (2009) proposed that Th1 cells can down-regulate Th2 and Th17 cells or vice versa (432). Thus, in the absence of IL-17 or IL-4, Th1 differentiation is augmented, and tissue damage in the gut and liver is preferentially exacerbated. In contrast, in the absence of IFN-γ, both Th2 and Th17 differentiation is augmented, and tissue damage in

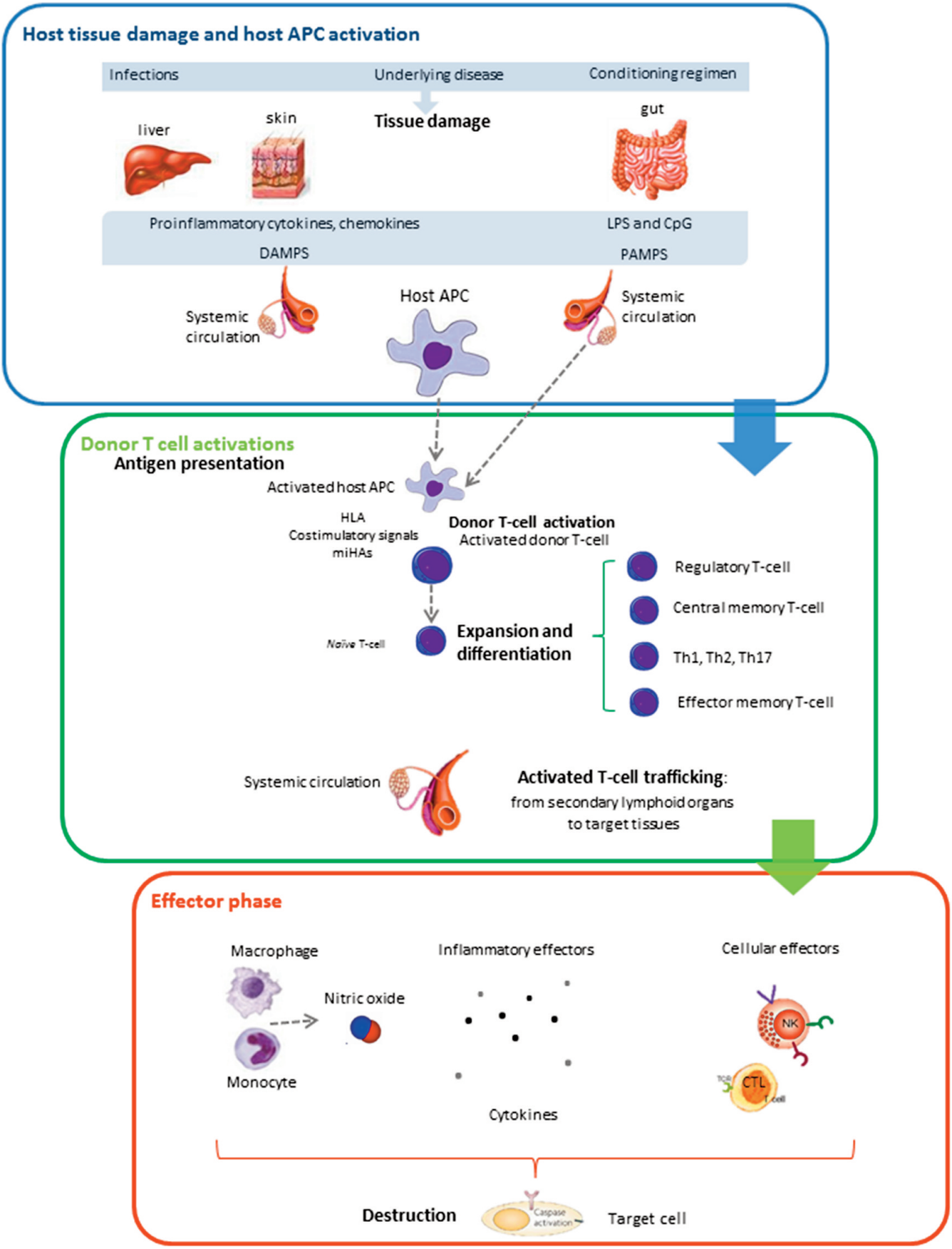

Figure 2. Pathophysiology of acute graft-versus-host disease (aGVHD).
The mechanisms implicated in the pathophysiology aGVHD are summarized below. Phase I: infections, the disease itself and the conditioning regimen damage host tissues (mostly liver, skin and intestinal mucosa). Phase II: activation of donor T cells against host antigens and subsequent clonal T-cell expansion. Phase III: release of inflammatory cytokines leading to further host tissue damage (104).
Symbol: ----→, danger signals. Abbreviations: APC, antigen-presenting cells; CpG, unmethylated cytosine-phosphate-guanine; CTL, cytotoxic T lymphocytes; DAMPS, damage-associated molecular patterns; HLA, human leukocyte antigen; LPS, lipopolysaccharide; NK, natural killer; PAMPS, pathogen-associated molecular patterns; Th1, T cell helper 1; Th2, T cell helper 2; Th17, T cell helper 17; NK, natural killer.

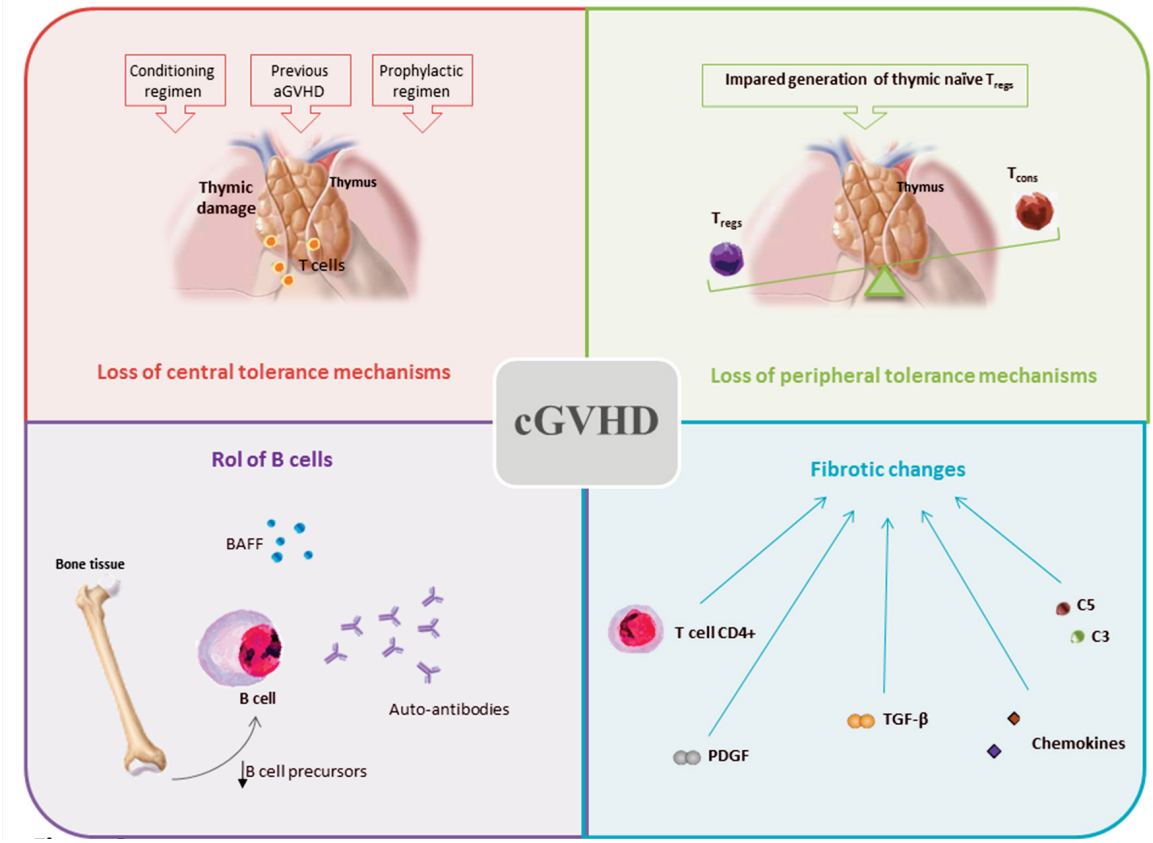

Figure 3. Pathophysiology of chronic graft-versus-host disease (cGVHD).
The mechanisms implicated in the pathophysiology cGVHD are summarized below. First, thymic damage alters the central tolerance mechanisms during immune reconstitution, producing cGVHD. Further, the thymus production of naïve regulatory T cells (T_{regs}) is impaired, also causing cGVHD as well as loss of peripheral tolerance. As for the implication of B cells, patients with cGVHD have high levels of B cell activating factor (BAFF), a high prevalence of auto-reactive antibodies, poor recovery of B cell levels, and prolonged immunodeficiency. Finally, fibrotic changes also occur due to several factors such as complement factor 3 and 5, chemokines, CD4+ T lymphocytes, transforming growth factor (TGF)-β1 and deregulation of platelet-derived growth factor (PDGF).
Abbreviations: BAFF, B-cell activating factor; C3, complement component 3; C5, complement component 5; T_{cons}, conventional T cells: TGF-β, transforming growth factor beta; Tregs, regulatory T cells; PDGF, platelet-derived growth factor.

lung and skin is exacerbated (432). Absence of both IFN-γ and IL-17 leads to further augmentation of Th2 differentiation and exacerbated lung damage (idiopathic pneumonia) (432). Lack of both Th1 and Th2 cells further augments Th17 differentiation and exacerbates skin damage. Therefore, the balance among Th1, Th2 and Th17 effector subsets plays an important role in regulating T-cell immune response and, neutralizing either Th1, Th2 or Th17 cytokines may lead to biased Th1, Th2 or Th17 differentiation and thus can cause organ-specific tissue damage (432).

II.3. T cell trafficking
Activated T cells migrate to secondary lymph organs and target tissues through a combination of chemokine-receptor, selectin-ligand and integrin-ligand interactions (73, 96, 267, 268, 285, 324, 394, 405, 414, 426, 427). For example, chemokine ligand 2 (CXCL2)-5, CCL9, CCL11, CCL17 and CCL27 are overexpressed in the liver, spleen, skin and lungs during aGVHD (427, 428). T cells with chemokine receptors (CCR)3 and CCR5 cause aGVHD in the gut and liver (95, 268, 426).

Phase III. Cellular and inflammatory effects
GVHD culminates with cytotoxic effects mediated by different processes:

III.1. Cellular effectors
The main cellular effectors of aGVHD are cytotoxic T lymphocytes and natural killer (NK) cells (182, 391). The effector lysis mechanisms employed by cytotoxic T lymphocytes are the pathways FAS/FASL, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and perforin/granzyme B (42, 45, 46, 182, 329, 331, 391, 413, 429, 439), though other pathways may exist (81).
III.2. Inflammatory effectors
Cell damage is aggravated by inflammatory mediators including IFN-γ, TNF-α (45, 156, 300) and IL-1 (2). Microbial products filtered by the gut mucosa trigger the production of cytokines (156, 171).
III.3. Other factors
Nitric oxide produced by monocytes/macrophages impairs the cell proliferation needed for damaged epithelial tissues to heal (206, 273).

Pathophysiology of cGVHD

The pathophysiology of cGVHD is summarized in **Figure 3**. Chronic GVHD was first described as a disease mediated by Th2 lymphocytes (307, 372), although this idea is not fully supported because it may be caused by Th1 cytokines (22, 111, 279, 314, 438). While several studies have revealed the

important role played by the Th1/Th2 balance (44, 167, 272, 276, 306, 340, 413), others suggest different cytokine profiles depending on the disease stage (320). Th17 cells (84, 230, 277) and auto-antibodies (436) can also cause cGVHD, though the spectrum of Th17 cells varies according to whether the cells are classic or alternative Th17 cells (4, 86, 130, 223, 297). To some extent, the pathophysiology of cGVHD resembles that of an autoimmune disease in which auto- and alloreactive T and B lymphocytes intervene (107). However, it differs from the pathophysiology of aGVHD (384) and several theories for its development exist.

Central tolerance failure. The thymus damage caused by the conditioning regimen, aGVHD or the prophylaxis regimen lead to dysregulation of the patient's central tolerance mechanisms during immune reconstitution following cell transplant, giving rise to cGVHD (356). During the early recovery stage, mature T cells obtained from the donor and expanded in a thymus-independent manner are responsible for the development of the disease. During late recovery, T cells generated *de novo* from the donor's hematopoietic stem cells through the host's thymus gland will condition the pathophysiology of cGVHD (160, 203). Although the T cells produced in this way should not attack tissues expressing autoantigens, impaired immunological tolerance to these autoantigens leads to the autoimmune characteristics of cGVHD (325). Although the $CD4^+$ T cells generated *de novo* from the donor stem cells seem to

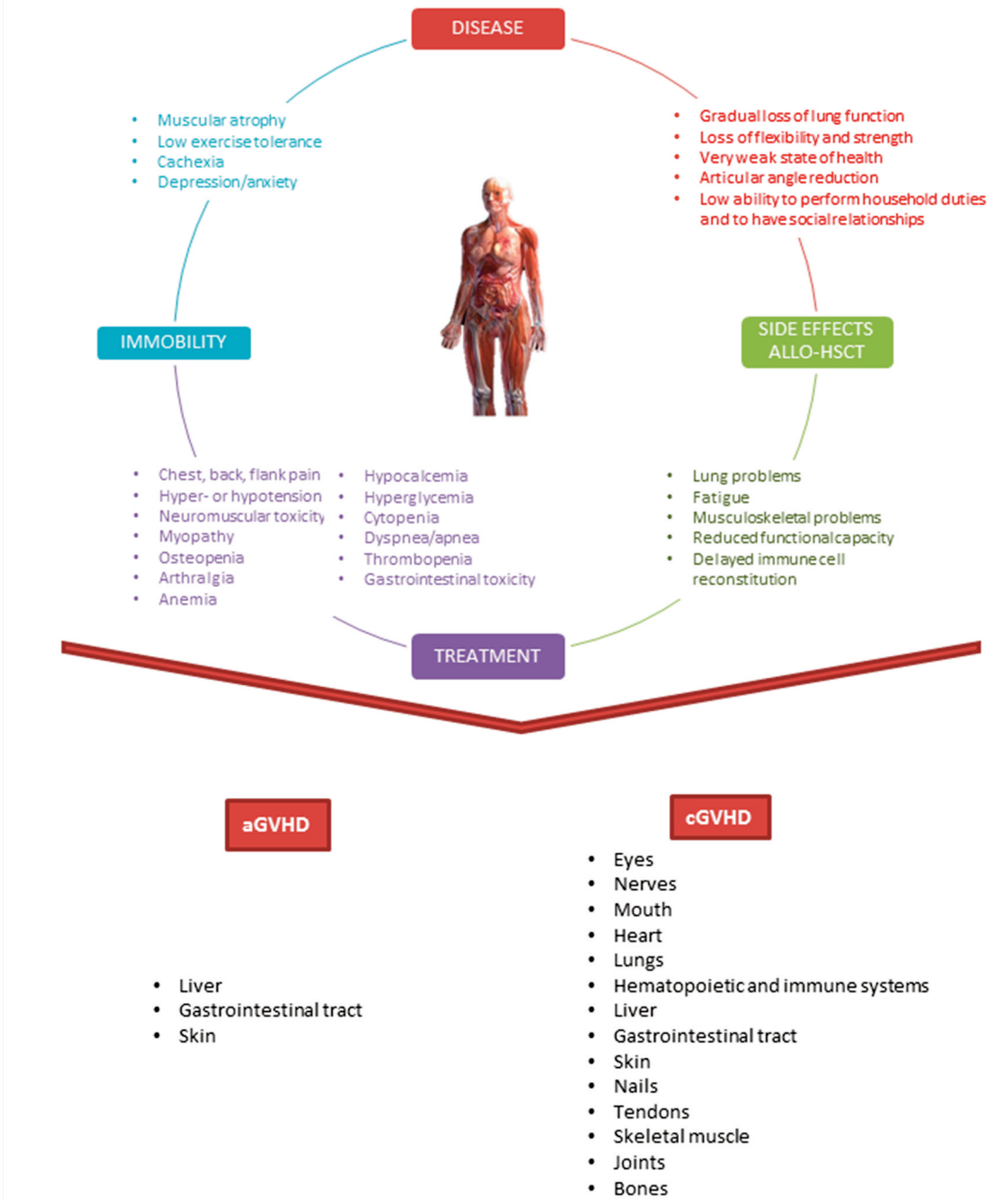

Figure 4. Main clinical features of acute (aGVHD) and chronic graft-versus-host disease (cGVHD). Abbreviation: allo-HSCT, allogeneic hematopoietic stem cell transplant. Patients with GVHD are affected at the multi-system level, which leads to a debilitated physical condition with a subsequent decrease in the ability to cope with activities of daily living. Patients' health status and physical condition further deteriorate in the mid and long-term by the pharmacological treatment they receive, which induces muscle toxicity (i.e., due to high doses of immunosuppressant drugs). The muscle tissue also deteriorates as a result of bed rest, resulting in muscle atrophy and eventually in cachexia. All together, these phenomena severely impair the patients' well-being and quality of life.

mediate conversion from aGVHD to cGVHD (97), the latter is also produced without being preceded by aGVHD. However, the host's thymus is not needed to induce cGVHD, because autoreactive quiescent T and B cells in transplants from non-autoimmune donors may also be activated and expanded to cause cGVHD (436).

Regulatory T cells (T_{regs}) and cGVHD. T_{regs} are a T-cell subset marked by a $CD4^+$ $CD25^{hi}$ $Foxp3^+$ phenotype. Its deterioration has been associated with peripheral tolerance loss, autoimmunity and with cGVHD (48, 76, 436, 440). During the lymphopenia period, thymus production of naïve T_{regs} is impaired and the T_{regs} generated show a memory phenotype (374). Initially T_{regs} undergo greater proliferation than conventional T cells (T_{cons}), but this expansion is offset by their greater susceptibility to apoptosis (252). This determines that in patients who show chronic $CD4^+$ cell reduction, the balance T_{regs}/T_{cons} is disrupted, and this has been linked to a high incidence of extensive cGVHD and peripheral tolerance loss (252).

B lymphocytes and cGVHD. The role of B lymphocytes in cGVHD has been identified in mice (336) and humans (55, 72, 180, 189, 190, 195, 308, 309, 403, 434), and several authors have described the factors that affect B cell subsets in the development of this disease (101, 259, 289, 352, 382, 436, 441). Patients with cGVHD show high levels of B cell activating factor (BAFF) (43, 59, 121, 327, 328) such that the BAFF/B cell ratio is elevated (210, 328). Several factors have been correlated with the presence and severity of cGVHD: a high prevalence of autoreactive antibodies (19, 27, 43, 75, 93, 105, 121, 142, 186, 192, 249, 260, 264, 289, 304, 317, 350, 365, 373, 398, 410), the relationship among genotypes of the Fc receptor-like 3 gene *(FCRL3)* (345) (335) and an increased number of B cells strongly expressing TLR-9 (344). Moreover, these patients show poor recovery of B cell numbers and prolonged immunodeficiency (9, 62, 101, 360, 361). However, a return to homeostasis is essential (261) and B cell precursor depletion can be a predictor of cGVHD development. In addition, elevated numbers of these cells in the bone marrow or an increase in *naïve* B and transition cells in blood can predict the success of allo-HSCT (101, 225, 328). Finally, depleted *naïve* B and transition cell compartments enhance the autoreactivity of B cells with antigenic experience in these patients (16, 172, 328).

Fibrotic changes. Various soluble factors play a role in the course of cGVHD. Complement factor 3 is deposited at the dermal-epidermal interface in patients with cGVHD (386), and complement factor 5 has been identified as a quantitative trait that modifies liver fibrosis (157). Chemokines have been attributed a role in the pathogenesis of systemic sclerosis as potent chemoattractants of leukocytes and collaborators of pro-fibrotic cytokines (18, 275, 438). $CD4^+$ T lymphocytes produce fibrotic lesions in the skin, liver, exocrine glands and thymus (1, 8, 88, 94, 126, 371, 389, 425). Elevated plasma levels of transforming growth factor (TGF)-β1 have been correlated with the development of hepatic and pulmonary fibrosis (12, 21, 269). In addition, skin fibrosis and the overregulation of TGF-β1 and mRNA for collagen have been observed in human and murine models of scleroderma (175). In the mouse, TGF-β has been related to sclerodermal skin changes (82, 254), and in humans, elevated levels of this growth factor have been detected in patients with cGVHD (227). TGF-β plays a role in the generation and maintenance of peripheral T_{regs} and in improving their suppressive actions (321). Finally, the dysregulation of platelet-derived growth factor (PDGF) signaling has been related to atherosclerosis, pulmonary hypertension and fibrosis. Anti-PDGF receptor antibodies recognize the native PDGF receptor inducing tyrosine phosphorylation, the build-up of reactive oxygen species, the expression of collagen type I genes and conversion of the myofibroblast phenotype in normal human primary fibroblasts, which leads to sclerosis (27). These antibodies have been detected in patients with scleroderma (27) and in all patients with extensive cGVHD (365).

CLINICAL FEATURES

GVHD features a heterogeneous pattern of clinical presentation (see **Figure 4** for a schema). The three main target organs of aGVHD are the skin, gastrointestinal tract and liver (85, 105, 107, 122, 134, 156, 245, 255, 274, 353, 354, 400) though the thymus (60, 207, 312, 411) and lungs (81); eyes and kidneys (362) may also be affected. In contrast, the clinical manifestations of cGVHD resemble those observed in autoimmune diseases (141, 277). Although considered to be a multi-organ disorder, initial signs of disease appear in the oral mucosa before affecting other organs such as the skin, nails, eyes, muscles, lungs, tendons, gut, liver, joints, nerves, serosal surfaces, heart and immune system (79, 100, 110, 129, 141, 143, 153, 154, 204, 280, 307, 349, 350, 395).

Pharmacological treatment

All allogeneic transplant patients receive prophylaxis against GVHD. The commonly used regimens for prevention of aGVHD consist of a combination of a calcineurin inhibitor, either cyclosporine-A (CsA) or tacrolimus, and an antimetabolite (359). However, these interventions that prevent aGVHD are not effective in preventing cGVHD. Strategies using anti-thymocyte globulins for *in-vivo* T-cell depletion show promise but no benefit on survival (112). Despite prophylaxis, many patients suffer from acute or cGVHD.

Corticosteroids (prednisone/6-methylprednisolone) are the standard-of-care first-line treatment for both acute (158, 236, 245, 258, 393, 412) and chronic forms of GVHD (110, 153, 364, 398, 421). The treatment protocol for each patient varies in terms of the dose, regimen and length of therapy. First-line treatment produces a response in fewer than 50% of patients with aGVHD and in 40–50% of patients with cGVHD depending on initial disease severity (127). This has meant that research efforts have been directed towards developing additional therapies combining corticosteroids with other agents (see **Table 1** for more detailed information). However, trials performed to date have shown no benefits when other agents are added to corticosteroids (355). Moreover, steroids have numerous side effects (e.g., osteoporosis, osteonecrosis, diabetes mellitus, hypertension, and can be detrimental in a growing child), which compromise the QoL of patients (5, 120, 127, 219, 337). There is no standard second-line treatment for aGVHD or cGVHD. Numerous therapeutic agents have been assessed to treat aGVHD and cGVHD (see **Table 1** for more details) but no single treatment has proven better

Table 1: Summary of pharmacological therapies against acute (aGVHD) and chronic graft-*versus*-host disease (cGVHD)

	aGVHD		**Refs.**
First line therapies	Standard of care	Corticosteroids (prednisone/6-methylprednisolone	(158, 236, 245, 258, 392, 393, 412)
	Methylprednisolone combined with either	Etanercept	(6, 41, 224, 388)
		Mycophenolate mofetil (MMF)	(7, 28, 193)
		Denileukin	(7, 343)
		Pentostatin	(7, 40)
		Infliximab	(67)
		Antibodies against IL-2R	(54, 222)
		Horse anti-thymocyte globulin (ATG)	(40, 70)
		Mesenchymal stem cells (MSC)	(187)
Second-line therapies	Alone	ATG	(15, 191, 233-235, 246, 319, 392)
		Alefacept	(383)
		Alemtuzumab (Campath)	(136, 250, 333, 334)
		Beclomethasone	(162)
		IL2 receptor antagonists such as daclizumab	(10, 217, 291, 302, 418)
		Inolimomab	(32, 71, 125, 301)
		Denileukin diftitox	(161, 343)
		Basiliximab	(123, 251, 332, 407)
		Antitumour necrosis factor antibodies such as infliximab	(67, 69, 290)
		Etanercept	(7, 51, 67)
		ECP	(64, 144, 293, 326, 390)
		MMF	(124, 144, 146, 193, 205, 257, 293, 298)
		Sirolimus	(33, 163)
		Pentostatin	(40, 299)
		MSC	(99, 152, 187, 213, 214, 271, 292, 313, 401, 402, 404)
	Combinations	Horse ATG + etanercept with or without MMF	(188)
		Daclizumab, infliximab and horse ATG	(357)
		Daclizumab + etanercept	(422)
		Daclizumab + infliximab	(305)

	cGVHD		**Refs.**
First line therapies	Corticosteroids alone or combined with:	Calcineurine inhibitors	(17, 198, 363, 364)
		Thalidomide	(17, 197)
		Sirolimus	(46, 58, 308)
		MMF	(50, 231, 247)
		Pentostatin	(40, 135)
		Rituximab	(72, 403, 434)
		Hydroxychloroquine	(133)
		Methotrexate (MTX)	(133)
		Extracorporeal photophoresis (ECP)	(66, 117, 118, 147, 184)
Second-line therapies	Alone	Azathoprine	(98)
		Alemtuzumab	(322)
		Alefacept	(341)
		Etanercept	(51)
		Infliximab	(351)
		Oral beclomethasone	(162)
		Hydroxychloroquine	(133)
		Thalidomide	(47, 209, 287, 318, 399)
		Clofazimine	(221, 323)
		Cyclophosphamide	(303)
		Steroid pulse	(5)
		Sirolimus	(69, 180, 181)
		ECP	(13, 36, 39, 53, 66, 80, 83, 117, 118, 128, 138, 145, 147, 183, 184, 257, 284, 294-296, 316, 326, 330, 337, 390)
		Imatinib	(238, 239, 265, 282, 283, 358)
		MMF	(28, 29, 50, 52, 124, 193, 205, 220, 231, 263)
		Rituximab	(55, 56, 72, 190, 195, 262, 281, 308, 309, 346, 370, 375, 403, 434)
		mTOR inhibitor	(178, 310)
		MSC	(415)
		Thoracoabdominal irradiation	(49)
		Pentostatin	(173, 174, 299)
		Retinoids (Am80, etretinate/isotretinoin)	(242, 277)
		Calcineurin inhibitors	(58, 387)
		MTX	(87, 132, 166, 168, 179)
	Combinations:	Prednisone + MMF + sirolimus or ECP	(50, 68, 117, 147, 180, 231)
		Isotretinoin + PUVA	(131)
		Pulse cyclophosphamide + MMF + steroids	(253)
		Infliximab + daclizumab	(315)

than others. All are associated with high failure rates and cause severe toxic effects (127, 243, 244). The evaluation of therapeutic options is complicated by the heterogeneous nature of the patient group (in terms of organ involvement, age, conditioning regimens, GVHD prophylaxis), the lack of a clear definition of corticosteroid-refractory disease, availability of therapies, financial considerations, inconsistent treatment end points, preferences and experience of treating physicians, and secondary effects of treatment. The outcome of refractory aGVHD is poor, including a high morbidity and mortality figures approaching 80% (90, 392). Response rates to agents against cGVHD range from 20% to 70% (420).

Non-pharmacological treatment: rationale for exercise interventions

Clearly there is an urgent clinical requirement to optimize current therapies and develop novel treatments for GVHD based on the patient's individual needs. The heterogeneous nature of its manifestations calls for a multidisciplinary approach to patient management including input from physiotherapists, microbiologists, occupational therapists, dieticians, pharmacists and psychologists. There is strong epidemiological evidence that regular physical exercise (e.g., brisk walking, jogging) leads to a lower risk of all-cause mortality, cardiovascular disease, hypertension, stroke, metabolic syndrome, type 2 diabetes, breast cancer, colon cancer, depression and falls (216). Exercise has therapeutic benefits on many systems in the body because working skeletal muscles produce numerous secreted factors ('myokines') with potential drug-like effects such as IL-6 (an anti-inflammatory cytokine when released during exertion), secreted protein acidic and rich in cysteine (SPARC) or calprotectin (with potential anti-tumorigenic effects) (see Fiuza-Luces et al. for an extensive review (114)). Exercise also stimulates the release of stem cells with a strong regenerative potential from their source of origin (e.g., bone marrow) to the bloodstream (114). Moreover, the beneficial effects of moderate-intensity exercise on immune function, at least in non-immunocompromised individuals, have been well established (406). Because regular physical exercise has positive effects on the chain of interactive events that occur from the time of central nervous system stimulation to skeletal muscle contraction, it increases a person's ability to cope with activities of daily living, and improves cardiorespiratory capacity (commonly expressed as peak oxygen uptake, VO_2peak) in virtually all population groups (232). Finally, exercise is a lifestyle intervention that is also recommended for all patient groups, including children and adult recipients of HSCT (423). Thus, it is of medical interest to assess the effects of exercise in GVHD.

Exercise interventions in GVHD (I): Murine model studies

The present authors sought to determine the effects of a moderate-intensity exercise (treadmill running) program on GVHD in mouse models of aGVHD (115) and cGVHD (113, 115, 116). No other data are available on exercise and murine models of GVHD. In one our studies (115), we addressed the effects of exercise (treadmill running) in the absence of CsA or any immunosuppressive treatment in a murine model of aGVHD and one of cGVHD. In the setting of aGVHD, mice subjected to 12 weeks of training showed an improved functional capacity and clinical course of disease relative to controls. At the muscle level, these mice featured higher citrate synthase activity (a classic indicator of mitochondrial oxidative capacity), although no effects were detected on the phospho-p70 S6 kinase/p70 S6 kinase ratio (an indicator of muscle anabolic state). However, both experimental animals and controls showed a similar response throughout the study in terms of rates of survival, immune cell recovery, systemic inflammation and target organ (skin, liver, intestine) damage. In the cGVHD model, the exercise group showed less worsening of physical capacity, accompanied by increases in citrate synthase activity. In addition, immune recovery was unmodified, such that no detrimental effects were produced on the GVT effect or on infections provoked by the immunocompromised state of the mice. These benefits did not appear to be linked to a possible anti-inflammatory effect of exercise, though reduced IL-6 levels were recorded in the exercise intervention group. However, the exercise intervention failed to affect variables such as survival, disease progression or target organ histological findings.

In another of our studies (116), we reported our analysis of the effects of exercise added to the standard immunosuppressive therapy used for this disease (CsA) in the same murine model of cGVHD. Mice in the intervention group showed significantly higher survival rates, a reduced resting heart rate (an indicator of cardiovascular fitness), and an improved disease course compared to control animals. Further, the exercise program led to lower TNF-α and IL-4 levels, reflecting a weaker inflammatory state. Immune reconstitution was improved, with expanded B lymphocytes and CD4 T lymphocyte compartments. At the muscle tissue level, citrate synthase, respiratory chain complex activities and the phospho-p70 S6 kinase/p70 S6 kinase ratio failed to show an improvement with exercise training, probably due to the detrimental muscle effects of CsA. Finally, similar histological observations were made in the disease's target organs in mice surviving the study period.

In another study (113), we examined the role of autophagy as a possible mechanism for cardiac adaptations produced in response to exercise in mice with cGVHD that survived until the end of the study described above (116). Autophagy is an intracellular quality control mechanism of degradation and recycling of damaged macromolecules and organelles that is currently gaining attention because of its potential involvement in longevity and defense against chronic diseases. After 12 weeks of training, levels of several markers of autophagy (autophagy related gene 12 (Atg12), microtubule-associated protein 1 light chain 3 alpha (LC3B), unc-51-like kinase 1 phosphorylated at serine 555 (phospho-ULK1 S555) and sequestosome 1 (SQSTM1/p62), were elevated, as were the activities of the antioxidant enzymes catalase and glutathione reductase relative to those recorded in control mice. These benefits of exercise were observed in the absence of modifications to the proteins involved in mitochondrial dynamics and heart muscle contraction, and thus failed to affect cardiac structure and function. No significant differences were detected in control and experimental animals in terms of electron transport chain complexes or citrate synthase activity.

Exercise interventions in GVHD (II): Human studies

Patients experience considerable levels of physical and psychological distress before, during and after allo-HSCT. In addition to GVHD, muscle atrophy, decrements in physical performance, cachexia, pneumonia, psychological impairments and mortality are more pronounced in the allogeneic compared to the autologous transplant setting (159, 200, 408, 431). Physical exercise has recently been purported to ameliorate some of these treatment-related side effects and enhance the rehabilitation process in allo-HSCT patients (419). Despite this, however, no research effort to date has characterized the effects of exercise in patients with GVHD. Existing exercise training interventions have targeted patients undergoing allo-HSCT, among whom patients with GVHD have sometimes been included **(Table 2 see next side)**. Among the

beneficial effects of exercise reported in these studies were positive effects on QoL (30, 177, 419), improvements in endurance/aerobic capacity (30, 31, 74, 177, 419), muscular strength (30, 31, 74, 177, 256, 419), functional capacity (30, 177), and perceptions of fatigue, physical emotional and social well-being (419). Exercise training has also been shown to reduce perceived pain scores and subdue anxiety, depression and aggressive or hostile behavior (419). Although the patient cohorts and experimental designs employed in these studies were very heterogeneous, both aerobic and resistance based exercise appeared to positively influence various outcomes in allo-HSCT inpatients, as well as outpatients.

The vast majority of studies that have examined the effects of exercise after allo-HSCT have involved inpatient cohorts **(Table 2 see next side)**. In a small retrospective study of allo-HSCT patients that received myeloablative conditioning regimens (i.e., chemotherapy, irradiation), an inverse correlation was found between the level of physical activity performed during hospitalization (number of steps taken daily by the patient) and time to discharge (170). This relationship was observed regardless of whether or not the patient experienced GVHD, infections or cytomegalovirus reactivation. Interestingly, however, physical activity did not impact the length of hospitalization in patients receiving non-myeloablative conditioning regimens.

Exercise training studies involving allo-HSCT outpatients are relatively uncommon. A home-based aerobic exercise training intervention was administered to cancer survivors (at least 6-months post allo-HSCT), although only ~31% of these patients received an allo-transplant (417). Patients performed 20-40 minutes of activity at 40-60% of predicted heart rate reserve 3 to 5 times per week for 12 weeks. Although lacking a control group, scores on aerobic fitness, fatigue severity and physical well-being improved after exercise training, with no adverse events being reported. Another study involving allo-HSCT outpatients (385) enrolled 10 patients with severe cGVHD and *bronchiolitis obliterans* syndrome, which is the most common and serious pulmonary complication of cGVHD (63, 65). The 8-week pulmonary rehabilitation program, which involved both strength and aerobic based exercise session ~3 times per week, improved 6-minute walk distance, exercise tolerance, subjective symptoms of dyspnea and QoL scores (385). To our knowledge this is the only study conducted to date in which all participants had some form of GVHD (385).

The diverse nature of the exercise training studies involving allo-HSCT patients makes it difficult to draw any firm conclusions pertaining to how physical exercise may benefit a patient with GVHD. Although no study reported an adverse event as a direct result of testing or exercising, safety issues or the feasibility of performing exercise training interventions in patients with GVHD were not clearly confirmed in most of these studies. Combining these studies is challenging due to study limitations and the disparate nature of the patient cohorts and experimental designs. These include small sample sizes, a wide range of different outcomes and measurements, varying types of interventions, different starting and end points, the duration, frequency and intensity of the different exercise components, different proportions and severity of GVHD patients, the presence or absence of a control group, or varying standard care regimes used in controls, among others.

Although exercise training shows promise, there is a critical need for more randomized clinical trials to determine if exercise is capable of ameliorating the detrimental effects of both acute and cGVHD in humans. Although exercise has been shown to have profound immunologic effects and GVHD is primarily an immunoreactive disorder, it is surprising that very few studies have focused on the effects of exercise on immunological outcomes in the allogeneic transplant setting. In one randomized control trial involving allogeneic bone marrow transplant patients, a series of bed exercises (performed 30 minutes daily for 6-weeks) increased total lymphocyte counts by 40.9 cells/μl compared to a decrease of 640.7 cells/μl in the non-exercising control group (194), without affecting the composition of $CD4^+$ and $CD8^+$ T-cell subsets. As immune reconstitution is a major determinant of prognosis and progression-free survival after allo-HSCT (89, 376), further randomized control trials exploring the effects of exercise training on immune reconstitution against viruses and tumors in patients with both acute and cGVHD would be illuminating.

CONCLUSION

Since exercise training as an adjunct therapy to improve health outcomes after allo-HSCT shows promise, there is a critical need for more randomized control trials that focus specifically on GVHD. While outcome measures such as hospitalization time, exercise tolerance, physical functioning and emotional and social well-being are all important, there is also a need to explore potential mechanisms underpinning the beneficial effects of exercise. For instance, it remains to be seen in human models of GVHD if exercise training can alter cytokine profiles and regulatory T-cell function, improve immune reconstitution to viruses and tumors, or dampen the activity of alloreactive T-cells.

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Table 2: Summary of exercise interventions in graft-versus-host disease (GVHD)

REFERENCE	DESIGN	PATIENTS			INTERVENTION	CONTROL	OUTCOME VARIABLES	MAIN RESULTS
Baumann, Kraut et al., 2010 (30)	Pilot RCT with inpatients		**Intervention group**	**Control group**	**Aerobic training** (cycle ergometer) Total duration: from day -6 until 1 day before the hospital discharge Frequency: daily during aplasia (2 sessions/day) and 5 sessions/week after engraftment. Time/session: 10-20 min. Intensity: 80% of peak wattage in WHO-test. **ADL-training** (walking, stepping and stretching) Total duration: during conditioning, not during aplasia, and after engraftment. Frequency: 5 sessions/week Time/session: 20 min Intensity: "slightly strenuous" to "strenuous" (Borg scale).	**Passive and active mobilization** (gymnastics, coordination training, stretching, massages) Total duration: from day +1 until 1 day before the scheduled discharge from hospital. Frequency: 5 sessions/week. Time/session: 20 min. Intensity: "not strenuous" (Borg scale).	**Endurance performance** peak wattage in modified WHO test. **Muscle strength** (isometric test in knee extensor muscles) **QoL** EORTC QLQ-C30 questionnaire **Hematological variables** leucocytes, platelets, hemoglobin and neutrophil engraftment **Lung function** IVC and FVC	Endurance performance (peak watts, test time) remained nearly constant in the intervention group but decreased in controls (p=0.004). The *relative endurance* (watts·kg^{-1}) increased in the former but decreased in the controls (p=0.031). The muscle strength performance decline was greater in the controls (-24%) than in the intervention group (-10%) during hospitalization. At the time of discharge, the intervention group showed 13% higher strength compared to the controls (p=0.002). At the time of discharge, the intervention group rated higher their global QoL (p=0.037) and physical functioning (p=0.039) compared to the controls. The hematological variables declined in both groups but there was no difference between them. The lung function values did not change during the study period in either group.
		Sample size (N)	32	32				
		Age (years; mean±SD)	44.9±12.4	44.1±14.2				
		Sex (N; male/female)	21/11	14/18				
		HSCT (N) Autologous Related allogeneic Unrelated allogeneic	 9 5 18	 9 11 12				
		Conditioning regimen (N) TBI-based Not TBI-based Full intensity Reduced intensity	 17 6 18 5	 14 9 15 8				
		Patients with GVHD (N, %)	13 (41%)	9 (28%)				
Baumann, Zopf et al., 2011 (31)	RCT with inpatients (included some patients from the study by those *Baumann et al., 2010*)		**Intervention group**	**Control group**	**Aerobic training** (cycle ergometer) Total duration: from day -6 until 1 day before the hospital discharge Frequency: daily during aplasia (2 sessions/day), 5 sessions/week during chemotherapy and 1 session/day after engraftment. Time/session: 10-20 min Intensity: 80% of peak wattage in WHO-test. **ADL-training** (walking, stepping, coordination, strength and stretching) Total duration: during conditioning, not during aplasia, and after engraftment. Frequency: 1 session/day Time/session: 20-30 min Intensity: "slightly strenuous" to "strenuous" (Borg scale).	**Passive and active mobilization** (gymnastics, massages, coordination training). Total duration: from +1 day until 1 day before hospital discharge. Frequency: 5 days/week Duration: 20 min Intensity: "not strenuous" in Borg scale.	Same as above + anthropometric assessments (weight, height and BMI).	*Relative endurance* (watts/kg) increased in the intervention group (p=0.023) and decreased in the controls (p=0.032). There were no other significant differences between groups.
		Sample size (N)	24 → 17*	23 → 16*				
		Age (years; mean±SD)	41.4±11.8	42.8±14.0				
		Sex (N; male/female)	11/6	5/11				
		HSCT (N) Related allogeneic Unrelated allogeneic	 4 13	 6 10				
		Patients with GVHD (N, %)	13 (76%)	9 (56%)				

Chamorro-Viña et al., 2010 (74)	Controlled (but not randomized) trial with inpatients		**Intervention group**	**Control group**	**Aerobic + resistance training** Total duration: from the beginning of the conditioning phase to the end of the neutropenic phase. Frequency: 5 sessions/week (3 aerobic sessions and 2 aerobic + resistance sessions). Time/session: ~50 min Aerobic training (cycle ergometer): Time/session: 25-30 min Intensity: 50%-70% of age-predicted maximum heart rate. Resistance training (arm curl, elbow extension, bench press, leg extension, half squat, abdominals, supine bridge, and rowing) 1 set of 12-15 repetitions per exercise Intensity: their own body weight, *therabands* with resistance gradually increasing over the program and barbells	'Historical' control group from hospital records not performing any type of exercise	**Anthropometric assessments** body mass, BMI and estimated fat-free mass. **Immune cell recovery** blood counts of leukocytes, monocytes, and lymphocytes and main lymphocyte subpopulations (T lymphocytes, natural killer cells, NK T, CD4+ and CD8+) and dendritic cells.	The intervention resulted in decreased resting heart rate (p=0.034) and increased muscle strength, as reported by gradually increasing loads (in *therabands* and barbells) as training progressed (p=0.018). The intervention group showed increased weight (p<0.001), BMI (p<0.001), body fat (p=0.006), and fat-free mass (p=0.021), as opposed to controls. There was a significant training effect for dendritic cells, (p=0.045 for the interaction effect group x time) which became non-significant after adjustment for multiple comparisons.
		Sample size (N)	7	13				
		Age (years; mean±SD)	8±3	7±3				
		Sex (N; male/female)	5/2	9/4				
		HSCT (N) Related Allogeneic Unrelated Allogeneic	 4 3	 9 4				
		Conditioning Nonmyeloablative	7	13				
		Patients with GVHD (N, %)	2 (29%)	6 (46%)				
Inoue et al., 2010 (170)	Non-controlled trial with inpatients		**Intervention group A**	**Intervention group B**	**Stretching** (exercises for shoulder, elbow, hip, knee, and ankle joints), **aerobic training** (cycle ergometer) and **resistance training** (exercises for upper/lower limbs and abdominal muscles) Total duration: from the neutrophil engraftment to the last day of hospitalization. Frequency: 5 sessions/week. Time/session: 20-40 min Aerobic training intensity: 60% of age-predicted maximum heart rate		**Physical activity level** (daily steps) **Duration of hospitalization** **Daily steps versus duration of hospitalization**	No significant differences (1,710.4 steps/day (range=301.8 – 3,444.7) in group A and 2,093.0 steps/day (range = 571.6 – 3,251.6) in group B (p=0.90). Hospitalization was longer in group A than in group B (p=0.0001). The correlation coefficients between mean daily steps and duration of hospitalization were -0.71 (p=0.0071) in group A and 0.09 (p=0.77) in group B. Increased physical activity levels through early rehabilitation prevented deconditioning and shortened the duration of hospitalization after allogeneic-HSCT among the patients of group A.
		Sample size (N)	13	13				
		Age (years; mean (range))	43.0 (20-55)	54.0 (27-62)				
		Sex (N; male/female)	7/6	5/8				
		HSCT (N) Related and unrelated allogeneic	n.a.					
		Conditioning (N) Myeloablative With TBI Without TBI Nonmyeloablative With TBI Without TBI	 12 1	 6 7				
		Patients with GVHD (N, %)	5 (38%)	0				

Jarden, Baadsgaard et al., 2009 (177)	Prospective RCT with inpatients		**Intervention group**	**Control group**	**Usual care and multimodal intervention** (aerobic and resistance exercises, relaxation and psycho-education) Total duration: from the first day of admission (day -7) until the day of discharge. Frequency: 5 sessions/week. Time/session: 1 hour±10 min. **Aerobic training** (cycle ergometer) Frequency: 5 days/week. Time/session: 15-30 min Intensity: 50-75% of age-predicted maximum heart rate and 10-13 in the Borg scale. **Resistance training** (free hand and ankle weights, biceps curl, shoulder press, triceps extension, chest press, flyers, squat, hip flexion, knee extension, and leg curl and extension) Frequency: 3 days/week Time/session: 15-20 min 1-2 sets of 10-12 reps (intensity: 10-13 in the Borg scale). **Relaxation** (5 sec of muscle tensing and 30 sec of muscle relaxation) Frequency: 2 days/week. Time/session: 20 min. Intensity: 6-9 in the Borg scale. **Psycho-education** Ongoing	Usual care	**Physical functioning** Karnofsky performance scores. VO_2max (Astrand-Rhyming cycle test). Muscle strength Functional performance (2-min chair climb test). **QoL:** EORTC QLQ-C30 **Psychological well-being and distress** Hospital Anxiety and Depression Scale (HADS) **Fatigue** Functional Assessment of Cancer therapy-Anemia scale FACT-An scale **Adherence to the intervention** "monitoring logbook" **Several clinical outcomes**	There was a significant effect in favor of the control group at post-testing for: VO_2max (p=0.0001), chest press (p=0.0001), leg extension (p=0.0003), isometric right elbow flexors' strength (p=0.0009), isometric right knee extensors' strength (p=0.0001) and the stair test (p=0.0008). The intervention group reported having less diarrhea, over the time than the controls (p=0.014) and received parental nutrition for fewer days than the controls (p=0.019). There was no significant effect on QoL, fatigue, psychological well-being or physical activity levels, but there were longitudinal trends that favored the intervention group. Though not significant, there was a 19% decrease in the occurrence of aGVHD in the intervention group.
		Sample size (N)	21	21				
		Age (years; mean±SD)	40.9±13.3	37.4±11.1				
		Sex (N; male/female)	13/8	13/8				
		HSCT (N) Related Allogeneic Unrelated Allogeneic	 11 10	 12 9				
		Conditioning agents (N) CY/ATG CY/BU CY/TBI Et/TBI CY/ATG/TBI	 2 6 6 4 3	 3 12 5 1				
		TBI (N) No Yes	 8 13	 3 18				
		Patients with GVHD (N, %)	5 (24%)	9 (43%)				
Kim and Kim 2006 (194)	RCT with inpatients		**Intervention group**	**Control group**	**Routine care (GCSF injection and aseptic care) with bed exercises** All performed in the supine position, consisting of joint mobility exercises, breathing exercises, stretching and relaxation techniques Total duration: 6-weeks Frequency: 1 session/day Time/session: 30-minutes	**Routine care without exercise**	Blood lymphocyte count T-cell subset percentages CD4/CD8 T-cell ratio	Total lymphocyte count changed by +40.9 and -640.7 cells/µl in the exercise and control groups respectively There were no significant group differences in the relative proportion of CD4+ and CD8+ T-cell subsets or in the CD4/CD8 T-cell ratio *Although the exercise group demonstrated an increase and the control group a decrease in the total lymphocyte count following the 6-week intervention, it is important to note that the total lymphocyte count was close to being* *significantly lower in the exercise group (p=0.051) at baseline (1048.8*±453 vs. 1424.1±616.2 cells/µl)
		Sample size (N)	18	17				
		Age (years; mean±SD)	32.9±7.0	34.3±7.8				
		Sex (N; male/female)	8/10	9/8				
		HSCT/BMT (N) Autologous Allogeneic (BM) Allogeneic (BM + PBSC)	 0 15 3	 0 14 3				
		Diagnosis (N) AML ALL SAA	 10 4 4	 8 4 4				
		Patients with GVHD (N, %)	Not reported	Not reported				

Mello, Tanaka and Dulley, 2003 (256)	Controlled (but not randomized) trial with inpatients		**Intervention group**	**Control group**	**Active exercises, muscle stretching and a walking-based program on a treadmill** Total duration: exercise initiated during the inpatient period after bone marrow engraftment and concluded in the outpatient facility (over 6 weeks). Frequency: 5 days/week. Time/session: 40 min. Intensity 70% of age-predicted maximum heart rate.	No exercise program. Usual care.	**Muscle strength performance** Maximum voluntary isometric contraction (assessed with a dynamometer) in upper and lower limb muscles.	1st assessment (prior to HSCT). No difference between groups for all muscle groups, except for the dominant elbow flexors (p=0.042) and the dominant hip abductors (p=0.035), with higher values in the controls. 2nd assessment (post-HSCT). Both groups had similarly decreased values. 3rd assessment (6 weeks after exercise training or normal life). The intervention group showed a trend towards higher values than the control group for all muscle groups tested, with a significant difference for non-dominant hip flexors (p=0.011).
		Sample size (N)	n.a.→ 9**	n.a.→ 9**				
		Age (years; range)	27.9 (18-39)	30.2 (18-44)				
		Sex (N; male/female)	5/4	3/6				
		HSCT (N) Related and unrelated allogeneic	n.a.	n.a.				
		Conditioning agents (N) BU + melphalan BU + CY	 8 1	 8 1				
		Patients with GVHD (N, %)	6 (67%)	7 (78%)				
Tran et al., 2012 (385)	Not controlled trial with outpatients		**Intervention group**		**Pulmonary rehabilitation program** (breathing techniques), and **strength** (free weights and weight machines; upper and lower body exercises) **and aerobic training** (recumbent bike, treadmill or step machine and upper body bike). Total duration: exercise intervention initiated during the outpatient period (8-12 weeks). Frequency: ~3 days/week. Time/session: ~65 min. Intensity: individualized and gradually increasing throughout the intervention.		Spirometry/pulmonary function tests. 6 minute walk tests. QoL: SF-36 survey.	All patients with pre-HCST pulmonary function tests had a drop of at least 10% in FEV1 after HCST, and most had a drop >25%. There was no significant change in spirometry when comparing pre and post rehabilitation values (p=0.446 for FEV1, and p=0.822 for FVC). Patients who completed the pulmonary rehabilitation improved their 6 minute walk distance (p=0.005) an average of 307 feet post-rehabilitation. There was a significant improvement in the physical functioning score by a mean of 14.4 points (p=0.029).
		No (N)	11 → 10*					
		Age (average)	48					
		Sex (N; male/female)						
		HCST (N) Related Allogeneic Unrelated Allogeneic	 6 5					
		Conditioning agents (N) Cytoxan + TBI Fludarabine + busulfan + ATG Fludarabine + busulfan Busulfan + cytoxan Busulfan + CY	 3 4 2 1 1					
		Patients with GVHD (N, %)	10 (**100%**)					
Wilson et al., 2005 (417)	Pilot not controlled with outpatients		**Intervention group**		**Home-based aerobic training** (walking, swimming, cycling, exercise tapes) Total duration: 12-weeks Frequency: at least 3 times weekly Time/session: 20 continuous minutes in training zone Intensity: 40%-60% of age-predicted heart rate reserve	No control group but training diaries were monitored weekly by telephone contact. Nine of 13 subjects reported completing 273 (84%) of the 324 exercise sessions assigned at the prescribed intensity and duration	**Aerobic fitness** Submaximal treadmill graded exercise test (Stanford protocol) **Fatigue** Fatigue Symptom Inventory **QoL** SF-36	Aerobic fitness (defined as the oxygen uptake at the ventilatory threshold) was poor at baseline but increased >15% after the intervention Fatigue levels at baseline were modest; symptom severity scores but not fatigue symptom duration or interference scores, improved significantly ($p<0.05$) after the intervention. At baseline, reported levels of physical functioning and physical role functioning were substantially lower (>0.5 SD) than those reported for the normal US population. Statistically significant improvements in the SF-36 Physical Functioning and Physical Role Functioning subscales were observed after the intervention.
		Sample size (N)	17 (13 completed)					
		Age (years; mean±SD)	48.9±10.4					
		Sex (N; male/female)	6/11					
		HSCT/BMT (N) Autologous Allogeneic	 13 4					
		Time since transplant (months; mean±SD)	16.9±8.3					
		Patients with GVHD (N, %)	Not Reported, although 3 of the 4 allogeneic-BMT patients were using cyclosporine and corticosteroids, presumably for GVHD.					

Wiskeman n et al., 2011 (419)	RCT with in- and outpatients		**Intervention group**	**Control group**	**Self-administered exercise outpatient intervention and partly supervised inpatient intervention (aerobic and resistance program)** Total duration: from 1-4 weeks before admission to 6-8 weeks after discharge from the hospital. Aerobic training (outpatient: walking; inpatient: bicycling and treadmill walking) Time/session: 20-40 min. Frequency: 3 sessions/week (up to 5 during hospitalization). Intensity: 12-14 Borg scale. Resistance training (exercises for the upper and lower extremities with or without stretch bands). Frequency: 2 sessions/week 2-3 sets of 8-20 repetitions Intensity: 14-16 Borg scale.	Outpatient setting: daily steps Inpatient setting: possibility to have physiotherapy (3 session/week, 30 min/session) or to use stationary cycles and treadmills.	**Fatigue** MFI and POMS **QoL** EORTC QLQ-C30 questionnaire. **Psychological well-being** HADS. **Distress** National Comprehensive Cancer Network Distress Thermometer. **Physical capacity** Endurance performance: 6-minute walk test. Hand-grip test. **Physical activity levels** Number of steps.	The intervention group had less fatigue at 6-8 weeks after discharge from the hospital than the controls in MFI scales general fatigue (p=0.009), physical fatigue (p=0.01) and in the POMS scale (p=0.004). EORTC physical functioning was higher in the intervention group than in the controls (p=0.03) at the end of the intervention (p=0.007). HADS anxiety, and global distress was higher (p = 0.01) at the end of the intervention and lower (p=0.05) at discharge from the hospital, respectively, in the intervention group than in the controls. Endurance capacity post-intervention (p=0.02) and strength of the lower extremities from baseline to discharge (p=0.03) improved in the intervention group but not in the controls. Physical capacity was inversely correlated with general fatigue (p=0.01-0.02).
		No (N*)	52	53				
		Age years; mean (range)	47.6 (18-70)	50 (20-71)				
		Sex (N; male/female)	32/21	39/13				
		HSCT (N) HLA-identical (related) HLA-matched/unrelated HLA-mismatched/unrelated	 13 26 13	 15 30 8				
		Intensity of Conditioning regimens Myeloablative Reduced intensity TBI	 11 41 18	 13 40 18				
		Patients with GVHD (N, %)	21 (40%)	18 (34%)				No differences were found in pedometer steps and coordination tasks.

*The data and analyses were focused on the final N (i.e., final number of participants completing the study).

** Same as above + Initial N per group not provided (initial total N=32)

Abbreviations: ADL, activities of daily living; aGVHD, acute graft versus host disease; ALL: acute lymphocytic leukemia; AML: acute myeloid leukemia; ATG, anti-thymocyte globulin; BMI, body mass index; BMT, bone marrow transplant; BU, busulphan; cGVHD, chronic graft versus host disease; CY, cyclophosphamide; EORTC, European Organization for Research and Treatment of Cancer; Et, etopofos; FACT-An, Functional Assessment of Cancer Therapy-Anemia; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; GCSF: granulocyte colony stimulating factor; GVHD, graft versus host disease; HADS, Hospital Anxiety and Depression Scale; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplant; IVC: inspiratory vital capacity; QoL, quality of life; MFI: Multidimensional Fatigue Inventory; n.a., not available; NK, natural killer; PBSC: peripheral blood stem cell; POMS: Profile of Mood States; RCT, randomized controlled trial; SAA: severe aplastic anemia; SF-36, Short Form-36; TBI, total body irradiation; VC, vital capacity; VO_2max, maximal oxygen uptake.

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Circulatory endotoxin concentration and cytokine profile in response to exertional-heat stress during a multi-stage ultra-marathon competition

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ABSTRACT

Exertional-heat stress has the potential to disturb intestinal integrity, leading to enhanced permeability of enteric pathogenic micro-organisms and associated clinical manifestations. The study aimed to determine the circulatory endotoxin concentration and cytokine profile of ultra-endurance runners (UER, n=19) and a control group (CON, n=12) during a five stage 230km ultra-marathon (mean ± SD: 27h38min ± 3h55min) conducted in hot and dry environmental conditions (30ºC to 40ºC and 31% to 40% relative humidity). Body mass and tympanic temperature were measured, and venous blood samples were taken before (pre-stage) and immediately after (post-stage) each stage of the ultra-marathon for the analysis of gram-negative bacterial endotoxin, C-reactive protein, cytokine profile (IL-6, IL-1β, TNF-α, IFN-γ, IL-10, and IL-1ra), and plasma osmolality. Gastrointestinal symptoms and perceptive thermal tolerance rating were also monitored throughout competition. Mean exercise-induced body mass loss over the five stages ranged 1.0% to 2.5%. Pre- and post-stage plasma osmolality in UER ranged277 to 282mOsmol/kg and 286 to 297 mOsmol/kg, respectively. Pre-stage concentrations of endotoxin (peak: 21% at Stage 5), C-reactive protein (889% at Stage 3), IL-6 (152% at Stage 2), IL-1β (95% at Stage 5), TNF-α (168% at Stage 5), IFN-γ (102% at Stage 5),IL-10 (1271% at Stage 3), and IL-1ra (106% at Stage 5) increased as the ultra-marathon progressed in UER; while no changes in CON were observed (except for IL-1β, 71% at Stage 5). Pre- to post-stage increases were observed for endotoxin (peak: 22% at Stage 3), C-reactive protein (25% at Stage 1), IL-6 (238% at Stage 1), IL-1β (64% at Stage 1), TNF-α (101% at Stage 1), IFN-γ (39% at Stage 1), IL-10 (1100% at Stage 1), and IL-1ra(207% at Stage 1) concentrations in UER. Multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia by post-Stage 1, both of which were sustained throughout competition at rest (pre-stage) and after stage completion. Compensatory anti-inflammatory responses and other external factors (i.e., training status, cooling strategies, heat acclimatization, nutrition and hydration) may have contributed towards limiting the extent of pro-inflammatory responses in the current scenario.

Keywords: endurance, running, heat, inflammation, gastrointestinal.

INTRODUCTION

The epithelial lining along the gastrointestinal tract acts as a protective barrier between the internal and external environment, playing a significant role in preventing the penetration of enteric pathogenic microorganisms into portal and systemic circulation (23). Prolonged physical exertion, particularly running exercise, appears to impact upon intestinal epithelial integrity through redistributing blood flow to the working muscles and peripheral circulation (i.e., aiding thermoregulation), inevitably leading to splanchnic hypoperfusion and hypoxia (53, 58). Additionally, alterations to intestinal motility and mechanical trauma (i.e., repetitive jarring associated with running) can further promote intestinal mucosa and epithelial damage and (or) dysfunction(41).

When acute bouts of prolonged strenuous exercise are performed in hot (>30ºC) environmental conditions, enhanced thermoregulatory strain, increased body water losses and accompanying hypovolaemia are commonly observed (62), and have the potential to further promote splanchnic hypoperfusion and disruption to intestinal epithelial integrity (24, 57). Such perturbations have been linked to increased intestinal permeability of localised gram-negative bacteria (e.g.,terminal ileum colonized lipopolysaccharide), primarily due to deterioration of the protective mucosal lining and widening of

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epithelial tight junction spaces. Subsequently, this leads to endotoxaemia and a responsive cytokinaemia (5, 6). Endotoxin induced cytokinaemia has previously been implicated in the aetiology of heat stroke and septic shock (26, 34); whereas its potential link to autoimmune disease, gastrointestinal disease, and chronic fatigue in high risk individuals (i.e., genotype predisposition) is of current research interest (7, 29, 43). In extreme cases, where by systemic endotoxin proliferation is evident, over-exaggerated immune activation (i.e., innate immune cell proliferation and function, and cytokine responses) and increased pro-coagulant factors results in tissue hypoperfusion, intravascular coagulation, endothelial injury, and the end-point being refractory shock (19).

It is well established that moderate levels of exercise elicit favourable changes in cytokine profile; such as suppression of low-grade inflammation and enhanced anti-inflammatory cytokine responses (37, 59). Indeed, the classical cytokine response to moderate exercise in thermoneutral conditions (body temperature increase <1°C) results in raised circulatory interleukin (IL)-1 receptor antagonist (ra), IL-10, and muscle derived IL-6 concentrations; while pro-inflammatory cytokine IL-1β and tumour necrosis factor (TNF)-α responses are generally minimal (59). For example, 2 hours of running at 75% of maximum oxygen uptake (VO_{2max}) in 20ºC ambient temperature resulted in an approximate 150%, 570%, and 1490% increase in IL-1ra, IL-10, and IL-6, respectively; while no changes in IL-1β and TNF-α were observed (11).

On the contrary, excessive strenuous exercise (e.g., long distance running), especially in hot ambient conditions (body temperature increase >1°C), results in enhanced enteric endotoxin translocation and a cytokine-mediated systemic inflammatory response similar to the cytokine profile of an acute infectious episode (e.g., sepsis, trauma, fever) (3, 15, 22, 47). Previous laboratory controlled studies have observed elevated pro- (e.g., TNF-α) and anti-inflammatory (e.g., IL-10) cytokine production after prolonged exercise in hot (32°C to 40°C) compared with thermoneutral (15°C to 22°C) environmental conditions (9, 42, 47, 49) indicating ambient temperature plays a crucial role in the degree of cytokinaemia observed after physical exertion. Such systemic endotoxin and cytokine responses have also been associated with symptomatic manifestations of gastrointestinal symptoms; a commonly observed feature in individuals exposed to prolonged periods of exertional-heat stress (22, 23, 33, 36, 56).Taking into account that previous research has predominantly focused on single bouts of exertional-heat stress, to date, it is still unclear the extent to which consecutive days of exertional-heat stress may impact on circulatory endotoxin and cytokine responses along the duration of exposure. Moreover, despite differences in sex and training status influencing such responses to acute exertional-heat stress through hormonal and adaptive factors (3, 47, 59, 61), it is unknown whether these sub-groups respond differently to consecutive repetitive exposure.

During exertional-heat stress, endotoxin induced systemic cytokinaemia appears to be a key feature in the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke) (26) with fatalities being acknowledged as resulting from systemic inflammatory response syndrome (SIRS), a condition known as a whole body inflammatory state (39). For example, fatal incidence of heat stroke in military personnel during infantry training in hot ambient conditions were reported to be due to septic shock, in which SIRS was a key feature (32, 39). From a practical perspective, given the substantial growth of ultra-endurance sports worldwide over the past decade and the environmental extremes of these events, competing in multi-stage ultra-endurance competition exposes ultra-endurance athletes to consecutive days of exertional-heat stress. This population may thus be predisposed to sub-clinical (e.g., gastrointestinal symptoms) and clinical (e.g., exertional-heat illnesses, sepsis, autoimmune diseases, gastrointestinal diseases, chronic fatigue) manifestations potentially originating from intestinal mucosa and epithelial damage and dysfunction. Indeed, mild circulatory endotoxaemia, cytokinaemia, and gastrointestinal symptoms have been reported after marathon (6) and Ironman triathlon (22) events, which were also associated with decrements in overall performance (38).

To date, exercise immunology research in ultra-endurance sports is limited (30), with no research exploring and tracking intestinal permeability of endotoxins and cytokine profile during multi-stage ultra-marathon. Besides the consecutive days of exertional-heat stress, such events are also accompanied by additional stressors that have previously been acknowledged as predisposing factors in the aetiology of fatal incidence of heat stroke and SIRS (2, 32, 39, 60). These include inadequate recovery opportunities, sleep deprivation, and acute periods of compromised hydration and (or) nutritional status (12, 13). Moreover, the predominant characteristics of ultra-endurance runners generally observed (e.g., recreationally active population, not acclimatised to environmental conditions, training status suboptimal for degree of physical exertion required, high body fat, high motivation, and situation of compromised immune status) are also reported to be aetiological predisposing factors (60).

The aims of the current study were to: 1) determine circulatory endotoxin concentration and cytokine profile of ultra-endurance runners throughout a five days (five stages) multi-stage ultra-marathon competition conducted in hot and dry environmental conditions; 2) determine the relationship between these responses with gastrointestinal symptoms and perceptive thermal tolerance rating; and 3) determine if sex and training status influence responses. Taking into account the consecutive days of exertional-heat stress, limited recovery time in-between stages, and acute periods of compromised hydration and (or) nutritional status throughout a multi-stage ultra-marathon competition, it was hypothesised that endotoxaemia would be seen by post-Stage 1 and progressively increase (both pre- and post-stage) along the ultra-marathon, which would be mirrored by a cytokinaemic response. It was also hypothesised that a correlation between circulatory responses with severe gastrointestinal symptoms (positive) and perceptive thermal tolerance rating (negative) would be seen. Additionally, it was hypothesised that no difference in responses would be seen between the sexes, and that faster runners with higher training status would show lower responses.

METHODS

Setting

The study was conducted during the 2011 Al Andalus Ultimate Trail (www.alandalus-ut.com), held during the 11th to

15th of July, in the region of Loja, Spain (Table 1). The multi-stage ultra-marathon was conducted over five stages (five consecutive days) totalling a distance of 230 km over a variety of terrains; predominantly off-road trails and paths, but also included steep and narrow mountain passes, and occasional road. Running intensity averaged 8.0, 8.1, 7.1, 7.0, and 7.5 metabolic equivalents (METs) (SenseWear 7.0, BodyMedia Inc., Pittsburgh, PA, USA) from Stages 1 to 5, respectively. Sleeping arrangements along the course included a combination of outdoor tent and village sports hall accommodation [sleep duration (mean ± SD) 8h10min±0h43min, 7h50min±0h36min, 8h32min±0h51min, 8h18min±1h05min; and sleep quality (rating scale, 1= very poor to 10= very good) 6 ±2 , 3 ± 2, 5 ± 2, 3 ± 1,from Stages 1 to 4, respectively].

Table 1: Multi-stage ultra-marathon characteristics, including stage times and average speed of ultra-endurance runners.

	Distance (km)	Altitude (m)	Ambient Temperature (°C)	Ambient Relative Humidity (%)	Running Time (hours:minutes) and Speed (km/h)	Predominant Course Terrain
Stage 1	37	503 to 1443	30 to 32	31 to 32	4:41 ± 0:37 7.9 ± 1.1	Off-road trails and paths, steep and narrow mountain passes.
Stage 2	48	830 to 1338	30 to 34	32 to 33	6:56 ± 1:03 6.9 ± 1.1	Off-road trails and paths, steep and narrow mountain passes, and occasional road.
Stage 3	38	689 to 1302	32 to 38	35 to 37	4:51 ± 0:42 7.8 ±1.2	Off-road trails and occasional road.
Stage 4	69	671 to 1152	32 to 40	31 to 33	7:11 ± 1:12 9.6 ± 1.7	Off-road trails and road.
Stage 5	38	473 to 1065	36 to 40	37 to 40	4:42 ± 0:43 8.1 ± 1.2	Off-road trails and road.

Mean ± SD or range (n= 19).

Participants

After ethical approval from Coventry University Ethics Committee that conforms with the 2008 Helsinki declaration for human research ethics, 19 out of the total 69 ultra-endurance runners (UER) who entered this ultra-marathon competition volunteered to participate in the study [males n= 13: age 41 ± 8 y, height 1.77 ± 0.05 m, body mass 76 ± 7 kg, body fat mass 14 ± 5%; females n= 6: age 49 ± 4 y, height 1.65 ± 0.05 m, body mass 62 ± 6 kg, body fat mass 21 ± 3%]. For comparative purposes, 12 individuals who accompanied the ultra-runners (on-location for pre- and post-stage measurements and sampling, slept at the ultra-marathon location with the competition participants, but drove around the course spectating the event) along the ultra-marathon course, but did not compete (absence of exertional stress), volunteered to participate in the study as part of the control (CON) group [males n= 5: age 41 ± 10 y, height 1.76 ± 0.10 m, body mass 76 ± 14 kg, body fat mass 18 ± 5%; females n= 7: age 31 ± 13 y, height 1.60 ± 0.04 m, body mass 62 ± 13 kg, body fat mass 25 ± 7%]. All participants arrived at location ≤48 hours prior to the start of Stage 1. Only n= 2 participants resided in countries with hot ambient conditions similar to those of the race location (≥30°C T_{amb}) at the time of competition; the remaining participants resided in countries with cold or thermo-neutral environmental conditions (≤20°C). No participant reported any incidence of illness and/or infection in the 12 weeks leading up to the ultra-marathon.

Oral anti-inflammatory agents

The use of non-steroidal anti-inflammatory drugs (NSAIDs) and other anti-inflammatory agents amongst UER included: paracetamol (500-1000mg), ibuprofen (400mg), cocodamol (500-1000mg), Celebrex (200mg), and fish oils (1-2g omega 3 fatty acids). Anti-inflammatory agent usage by UER was n= 2, n= 5, n= 3, n= 4, and n= 6 from Stage 1 to 5, respectively. No form of oral anti-inflammatory agents were used by n= 13 UER and CON (n= 12) throughout the ultra-marathon.

Study design and data collection

Following participant recruitment and informed consent, a preliminary session was completed to determine baseline body mass, height, and body fat mass. Height was measured by a wall-mounted stadiometer. Baseline body mass was determined using calibrated electronic scales (BF510, Omron Healthcare, Ukyo-ku, Kyoto, Japan) placed on a hard levelled surface. Waist and hip circumferences were measured using a standard clinical tape measure by trained researchers, in accordance with ISAK international standards for anthropometric assessment. Body mass and circumference measures were used when conducting multifrequency bioelectrical impedance analysis (MBIA; Quadscan 4000, Bodystat, Douglas, Isle of Man, UK) to estimate body composition. The current ultra-marathon was semi self-sufficient, whereby participants (including CON) planned and provided their own foods and fluids (except plain water) along the five days of competition. Participants' equipment and sustenance was transported to each stage section by the race organisation. *Ad libitum* water was provided by the race organisers during the rest phase throughout competition. Additionally, aid stations along the running route were situated approximately 10 km apart, and only provided plain water, fruit (oranges and watermel-

on), and electrolyte supplementation that was used by n= 9 UER as per manufacturer's instruction (2.46 ml/896 ml fluid; Elete electrolyte add-in, Mineral Resources International, South Ogden, Utah, US). Participants were advised to adhere to their programmed habitual dietary practices throughout the entire competition.

Each day, for five consecutive days, running stages commenced at either 08:00 or 09:00. Within the hour prior to the start of each running stage, body mass measurement was determined using calibrated electronic scales placed on a hard levelled surface. Participants were then required to sit in an upright position for 10 minutes before tympanic temperature (T_{tymp}; Braun Thermoscan, Kronberg, Germany) was determined and whole blood collected. To determine T_{tymp}, participants were asked to position their head in the Frankfort plane and avoid head movement until T_{tymp} measurement was completed. A disposable thermometer tip cover was placed on the sensor; the right auricle was then gently pulled up and back before the sensor was insertion into the right external auditory canal for five seconds, without touching the tympanic membrane. All measurement techniques and samples were consistently conducted and collected in a large partitioned research field tent (four sections, 3 m x 3 m) or sports hall facility. Body mass was re-measured in those participants who needed to urinate prior to the stage start.

Immediately post-stage and before any foods or fluids could be consumed, body mass and T_{tymp} were measured, followed by whole blood collection. For consistency, the order, positioning and technique of measurements and sampling were similar pre- and post-stage for all stages, and were taken by the same trained researcher throughout. At the end of each competition day (20:00 to 22:00) on Stages 1 to 4, researchers explored severe gastrointestinal symptoms (38) and perceptive thermal tolerance rating (20) through a rating scale (gastrointestinal symptoms: "no symptoms" to "extremely bad symptoms" and thermal tolerance rating: "cool" to "unbearable hot"). Exertional-heat illness symptoms were verified by a qualified Sports Physician.

Dietary analysis and hydration status

At the end of each competition day on Stages 1 to 4, trained dietetic researchers conducted a standardised structured interview (dietary recall interview technique) on participants to ascertain total daily food and fluid ingestion. Energy and water intake through foods and fluids were analysed on Dietplan 6 dietary analysis software (v6.60, Forestfield Software, Horsham, West Sussex, UK). A comprehensive description of the dietary assessment and analysis technique used can be viewed in Costa et al. (12, 13). Pre- and post-stage body mass values were used to determine exercise-induced body mass change. Pre- and post-stage plasma osmolality (P_{Osmol}) was determined from 50 µl lithium heparin plasma in duplicate by freezepointosmometry (Osmomat 030, Gonotec, Berlin, Germany). The coefficient of variation for P_{Osmol} was 3.5%.

Blood sample collection and analysis

Whole blood samples were collected by venepuncture without venostasis from an antecubital vein using a 21G butterfly syringe into one lithium heparin (6 ml, 1.5 IU/ml heparin; Becton Dickinson, Oxford, UK) and one K_3EDTA (6 ml, 1.6 mg/ml of K^3EDTA; Becton Dickinson, Oxford, UK) vacutainer tube. Blood samples were immediately centrifuged and plasma aliquoted into Eppendorf tubes and stored frozen initially at -20°C during the ultra-marathon competition, prior to transferring to -80°C storage after completion of the experimental procedure. Whole blood haemoglobin concentration and haematocrit were used to estimate changes in plasma volume (P_V) relative to pre-Stage 1. Haemoglobin concentration and hematocrit content of K_3EDTA blood samples (100µl) were determined using an automated cell counter (Coulter ACT Diff, Beckham Coulter, USA) immediately after sample collection. All blood parameters were corrected for changes in P_V (14).

Circulatory concentrations of C-reactive protein (CRP) (eBioscience, Hatfield, UK), IL-6, TNF-α, IL-1β, IFN-γ, IL-10, and IL-1ra (Invitrogen, Carlsbad, US) were determined by ELISA using K_3EDTA plasma as per manufacturer's instructions. Gram-negative bacterial endotoxin concentration was determined by limulus amebocyte lysate (LAL) chromogenic endpoint assay using K_3EDTA plasma (HIT302, Hycult Biotech, Uden, Netherlands) as per manufacturer's instructions. In short, 20 μl of sample was diluted in 380 μl of endotoxin-free water, and then incubated at 75°C for 10 minutes. Once at room temperature, 50 μl of standards, blank, positive control, and samples were added to plate wells in duplicate. To enhance assay validity, background plate reading without LAL reagent was performed at OD 405nm. 50 µl LAL reagent was then added. Plate was covered and incubated at 22ºC for 30 minutes, followed by reading at OD 405nm. Concentration was calculated by plotting the absorbance against standards in a linear regression curve and eliminating background error. The assay was performed using endotoxin-free and depryogenated consumables in a sterile laboratory. Each plasma variable was analysed on the same day, with standards and controls on each plate, and each participant assayed on the same plate. The intra-assay coefficient of variation for plasma variables analysed was ≤5.5%. In CON, blood-borne indices were determined on pre-Stages 1, 3 and 5 only.

Data analysis

Data in text and tables are presented as mean ± standard deviation (SD). Due to commonly large individual variation in immunological responses to exercise (59), data in figures (% change) are presented as individual participant responses. Prior to data analysis, outlying values for all variables were detected through box-plot analysis (SPSS v.20, Illinois, US). Participants that presented consistent outlying values throughout the ultra-marathon were removed. The data were examined using a two-way (stage x time) repeated-measures ANOVA (Friedman for gastrointestinal symptoms and perceptive thermal tolerance rating) (SPSS v.20, Illinois, US); except for energy, macronutrient, and water intake that was examined using a one-way ANOVA. Assumptions of homogeneity and sphericity were checked, and then appropriate adjustments to the degrees of freedom were made using the Greenhouse–Geisser correction method. Significant main effects were analysed using a post hoc Tukey's HSD test. For comparative purposes, a two-way ANOVA was also applied to sub-group analysis [UER vs CON, sexes (total and body mass corrected values), oral anti-inflammatory agent administration, and running speed (slow runners (SR, n= 11), who completed the entire distance of the ultra-marathon using a mixture of walk-

ing and running (overall mean speed <8 km/h) and fast runners (FR, n= 8), who completed the majority of the ultra-marathon distance running (overall mean speed ≥8 km/h)]. Pearson's coefficient correlation analysis was used to assess the associations between endotoxin with C-reactive protein and cytokine profile. Spearman's rank correlation analysis was used to assess the associations between blood-borne variables with self-reported gastrointestinal symptoms and perceptive thermal tolerance rating. The pro- to anti-inflammatory balance was determined by calculating the IL-1β:IL-10 and TNF-α:IL-10 ratios. The acceptance level of significance was set at $P < 0.05$.

RESULTS

Energy, macronutrient, and water ingestion

A difference in total daily energy intake was seen between stages in UER and CON ($P < 0.001$; Table 2). Total daily protein and carbohydrate intakes were higher ($P < 0.001$) in UER compared with CON at various stages of the ultra-marathon. Rate of carbohydrate intake during running did not differ between stages in UER. No difference in total daily water intake through foods and fluids was seen between stages in UER and CON (Table 2). Total daily water intake through foods and fluids was higher in UER on all stages compared with CON ($P < 0.001$). Rate of water intake through foods and fluids during running did not differ between stages in UER.

Body mass, plasma osmolality and volume change

Pre-and post-stage body mass did not significantly alter throughout competition in UER (pre-Stage 1: 71.7 ± 9.5 kg to pre-Stage 5: 71.2 ± 9.2 kg; and post-Stage 1: 69.8 ± 8.9 kg to post-Stage 5: 69.6 ± 9.5 kg) and CON (pre-Stage 1: 67.4 ± 15.0 kg to pre-Stage 5: 67.0 ± 14.8 kg. Stage 1 (2.5%) resulted in a greater exercise-induced body mass loss compared with Stages 2 to 5 in UER (2.0%, 1.0%, 2.2%, and 2.2%, respectively; $P < 0.001$). Pre-stage(range: 277 to 282 mOsmol/kg) and post-stage (range: 286 to 297 mOsmol/kg) P_{Osmol} did not differ between stages in UER. Pre-stage P_{Osmol} did not differ from CON throughout the ultra-marathon. Pre- to post-stage increases in P_{Osmol} ($P < 0.001$) were observed on all stages in UER. Relative to pre-Stage 1, resting pre-stage P_V increased significantly ($P < 0.001$) by Stage 2 (7.0 ± 1.4%) and peaked at Stage 5 (22.7 ± 2.0%) in UER; while no significant change in P_V was observed in CON. UER presented greater P_V change at pre-Stages 3 and 5 compared with CON ($P < 0.001$).

Tympanic temperature

Tympanic temperature (T_{tymp}) was within normal range pre- (overall mean: 36.3 ± 0.4°C) and post-stage (overall mean 37.0 ± 0.3°C) in UER. Pre-stage T_{tymp} gradually decreased ($P = 0.003$) in UER as the ultra-marathon progressed (pre-Stage 1: 36.5°C and pre-Stage 5: 36.0°C). No change in pre-stage T_{tymp} (36.7 ± 0.5°C) was observed for CON throughout the ultra-marathon. Pre- to post-stage increase (0.7°C; $P < 0.001$) in T_{tymp} was also observed in UER throughout the ultra-marathon. No difference in T_{tymp} was observed for sub-group comparisons.

Circulatory gram-negative bacterial endotoxin concentration

Pre-stage circulatory endotoxin concentration gradually increased ($P < 0.001$) in UER as the ultra-marathon progressed (Table 3, Figure 1A), peaking at Stage 5 (21%). No change in pre-stage circulatory endotoxin concentration was observed between Stages 1, 3, and 5 for CON. Pre- to post-stage increase ($P = 0.001$) in circulatory endotoxin concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 1B). No difference in circulatory endotoxin concentration was observed for sub-group comparisons.

Table 2: Energy, macronutrient, and water intake (through foods and fluids) of a control group and ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4	
	UER	CON	UER	CON	UER	CON	UER	CON
Total daily intake								
Energy (MJ/day)	16.3 ± 4.4^{aa}	10.4 ± 1.4	14.6 ± 4.2†	12.6 ± 1.1†	13.7 ± 4.0†	12.1 ± 1.7	15.2 ± 5.4^{aa}	12.6 ± 1.9†
Protein (g/day)	123 ± 42^{aa}	54 ± 8	109 ± 33^{aa}	77 ± 28	92 ± 31^{aa}	68 ± 6	106 ± 40^{aa}	81 ± 27
Carbohydrate (g/day)	590 ± 189^{aa}	400 ± 58	527 ± 190	487 ± 37	516 ± 145	469 ± 47	534 ± 184	484 ± 45
Fat (g/day)	116 ± 51	75 ± 12	106 ± 39	85 ± 10	93 ± 45	82 ± 27	120 ± 47	84 ± 23
Water (L/day)	7.5 ± 1.5^{aa}	2.8 ± 0.3	6.8 ± 2.9^{aa}	3.4 ± 0.2	6.6 ± 1.6^{aa}	3.3 ± 0.6	6.5 ± 2.8^{aa}	3.7 ± 0.7
During running								
Total carbohydrate (g)	121 ± 72‡		146 ± 60‡		137 ± 61‡		195 ± 91	
Carbohydrate intake rate (g/h)	27 ± 16		23 ± 10		29 ± 13		28 ± 14	
Total water (L)	3.7 ± 1.0‡		4.3 ± 1.9		3.6 ± 1.5‡		4.4 ± 1.7	
Water intake rate (ml/h)	819 ± 277		693 ± 269		797 ± 331		721 ± 256	

Mean ± SD: ultra-endurance runners (UER, n= 19) and control group (CON, n= 12). † $P < 0.05$ vs Stage 1, ‡ $P < 0.05$ vs Stage 4, ^{aa} $P < 0.01$ vs CON.

Table 3: Circulatory endotoxin, C-reactive protein concentrations, and plasma cytokine profile of a control group and ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment.

	Stage 1		Stage 2		Stage 3		Stage 4		Stage 5	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Gram-negative endotoxin (EU/ml)										
UER	2.8 ± 0.3	3.2 ± 0.8§	3.0 ± 0.4	3.1 ± 0.5	2.9 ± 0.4	3.5 ± 0.8**	3.3 ± 0.5††	3.6 ± 0.6*	3.4 ± 0.5††	3.6 ± 0.9
CON	3.0 ± 0.2				3.0 ± 0.4				3.1 ± 0.5	
C-reactive protein (μg/ml)										
UER	1.1 ± 1.7	1.6 ± 2.4	7.4 ± 5.3††	8.8 ± 5.4	10.0 ± 5.7$^{\dagger\dagger aa}$	9.6 ± 5.9	9.2 ± 5.9††	10.0 ± 6.7*	8.8 ± 5.6$^{\dagger\dagger aa}$	11.0 ± 6.4*
CON	1.4 ± 0.7				1.3 ± 0.8				1.3 ± 0.8	
IL-6 (pg/ml)										
UER	8.2 ± 4.5	27.9 ± 23.4**	20.8 ± 18.5††	20.7 ± 14.8	20.7 ± 16.8$^{\dagger\dagger aa}$	25.3 ± 24.3**	19.2 ± 14.1††	21.7 ± 12.6**	18.2 ± 11.6$^{\dagger\dagger aa}$	23.4 ± 13.1**
CON	7.5 ± 2.5				5.5 ± 7.1				6.5 ± 5.7	
IL-1β (pg/ml)										
UER	0.6 ± 0.3	1.0 ± 0.3**	1.1 ± 0.4††	1.1 ± 0.4	1.2 ± 0.4††	1.2 ± 0.4	1.1 ± 0.3††	1.4 ± 0.4**	1.2 ± 0.4††	1.4 ± 0.4*
CON	0.7 ± 0.2				1.2 ± 0.2†				1.3 ± 0.5††	
TNF-α (pg/ml)										
UER	3.1 ± 2.9	6.3 ± 5.0**	6.1 ± 4.5††	6.6 ± 3.7	6.9 ± 4.4$^{\dagger\dagger aa}$	6.1 ± 3.8*	6.5 ± 4.2††	8.1 ± 4.3**	7.1 ± 3.8$^{\dagger\dagger aa}$	8.3 ± 5.0
CON	1.3 ± 0.4				1.8 ± 0.7				2.3 ± 0.7	
IFN-γ (IU/ml)										
UER	9.3 ± 5.5	12.9 ± 6.0**	15.2 ± 6.8	16.9 ± 5.7	16.7 ± 6.7†	15.2 ± 5.2	16.2 ± 7.2†	19.9 ± 8.3**	18.8 ± 10.0††	22.7 ± 9.9**
CON	16.8 ± 5.5				14.3 ± 2.0				16.8 ± 5.1	
IL-10 (pg/ml)										
UER	0.7 ± 0.6	7.9 ± 10.1**	7.0 ± 10.8††	7.9 ± 9.1	9.0 ± 10.2$^{\dagger\dagger aa}$	8.0 ± 9.4	9.0 ± 12.2††	9.3 ± 10.1	8.2 ± 11.2$^{\dagger\dagger aa}$	10.9 ± 15.0*
CON	0.6 ± 0.1				1.4 ± 0.3				1.4 ± 0.7	
IL-1ra (pg/ml)										
UER	22.9 ± 8.0	70.3 ± 28.1**	39.8 ± 12.4††	61.0 ± 39.8**	45.5 ± 20.6$^{\dagger\dagger aa}$	53.9 ± 19.0*	37.9 ± 14.7††	56.3 ± 30.4**	47.1 ± 22.4$^{\dagger\dagger aa}$	63.2 ± 28.1**
CON	23.4 ± 7.3				36.4 ± 9.2				33.1 ± 9.3	

Mean ± SD: ultra-endurance runners (UER, n= 19) and control group (CON, n= 12). †† $P < 0.01$ and † $P < 0.05$ vs pre-Stage 1, ** $P < 0.01$ and * $P < 0.05$ vs respective pre-stage, § $P = 0.058$ vs respective pre-stage, ^{aa} $P < 0.01$ vs CON.

Plasma C-reactive protein concentration

Pre-stage plasma CRP concentration increased ($P < 0.001$) by Stage 2 in UER, and remained elevated thereafter (Table 3, Figure 2A), peaking at Stage 3 (889%). No change in pre-stage plasma CRP concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P = 0.014$) in plasma CRP concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 2B).Plasma CRP concentration was observed to be higher ($P < 0.001$) in males (pre-stage: 8.9 ± 3.6 µg/ml, post-stage: 10.1 ± 4.0 µg/ml) compared with females (pre-stage: 4.2 ± 2.6 µg/ml, post-stage: 4.3 ± 2.5 µg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass ($P < 0.001$). No differences in other sub-group comparisons were observed.

Plasma interleukin-6 concentration

Pre-stage plasma IL-6 concentration increased ($P = 0.006$) by Stage 2 (152%) in UER and remained elevated thereafter (Table 3, Figure 3A). No change in pre-stage plasma IL-6 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P < 0.001$) in plasma IL-6 concentration was also observed in UER (Table 3, Figure 3B). Post-stage plasma IL-6 concentration was observed to be higher ($P = 0.054$) in males (26.7 ± 20.5 pg/ml) compared with females (17.6 ± 5.7 pg/ml) throughout the ultra-marathon. However, when corrected for body mass no substantial difference was observed. There was also a tendency for higher ($P = 0.094$) pre-stage plasma IL-6 concentration in SR (20.4 ± 15.2 pg/ml) compared with FR (13.3 ± 5.4 pg/ml) throughout the ultra-marathon. No difference in plasma IL-6 concentration was observed for oral anti-inflammatory administration.

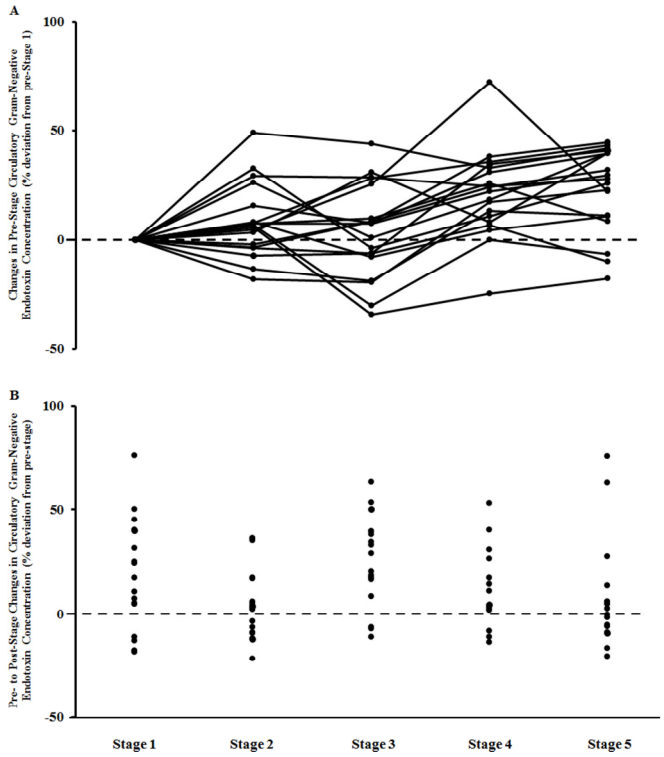

Figure 1. Individual changes in pre-stage resting (A) and pre- to post-stage (B) circulatory gram-negative endotoxin concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19).

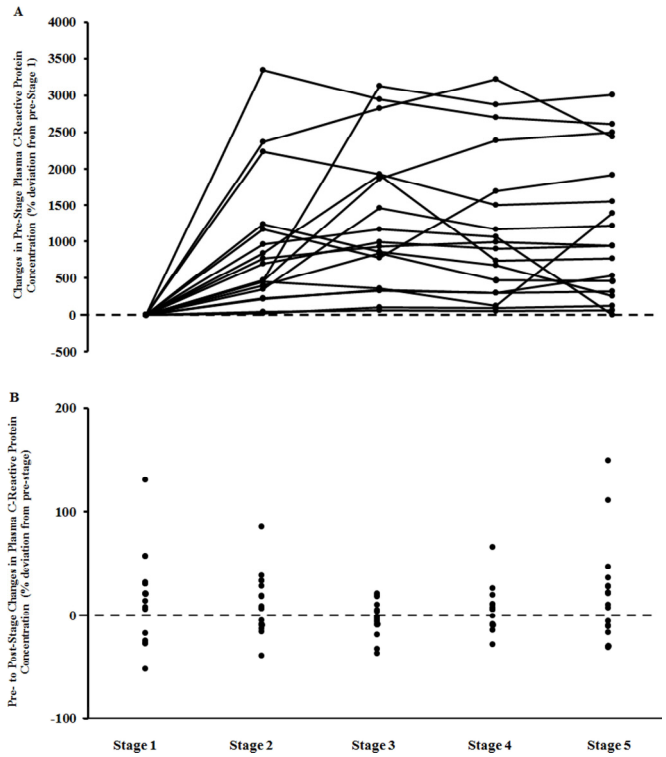

Figure 2. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma C-reacitve protein concentration of ultra-endurance runner participating in a 230 km mulit-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, n=1 at 1827%; Stage 2, n=1 at 429% and n=1 at 620%; and Stage 4, n=1 at 1192%

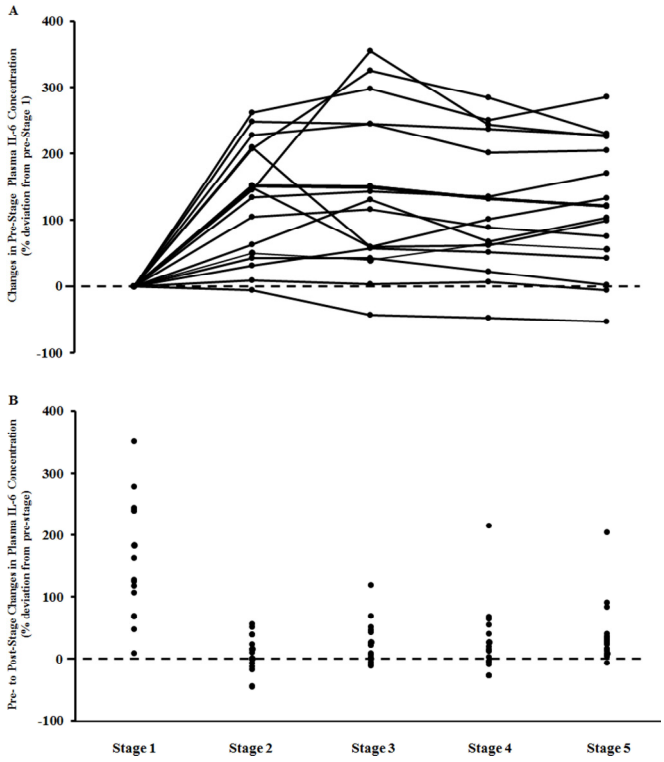

Figure 3. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-6 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes; Stage 1, n=1 at 605%, n=1 at 704%, and n=1 at 1205%

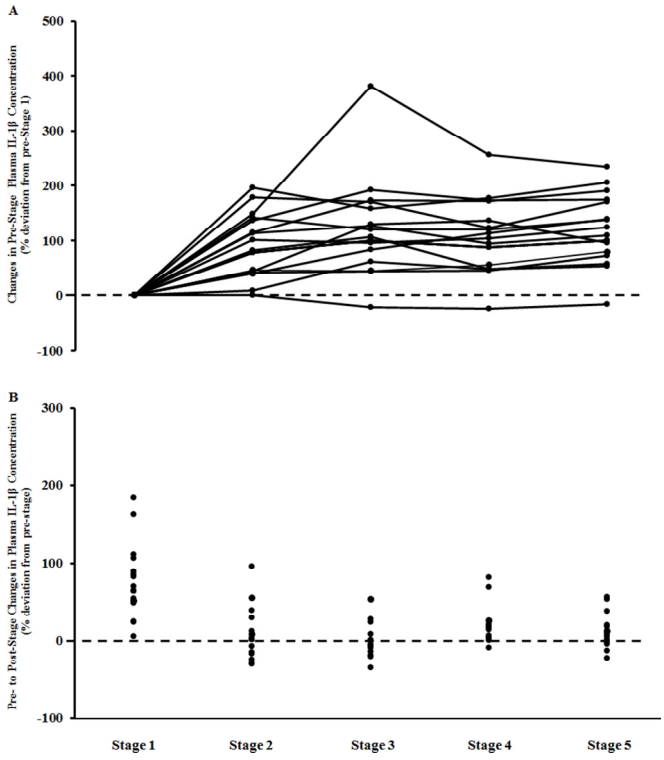

Figure 4. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-1β concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19).

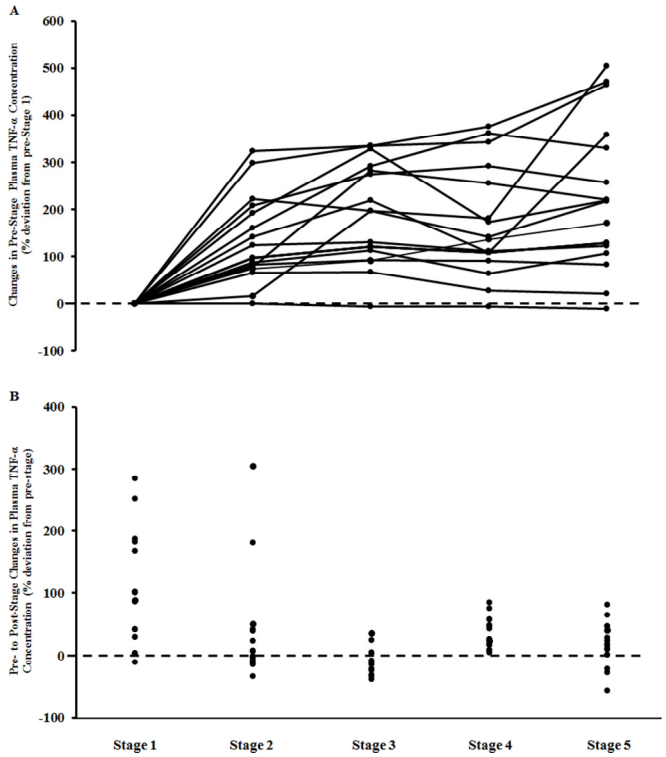

Figure 5. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma TNF-α concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19).

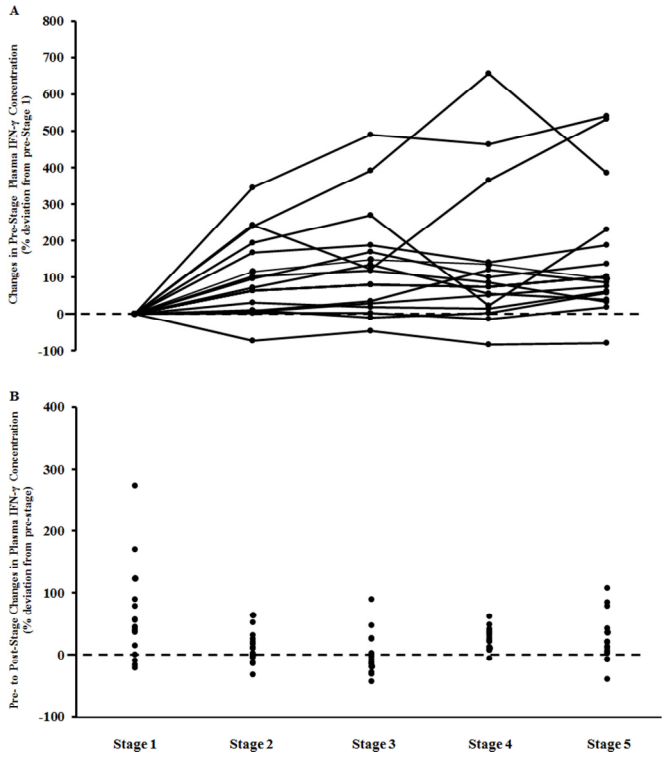

Figure 6. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IFN-γ concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, n=1 at 533%; and Stage 5, n=1 at 511%

Plasma interleukin-1β concentration

Pre-stage plasma IL-1β concentration increased ($P < 0.001$) by Stage 2 in UER (Table 3, Figure 4A) and remained elevated thereafter, peaking at Stage 5 (95%). While an unexpected increase was also observed for CON ($P < 0.001$), whereby plasma IL-1β concentration increased by Stage 3 and remained elevated there after. Pre- to post-stage increase ($P < 0.001$) in plasma IL-1β concentration was also observed in UER (Table 3, Figure 4B). Pre- and post-stage plasma IL-1βconcentration was observed to be lower ($P = 0.014$) in males (pre-stage: 1.0 ± 0.2 pg/ml, post-stage: 1.1 ± 0.2 pg/ml) compared with females (pre-stage: 1.2 ± 0.4 pg/ml, post-stage: 1.3 ± 0.4 pg/ml) throughout the ultra-marathon. However, when corrected for body mass no substantial difference was observed. There was a tendency for higher ($P = 0.054$) pre-stage plasma IL-1β concentration in SR (1.1 ± 0.2 pg/ml) compared with FR (0.9 ± 0.5 pg/ml) throughout the ultra-marathon. No difference in plasma IL-1β concentration was observed for oral anti-inflammatory administration.

Plasma tumour necrosis factor-α concentration

Pre-stage plasma TNF-α concentration increased ($P < 0.001$) at Stage 2 in UER (Table 3, Figure 5A) and remained elevated thereafter, peaking at Stage 5 (168%). No change in pre-stage plasma TNF-α concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P < 0.001$) in plasma TNF-α concentration was also observed in UER (Table 3, Figure 5B). Pre- and post-stage plasma TNF-α concentration was observed to be lower ($P < 0.001$) in males (pre-stage: 4.8 ± 1.8 pg/ml, post-stage: 6.1 ± 2.8 pg/ml) compared with females (pre-stage: 8.4 ± 6.1 pg/ml, post-stage: 9.3 ± 6.1 pg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (pre-stage: $P < 0.001$, post-stage: $P = 0.001$). Pre-stage plasma TNF-α concentration was observed to be higher ($P = 0.016$) in SR (6.9 ± 1.8 pg/ml) compared with FR (4.7 ± 6.1 pg/ml) throughout the ultra-marathon. No difference in plasma TNF-α concentration was observed for oral anti-inflammatory administration.

Plasma interferon-γ concentration

Pre-stage plasma IFN-γ concentration increased ($P < 0.001$) at Stage 3 in UER(Table 3, Figure 6A), peaking at Stage 5 (102%). No change in pre-stage plasma IFN-γ concentration was observed for CON. Pre- to post-stage increase ($P < 0.001$) in plasma IFN-γ concentration was also observed in UER (Table 3, Figure 6B). Pre-stage plasma IFN-γ concentration was observed to be higher ($P = 0.016$) in SR (16.7 ± 6.4 IU/ml) compared with FR (13.2 ± 9.2 IU/ml) throughout the ultra-marathon. No differences in other sub-group comparisons were observed.

Plasma interleukin-10 concentration

Pre-stage plasma IL-10 concentration increased ($P = 0.011$) by Stage 2 in UER, and remained elevated thereafter (Table 3, Figure 7A), peaking at Stage 3 (1271%). No change in pre-stage plasma IL-10 concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P = 0.020$) in plasma IL-10 concentration was also observed in UER (Table 3, Figure 7B). Pre- and post-stage plasma IL-10

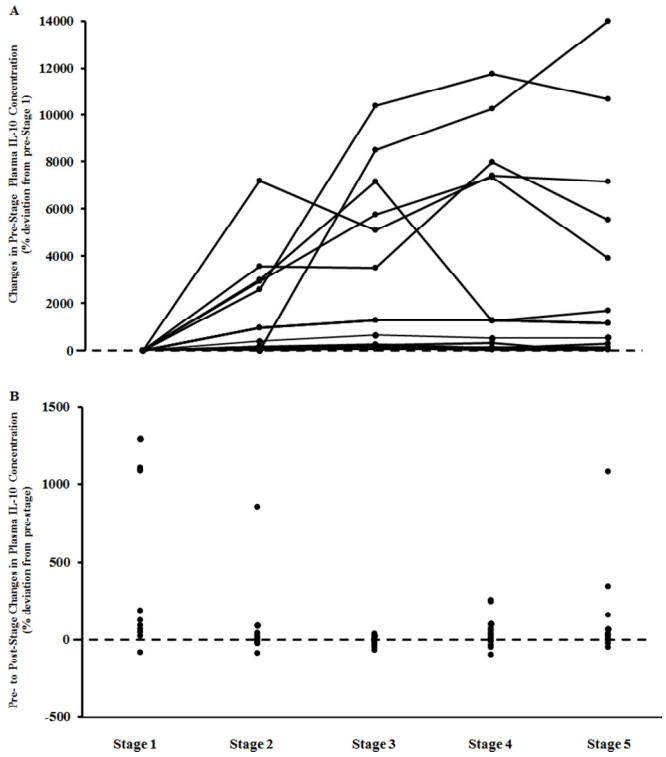

Figure 7. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-10 concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 1, n=1 at 2305%, n=1 at 3781%, n=1 at 4761%, and n=1 at 6226%; and Stage 3, n=1 at 1810%.

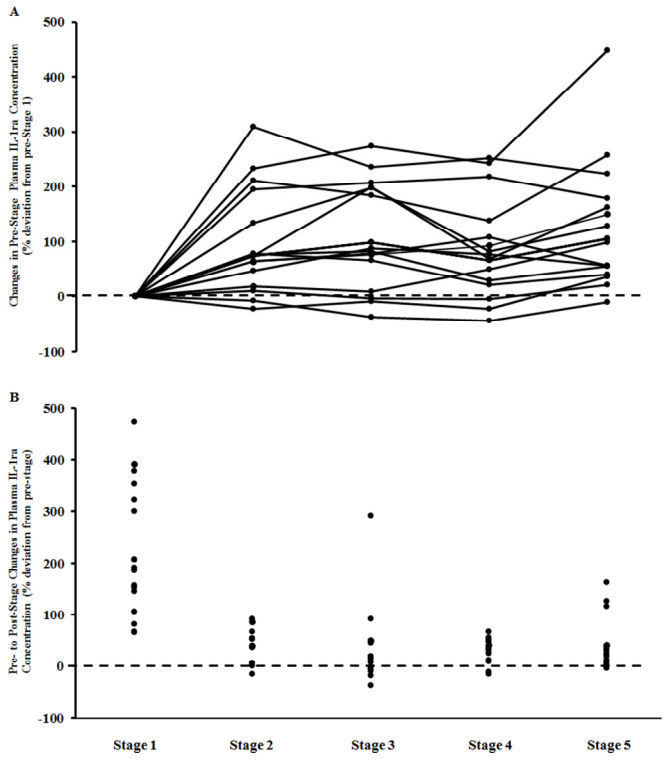

Figure 8. Individual changes in pre-stage resting (A) and pre- to post-stage (B) plasma IL-1ra concentration of ultra-endurance runners participating in a 230 km multi-stage ultra-marathon competition conducted in a hot ambient environment. Individual ultra-endurance runner responses (•; n=19). Additional individual responses outside the figure range for pre- to post-stage changes: Stage 2, n=1 at 509%; and Stage 4, n=1 at 696%

concentration was observed to be lower ($P < 0.001$) in male ultra-runners (pre-stage: 4.1 ±4.1pg/ml, post-stage: 6.3 ± 7.6pg/ml) compared with female ultra-runners (pre-stage: 12.6 ± 13.6 pg/ml, post-stage: 14.2 ± 14.3 pg/ml) throughout the ultra-marathon. This difference was also observed when corrected for body mass (pre-stage: $P < 0.001$, post-stage: $P = 0.001$). No differences in other sub-group comparisons were observed.

Plasma interleukin-1 receptor antagonist concentration

Pre-stage plasma IL-1ra concentration gradually increased ($P < 0.001$) in UER as the ultra-marathon progressed (Table 3, Figure8A), peaking at Stage 5 (106%). No change in pre-stage plasma IL-1ra concentration was observed between Stages 1, 3, and 5 for CON, and levels were lower than UER pre-Stages 3 and 5 ($P < 0.001$). Pre- to post-stage increase ($P < 0.001$) in plasma IL-1ra concentration was also observed in UER throughout the ultra-marathon (Table 3, Figure 8B). No difference in plasma IL-1ra concentration was observed for sub-group comparisons.

Pro-inflammatory to anti-inflammatory cytokine ratio

Pre-stage IL-1β:IL-10 and TNF-α:IL-10 ratios decreased in UER as the ultra-marathon progressed, but failed to reach significance ($P > 0.05$). Ratios in UER did not differ from CON pre-Stages 3 and 5. No change in pre- to post-stage IL-1β:IL-10 and TNF-α:IL-10 ratios were observed in UER. No differences in sub-group comparisons were observed for ratios.

Gastrointestinal symptoms and thermal tolerance rating

Gastrointestinal symptoms were a common feature amongst UER sampled for endotoxin and cytokine responses; with 58% reporting at least one severe gastrointestinal symptom (including 33% of sampled UER reporting nausea) during competition, while no gastrointestinal symptoms were reported by CON. No differences in the reported rates of severe gastrointestinal symptoms were observed between stages in UER. Perceptive thermal tolerance rating in UER improved as the ultra-marathon progressed ($P = 0.005$), with no change in CON. Additionally, no heat related illnesses were observed in UER and CON throughout the ultra-marathon.

Correlation analysis

Small but significant positive correlations were observed between pre-stage circulatory gram-negative bacterial endotoxin concentration with pre-stage plasma CRP ($r= 0.343$, $P = 0.001$), IL-6 ($r= 0.246$, $P = 0.019$), IL-1β ($r = 0.305$, $P = 0.003$), TNF-α ($r= 0.370$, $P < 0.001$), IFN-γ ($r= 0.282$, $P = 0.007$), IL-10 ($r= 0.309$, $P = 0.003$), and IL-1ra ($r= 0.268$, $P = 0.011$) concentrations; and between post-stage circulatory endotoxin concentration with post-stage plasma CRP concentration ($r= 0.213$, $P = 0.043$). No correlations were observed between circulatory gram-negative bacterial endotoxin and plasma cytokine concentrations with severe gastrointestinal symptoms (including nausea). A strong relationship between perceptive thermal tolerance rating and severe gastrointestinal symptoms was observed ($r= -0.665$, $P < 0.001$), whereby lower perceptive tolerance rating to heat was associated with greater reports of severe gastrointestinal symptoms in UER. However, no correlations were observed between circulatory gram-negative bacterial endotoxin and

plasma cytokine concentrations with perceptive thermal tolerance rating.

DISCUSSION

The current study aimed to determine circulatory endotoxin concentration and cytokine profile of ultra-endurance runners throughout a multi-stage ultra-marathon competition conducted in hot and dry environmental conditions; and determine the relationship between these responses with severe gastrointestinal symptoms and perceptive thermal tolerance rating. Findings confirm that consecutive days of exertional-heat stress resulted in a modest and sustained rise in both resting and post-stage circulatory gram-negative bacterial endotoxin concentration. Despite overnight recovery between stages, results show that pyrogenic pro-inflammatory cytokines (i.e., IL-6, IL-1β, TNF-α, and IFN-γ) increased in response to exertional-heat stress and remained elevated at rest throughout competition. These responses however were counteracted by compensatory anti-inflammatory cytokine responses that predominated throughout the ultra-marathon (1, 48). Strength of the findings is supported by the control group showing no change in circulatory endotoxin concentration and cytokine profile (except IL-1β) throughout the ultra-marathon period.

Although the characteristics of the cytokine responses are similar to that observed during an acute infectious episode and in accordance with the aetiology of exertional-heat illnesses (i.e., exertional-heat stroke and SIRS), no diagnosis of heat related illnesses by a qualified Sports Physician were established in UER along the ultra-marathon. Severe gastrointestinal symptoms reported by UER were generally high; but in contrast to our hypothesis, no relationship between severe gastrointestinal symptoms with circulatory gram-negative endotoxin and plasma cytokine concentrations were observed. Thermal strain (T_{tymp} and perceptive thermal tolerance rating) appeared to improve as the ultra-marathon progressed, and is in accordance with heat acclimatization (10). A strong relationship between severe gastrointestinal symptoms and perceptive thermal tolerance rating was evident, whereas no relationship between perceptive thermal tolerance rating with circulatory gram-negative endotoxin and plasma cytokine concentrations were observed. Even though ultra-endurance runners presented no heat related medical issues during the current study, the endotoxin and cytokine responses observed provide a novel and valuable insight into a potentially high risk situation, whereby individuals with predisposition and multiple risk factors for deranged pro-inflammatory and compensatory anti-inflammatory responses are likely to develop consequential clinically significant issues. For example, the degree of cytokinaemia observed has been linked to the aetiology of heat stroke, septic shock, autoimmune disease, gastrointestinal disease, and chronic fatigue (7, 34, 26, 29, 43).

Plasma CRP concentration, which is normally low and undetectable in the circulation of healthy populations, is an acute phase reactant that dramatically rises in the presence of inflammation (e.g., induced by trauma, bacterial infection, and (or) inflammatory responses). In the current study, plasma CRP concentrations of UER peaked by Stage 2 and remained elevated thereafter; while no change in CON was observed. These responses are similar to that observed during a six-days (total distance: 468 km) endurance mountain bike event (44), whereby plasma concentrations were significantly elevated at rest for the duration of the event. However, the level of rise in plasma CRP concentration was less pronounced in comparison to levels (1.9 to 18.4 mg/L) reported in ultra-endurance athletes after six-days of track based running race totalling 622 km (16), and after the 246 km Spartathlon ultra-marathon race (0.65 to 97.3 mg/L) (27). Taking into account the responsive nature of CRP to general inflammation, the wide variations in plasma concentrations observed in the current and previous ultra-endurance studies likely reflect multi-influential stimulating factors, such as intestinal originated bacterial endotoxin leakage into circulation ($r= 0.343$, $P = 0.001$) and soft tissue damage (e.g., exertional rhabdomyolysis) (8). It is possible that persistent elevations in CRP at rest in UER may contribute to progressive perceptions of fatigue and subsequent impaired performance over the given time course (31, 43). Interestingly, on this occasion, male ultra-runners showed high plasma CRP concentration throughout competition compared with female ultra-runners, suggesting greater general inflammatory presence in males. The reason for this observation is unclear; it is however likely to be attributed to muscle originated responses (50), since greater plasma IL-6 concentrations concomitant with lower IL-1β and TNF-α responses were observed in male ultra-runners compared with female ultra-runners. Due to practical limitations in monitoring parameters after competition (i.e., ultra-endurance athletes returning to country of origin after cessation of the ultra-marathon), the current study was not able to determine the recovery time course of CRP. However, such responses have been shown to remain elevated above pre-exercise values for a considerable period of time (i.e., up to nineteen-days after an Ironman triathlon event)(31), suggesting time course for full recovery of altered inflammatory status is considerably delayed.

In comparison with previous endurance and ultra-endurance studies observing mild (e.g., marathon, 160 km ultra-marathon, and Ironman distance triathlon: 5 to 15 pg/ml) (6, 22) and substantial (e.g., 89.4 km ultra-marathon whereby 81% of runners had concentrations >100 pg/ml and an ultra-distance triathlon reporting 81 to 294 pg/ml) (3, 4) increases in circulatory endotoxin concentrations, the current ultra-marathon resulted in modest increases in post-stage circulatory endotoxin concentrations throughout competition (i.e., 30 pg/ml average increase from pre- to post-stage, with the highest individual increase observed at 92pg/ml). A novel finding was the gradual increases in resting levels as the ultra-marathon progressed (i.e., 60pg/ml average increase from Stage 1 to 5, with 32% of UER showing concentrations >100 pg/ml and the highest individual increase observed at 130 pg/ml), possibly attributed to a delayed and sustained intestinal leakage upon exercise cessations, which is accompanied by splanchnic reperfusion (57). The cumulative affect observed as the ultra-marathon progressed suggests a reduced tolerance for exertional-heat stress induced endotoxin leakage, subsequent to anti-endotoxin antibodies not restoring to their optimal level on consecutive occasions (25). For example, depressed anti-endotoxin antibodies have been reported after a marathon race, which remained below pre-exercise values for 24 hours (6). More over, a100-fold range difference in

endotoxin neutralizing capacity in plasma has been observed between individuals (61), likely associated with training adaptations (3). Indeed, higher circulating concentrations of endotoxin and anti-endotoxin antibodies have been observed in untrained compared with trained individuals (22, 47).

The proposed gained adaptation to endotoxin tolerance in trained individuals is likely attributed to repetitive endotoxin challenge resulting from exercise-stress inducing endotoxin intestinal leakage and subsequent "self-immunisation" (3, 4). Therefore during the current ultra-marathon, it is possible that the experience level of ultra-runners and frequent endotoxin exposure induced as part of their competition preparation may have resulted in training adaptations favouring an attenuated circulatory endotoxin peak along competition (i.e., ultra-runners developing adaptations that enhance resistance and resilience to enteric pathogenic endotoxin exposure); such plausibility, however, warrants investigation. Favourable adaptations would reduce the risk of developing clinically significant issues associated with endotoxaemia and subsequent cytokinaemia during consecutive days of exertional-heat stress with or without additional stressors. Conversely, inadequate training and not being physically prepared for such an extreme event would potentially increase the risk. Even though no differences in endotoxin was seen between running speeds, pre-stage plasma IL-6, IL-1β, TNF-α, and IFN-γ concentrations were higher in SR throughout the ultra-marathon compared with FR; potentially suggesting greater intestinally originated endotoxin exposure above clearance capacity in less trained ultra-runners. This explanation however also warrants further investigation (e.g., role of intestinal originated endotoxin in training adaptions- immune competence), and may provide valuable findings into the role of endotoxin leakage in physiological adaptations to exercise stress, especially in environmental extremes. Moreover, it has also been suggested that plasma endotoxin concentrations may reach equilibrium during endurance exercise, whereby endotoxin influx from the gastrointestinal tract into circulation matches endotoxin clearance by anti-endotoxin antibodies (5, 47); which may in part explain why only modest fluctuations in circulatory endotoxin concentrations were observed.

The current study observed increases in resting pre-stage and pre- to post-stage plasma IL-6, IL-1β, TNF-α, and IFN-γ concentrations that remained elevated throughout competition; while no change in CON was observed (except for IL-1β). The cytokine profile of the current study mirrors that of an acute infectious episode, and is similar to pro-inflammatory cytokine responses seen after endotoxin (e.g., lipopolysaccharide) infusion in both animal (17) and human (55) models. These results are in accordance with previous endurance based (e.g., marathon) experimental designs observing modest increases in plasma IL-6, IL-1β, and TNFα concentrations (35, 51); which were also accompanied by compensatory anti-inflammatory responses (i.e., increase in plasma IL-10 and IL-1ra concentrations). The current ultra-marathon also resulted in substantial increases in resting pre-stage and pre- to post-stage anti-inflammatory cytokines that remained elevated throughout competition to a similar degree as compensatory anti-inflammatory syndrome (1, 48). It is possible the anti-inflammatory properties of IL-10, with adjunct IL-1ra, may have restricted the magnitude of pro-inflammatory cytokine production along competition. Interestingly, no differences in pro- and anti-inflammatory cytokine responses were observed between UER that ingested and did not ingest oral anti-inflammatory agents. This observation may suggest that exposure to exertional-heat stress induce by the event far outweighs any impact of inconsistent use of low dose anti-inflammatory medication on cytokine responses, and questions the efficacy of such inconsistent administration of anti-inflammatory pharmaceutical agents within medical management of ultra-runners during extreme events.

In well trained individuals, where exertional-heat stress is better tolerated (10), anti-inflammatory responses predominated, off-setting potential clinically significant episodes associated with cytokinaemia. It is however concerning that inadequately trained individuals may not present such competent anti-inflammatory responses, and may be a prime risk population for developing heat illness from immune aetiology (i.e., exertional-heat stroke, SIRS) (25, 32, 39). Indeed, SR presented a higher resting pro-inflammatory cytokine profiles compared with FR. It also needs to be taken into consideration that SR were on the course routes for a greater amount of time than FR; and thus SR may have been exposure to greater volumes of exertional-heat stress and a time-dependant effect on cytokine production during recovery may produce delayed anti-inflammatory responses in SR. Moreover, an age difference existed between UER and CON, but only in the female participants. It is well established that immune responses decline with age. Depressed responses are commonly observed in the elderly population, with and without medical issues, compared with the healthy adult population (59). It is, however, unlikely that the healthy recreational middle-aged ultra-endurance female population of the current study would present altered immune responses due to their age.

The recovery time course of cytokine responses after the ultra-marathon was not determined on this occasion due to practical limitations; however previous ultra-endurance studies (e.g., long-distance triathlon and ultra-marathon running) have observed variations in cytokine responses during the recovery period. For example, IL-6 and TNF-α returned to baseline by 24 hours after a 50 km ultra-marathon (28); whilst IL-6 returned to baseline values 16 hours, with no significant changes observed in TNF-α, after a long-distance triathlon (22). Furthermore, on cessation of two endurance events of similar duration (long-distance triathlon and 100 km run), IL-6, IL-10, and IL-1ra peaked after competition, returning to baseline values seven days after the events(18). Whereas after a long distance triathlon, IL-6 remained elevated on day one (345%) and day five (79%); while IL-10 was elevated on day one (37%), declining by 4% below pre-competition concentrations on day five (31).These observations suggest the time course for full recovery of altered cytokine profile in response to extreme events are considerably delayed, and may play a role in the aetiology of undefined underperformance and chronic fatigue syndromes (29, 43). The potential role of extreme event induced immune perturbations initiating autoimmune disease in individual with predisposition warrants attention, since chronic elevations in cytokine responses are reported in many autoimmune condition (e.g., systemic lupus erythematosus, fibromyalgia, myalgicencephalomyelitis, idiopathic inflammatory myopathies, arthritic conditions, and inflammatory bowel diseases) (7, 29, 52, 63).

Despite amplified cytokine responses similar to that of an acute infectious episode and in accordance with the aetiology of heat-related illnesses, none of the current n= 19 UER were diagnosed with heat related illnesses. Previously, only n= 1 ultra-endurance runner competing in the five-days 2010 Al Andalus Ultra-Trail race suffered heat-related problems (46), reported to be due to ultra-runners experience (e.g., training status), the hot environmental conditions, and the nature of the race course (e.g., limited shade availability). Perceptive thermal comfort rating improved as the competition progressed in the sampled population, and likely reflected heat acclimatization as evidenced by P_V increases and reductions in T_{tymp} as the ultra-marathon progresses, with no changes in CON being observed (10, 13). Interestingly, the two ultra-runners that originating from countries with hot ambient conditions at the time of competition showed similar circulatory endotoxin and cytokine responses to the main cohort, with substantial increases in P_V indicative of heat acclimatization still being observed in these ultra-runners (pre-stage 1 to pre-stage 5: ultra-runner 1 = 30.4% and ultra-runner 2 = 24.9%); suggesting exertional stress is an essential key feature of heat adaptions (10). In view of the unique and challenging characteristics of ultra-marathon competitions (i.e., prolonged physical exertion, sleep deprivation, environmental extremes, acute periods of under-nutrition and hypohydration) and associated factors (i.e., training status, inadequate rest, tolerated injury and trauma) having the potential to disturb intestinal integrity and promote cytokine-mediated inflammatory responses, the maintenance of hydration status in the majority of runners, thermoregulatory-induced adaptations, and cooling behaviours throughout competition may have contributed to improved heat tolerance despite prolonged exposure to exertional-heat stress (2, 10, 13, 54).

The systemic endotoxin and cytokine responses seen in the current study have previously been associated with symptomatic manifestations of gastrointestinal symptoms, commonly associated with prolonged exposure to exertional-heat stress (22, 23, 36, 38, 40, 56). For example, gastrointestinal symptoms, such as nausea and vomiting, have been observed in endurance athletes presenting endotoxaemia after an Ironman triathlon event (22). In contrast to previous studies, no associations between gastrointestinal symptoms with circulatory endotoxin and plasma cytokine concentrations were observed on this occasion. However, a strong relationship (r= -0.665) between severe gastrointestinal symptoms and perceptive thermal tolerance rating was confirmed ($P < 0.001$). These results suggest that severe gastrointestinal symptoms likely originate from heat stress during exercise, potentially through splanchnic hypoperfusion and hypoxia (i.e., exercising in the heat creating greater redistribution of blood flow away from the splanchnic area) (53, 57, 58). Such physiological changes in splanchnic blood flow, which have symptomatic outcomes, likely lead to disturbances in intestinal mucosal and epithelial integrity that enhances local enteric endotoxin leakage and subsequent cytokinaemia; and not necessarily that endotoxaemia and cytokinaemia induced gastrointestinal symptoms.

To date, it is still unknown how the degree of exertional stress, with or without environmental extremes and between different exercise modes, impacts overall gastrointestinal integrity. Additionally, does the nutritional and hydration status before exertional stress, and the changes that occurs to status during physical exertion, influence the degree of gastrointestinal disturbance? Conducting a set of controlled laboratory experiments assessing varying ambient temperatures, exercise intensities, durations and modes whilst assessing gastrointestinal integrity measures (57) would contribute substantially to the current knowledge base and provide a foundation to investigate potential strategies to overcome gastrointestinal complications associated with exertional-heat stress. For example, dietary strategies during physical exertion, development of gut training protocols, functional foods, heat acclimation protocols, external pre-cooling (e.g., cold water bath or cooling vest) and (or) during physical exertion internal cooling (e.g., cold beverages) are proposed strategies that may attenuate exertional-heat stress induced gastrointestinal perturbations. Indeed, due to gut plasticity, there is potential for the gastrointestinal tract to adapt to a challenge load ('training the gut') (21). Whereas, previous investigations have demonstrated favourable effect of prebiotic oligosaccharides (e.g., inulin and oligofructose) and probiotic bacteria (e.g., *Lactobacillus* casei and *Bifidobacterium*) on markers of gastrointestinal integrity; albeit within inflammatory diseases of the gut (45). Knowledge into the impact of such biotics on gut integrity during exertional-heat stress is, however, scarce. Anecdotal evidence during the current study highlighted that ultra-runners who consistently consumed commercial probiotic product in the week leading up to the ultra-marathon presented no incidence of gastrointestinal symptoms; suggesting further controlled investigation is needed to confirm any beneficial effects of biotics on gastrointestinal integrity in response to exertional-heat stress.

CONCLUSION

In conclusion, multi-stage ultra-marathon competition in the heat resulted in a modest circulatory endotoxaemia accompanied by a pronounced pro-inflammatory cytokinaemia and compensatory anti-inflammatory responses. No incidences of exertional-heat illnesses were evident throughout competition. Even though severe gastrointestinal symptoms were reported, no relationships with blood borne indices were identified. The expected exacerbated cytokine responses were possibly attenuated by the maintenance of hydration status in the majority of runners, and as well thermoregulatory-induced adaptations and behaviours adopted by participants. The findings from the current study suggest that appropriate informed training (e.g., physically trained to complete the required distance in environmental extremes) and competition preparation (e.g., effective and evidence-based heat acclimation protocols, hydration maintenance and (or) cooling strategies) may help prevent significant exertional-heat related sub-clinical and clinical manifestations from occurring in high risk ultra-endurance runners competing in extreme events.

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Changes of thioredoxin, oxidative stress markers, inflammation and muscle/renal damage following intensive endurance exercise

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ABSTRACT

Thioredoxin (TRX) is a 12 kDa protein that is induced by oxidative stress, scavenges reactive oxygen species (ROS) and modulates chemotaxis. Furthermore it is thought to play a protective role in renal ischemia/reperfusion injury. Complement 5a (C5a) is a chemotactic factor of neutrophils and is produced after ischemia/reperfusion injury in the kidney. Both TRX and C5a increase after endurance exercise. Therefore, it may be possible that TRX has an association with C5a in renal disorders and/or renal protection caused by endurance exercise. Accordingly, the aim of this study was to investigate relationships among the changes of urine levels of TRX, C5a and acute kidney injury (AKI) caused by ischemia/reperfusion, inflammatory responses, and oxidative stress following intensive endurance exercise. Also, we applied a newly-developed measurement system of neutrophil migratory activity and ROS-production by use of ex vivo hydrogel methodology with an extracellular matrix to investigate the mechanisms of muscle damage. Fourteen male triathletes participated in a duathlon race consisting of 5 km of running, 40 km of cycling and 5 km of running were recruited to the study. Venous blood and urine samples were collected before, immediately following, 1.5 h and 3 h after the race. Plasma, serum and urine were analyzed using enzyme-linked immunosorbent assays, a free radical analytical system, and the ex vivo neutrophil functional measurement system. These data were analyzed by assigning participants to damaged and minor-damage groups by the presence and absence of renal tubular epithelial cells in the urinary sediments. We found strong associations among urinary TRX, C5a, interleukin (IL)-2, IL-4, IL-8, IL-10, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1. From the data it might be inferred that urinary TRX, MCP-1 and β-N-acetyl-D-glucosaminidase (NAG) were associated with renal tubular injury. Furthermore, TRX may be influenced by levels of IL-10, regulate chemotactic activity of C5a and IL-8, and control inflammatory progress by C5a and IL-8. In the longer duration group (minor-damage group), circulating neutrophil count, plasma concentration of myeloperoxidase (MPO) and serum concentration of myoglobin were markedly increased. In the higher intensity group (damaged group), neutrophil activation and degranulation of MPO might be inhibited, because not only was ROS production observed to be higher, but also antioxidant capacity and anti-inflammatory cytokines were increased. Critically, the newly-developed ex vivo methodology corroborated the neutrophil activation levels in the two groups of participants.

Key words: TRX, C5a, ROS, antioxidant, anti-inflammation, acute kidney injury (AKI)

INTRODUCTION

Endurance exercise not only promotes the generation of reactive oxygen species (ROS), mainly as a result of increased oxygen utilization, ischemia-reperfusion and leukocyte activation, but also consumes endogenous antioxidants (2, 3, 54). This unbalanced state induces oxidative stress and cellular tissue damage in the body. Oxidative stress-induced injury and inflammation are important considerations for athletes.

Recently, it has been reported that aerobic exercise interventions can have a positive effect on chronic renal failure. In the patients with chronic kidney diseases including those undertaking dialysis therapies or in receipt of kidney transplant, it was demonstrated that aerobic exercise reduced oxidative stress and improved quality of life (18, 27). For

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these reasons, exercise is recommended for these patients (26). The underlying mechanisms for the direct improvement of renal functions due to aerobic exercise have not yet been identified. However, it has been suggested that moderate exercise may promote nitric oxide production, and inhibit the renin-angiotensin-aldosterone system, or improve renal blood flow by modifying dyslipidemia and intraglomerular pressure (29, 33, 48). However, the acute effects of exercise are different from the chronic effects of exercise training and therefore these exercise modes should be considered separately (33). In acute exercise, it is known that deterioration of glomerular filtration rate and oliguria are induced following endurance exercise (69), and that intensive endurance exercise may cause rhabdomyolysis-induced acute renal failure (10, 52). Rhabdomyolysis may manifest in acute renal failure due to acute tubular necrosis leading to deterioration of glomerular filtration rate. This is induced by glomerulus degeneration and reduced renal blood flow leading to a reduced supply of oxygen and energy, thereby resulting in ischemic vascular endothelial cell damage (6). In animal models of acute renal failure, ROS and lipid peroxides increase, whereas scavengers of ROS such as glutathione and superoxide dismutase (SOD) are decreased in renal tissue (51). It was reported that ROS, such as superoxide anion and hydroxyl radical, contributed to the onset of acute renal failure through reduced renal blood flow and disorder of tubular cells (55). Thus, acute endurance exercise-induced oxidative stress may cause renal failure, and therefore it is important to evaluate oxidative stress in this context.

Currently, oxidative stress is evaluated using metabolic oxidation end products, because the real time evaluation of this phenomenon is difficult. Recently, it was reported that thioredoxin (TRX) is secreted by renal tubular cells due to ischemia and oxidative stress (30). Furthermore, its usefulness as a specific biomarker of acute kidney injury (AKI) caused by ischemia and oxidative stress is being established (30). TRX is a small (12 kDa) multifunctional protein that contains a redox-active dithiol/disulfide in the conserved active site sequence: -Cys-Gly-Pro-Cys- (20). TRX is stress-inducible, and it protects cells from various types of stress (45, 46). TRX functions as an antioxidant, as an inhibitor of chemotaxis, and as a redox-regulating protein in signal transduction (12, 21, 22, 57). TRX eliminates hydrogen peroxide and acts as a radical scavenger, as demonstrated where recombinant TRX has a protective activity against hydrogen peroxide cytotoxicity (23, 38, 45). It is reported that elevated serum TRX in various diseases is associated with increased oxidative stress (25, 32, 40, 46, 47, 60, 61, 83). Moreover, serum levels and expression levels of TRX increase following endurance exercise (36, 70, 85). Recently, it was reported that the urine level of TRX is a specific marker for ischemia and oxidative stress-induced acute renal failure, because it is secreted from renal tubular epithelial cells in response to ischemia/reperfusion injury in renal tissue (30).

Another protein associated with AKI is complement 5a (C5a) (1). C5a is a multifunctional proinflammatory mediator, and a chemotactic factor, which increases the permeability of blood vessels and promotes the migration of leukocytes towards inflammatory sites and their generation of ROS (14, 35). It is reported that C5a increases and causes inflammation following marathon race (8). C5a is an important pathogenic factor in renal ischemia/reperfusion injury (1). The role of C5a in the tubulointerstitial component is demonstrated in an experimental model of progressive glomerulonephritis (80). Indeed, C5a receptor activation in glomerular mesangial cells has been shown to induce proliferation, produce cytokines and growth factors, as well as upregulate certain transcription factors and early response genes (81). The terminal complement complex in plasma and urine, and the anaphylatoxin C5a in plasma and urine, might have potential as an early and reliable marker for acute renal allograft rejection (44). In this regard, urinary C5a level is positively correlated with the severity of renal injury, which highlights the important role of C5a in renal damage of human anti-glomerular basement membrane disease (14, 35).

Given the stresses experienced by endurance athletes, the first aim of this study was to investigate relationships among urine levels of TRX, C5a and AKI caused by ischemia/reperfusion and oxidative stress following intensive endurance exercise. Furthermore, in our previous study, we reported urinary excretion of interleukin (IL)-2, IL-4, IL-8, IL-10, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 in stressed athletes suffering from renal tubular epithelial damage. The damaged kidney might be responsible, at least in part, for the kinetics of some cytokines after endurance exercise (59). Therefore, the second aim was to clarify associations between urine levels of TRX or C5a and those of IL-2, IL-4, IL-8, IL-10, IFN-γ, MCP-1 as well as urine albumin (ALB) and serum creatinine (Cr) as renal function markers. The final aim was to determine oxidative stress responses in the circulation after exercise. Here, we applied a newly-developed system of measurement for neutrophil migratory activity and ROS-production. This system uses *ex vivo* hydrogel methodology with an extracellular matrix as a means to investigate the mechanisms of tissue damage (28).

METHODS

Subjects

Fourteen male triathletes [age 28.7 ± 7.9 (mean ± SD) yr and body mass 63.2 ± 6.0 kg], volunteered to participate in this study. The subjects were seven professional triathletes and seven amateur triathletes. They completed a medical questionnaire and gave written informed consent prior to the study. None of the athletes had been ill in the previous month. The experimental procedure was approved by the institutional ethics committee of Waseda University.

Renal tubular epithelial cells and renal tubular epithelial casts were observed in the urinary sediments of seven subjects, among the fastest eight subjects for race time (59). In this study, according to the values of serum Cr in the AKI diagnosis criteria such as “Risk, Injury, Failure, Loss, End Stage Kidney Disease (ESKD): RIFLE criteria” (4) and “acute kidney injury network: AKIN” (34, 37), AKI following endurance exercise showed “Risk” or “StageI”at 0 h and 1.5 h after the race in the seven subjects with the existence of renal tubular epithelial cells in the urinary sediments. Immediately after exercise in the other seven subjects, there was no evidence of renal tubular epithelial cells in the urinary sediments. After this, the athletes were analysed as two subgroups that were divided according to the existence (damaged group,

n=7) or non-existence (minor-damage group, n=7) based on the levels of renal tubular epithelial cells in the urinary sediments (59).

Duathlon race

The present investigation was conducted in an official duathlon race held on the road course of Miyako Island, Okinawa, Japan as described previously (59). Briefly, the race consisted of 5 km of running, 40 km of cycling, and 5 km of running, and began at 14:00. The weather was fair, and the ambient temperature was 24.6 °C.

Research design

All participants agreed to avoid the use of vitamin/mineral supplements, herbs and medications from the previous day until after the last sampling point. All participants ate an identical breakfast at 08:30. The breakfast contained 574 kcal, with 22.1 g protein, 13.7 g fat and 88.8 g carbohydrate. The pre-race blood and urine samples (Pre) were collected at 10:30 while the participants were at rest. The athletes did not exercise for approximately 18 h before the pre-race blood and urine sampling. The post-race blood and urine samples were collected immediately (0 h), 1.5 h and 3 h after the race. Peripheral blood samples were drawn by antecubital venipuncture with the participants in the sitting position. Urine samples were collected into designated vessels. They ate lunch at 11:00. The lunch contained 211 kcal, with 9.3 g protein, 2.4 g fat and 38.6 g carbohydrate. All participants drank the same quantity of fluid during exercise. After a warm-up, they each drank 600 ml of fluid before the race. During the race, they each drank 1400 ml of fluid. Therefore, the total fluid intake for each individual was 2000 ml. They each drank 1500 ml of water until 3 h after the race.

Serum, plasma, urine sampling, urinary sediments and biochemical parameters

Approximately 7 ml of blood was drawn by a standard venipuncture technique from the antecubital vein using vacutainers containing no additive or sodium heparin and disodium EDTA as an anticoagulant to obtain serum and plasma samples, respectively. Collected blood samples containing no additives were allowed to clot at room temperature for 1 hour before centrifugation at 1000 × *g* for 10 min for serum preparation, whereas blood samples containing disodium EDTA were centrifuged immediately for plasma preparation. Plasma was stored at -80 °C until the day of analysis. Serum concentrations of Cr, myoglobin (Mb), uric acid (UA), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and plasma concentration of lactate were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan) (59).

Urine sample volume was measured and then approximately 8 ml was stored at 4 °C without centrifugation until analysis of sediments. Remaining urine samples were centrifuged immediately at 1000 × *g* for 10 min to remove sediments, and the supernatants were stored at -80 °C until the day of analysis. Urinary concentrations of Cr, ALB, UA, Mb and *β-N*-acetyl-D-glucosaminidase (NAG) activity were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan) (59). The urinary data are reported as the gross amount per minute (urinary excretion rate) as described previously (59).

MPO, TRX, C5a, cytokines and chemokines

Myeloperoxidase (MPO), IL-1 receptor antagonist (IL-1ra), IL-6, IL-8 and IL-10 were measured in plasma, and TRX, C5a, IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 were measured in urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (59). The following kits were used for all measurements: MPO (Hbt ELISA test, Hycult biotechnology, Uden, The Netherlands), TRX (TRX ELISA Kit, Redox Bio Science, Kyoto, Japan), IL-6 (Quantikine HS, R&D Systems, Inc., Minneapolis, MN), IL-1ra and MCP-1 (Quantikine, R&D Systems, Inc.), C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ (OptEIA, Beckton Dickinson Biosciences, San Diego, CA, USA) (59). For all assays, the absorbance was measured spectrophotometrically on a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) and the concentration of each cytokine was calculated by comparison with a standard curve established in the same measurement. The urinary data are reported as the gross amount per minute (urinary excretion rate).

Neutrophil function

Neutrophil function was measured using modified Mebiol (scaffold-thermoreversible galation polymer: S-TGP) gel (Mebiol Co., Hiratsuka, Kanagawa, Japan) and luminol as described previously (17, 28, 68). Peripheral blood samples were drawn in a 2 ml sodium-heparin tubes (Venoject II, Terumo Co., Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 µl luminol-blood samples were layered on 50 µl S-TGP gel prepared in a tube at 37°C, and was promptly measured by relative light unit (RLU) using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Chiba, Japan). The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored in a kinetic mode for 60 min. After measurement of luminol-dependent chemiluminescence (LmCL) for 60 min, luminol-blood samples were removed. The tubes with 50 µl S-TGP gel in which neutrophils migrated were washed three times with PBS at 37°C. Then, the tubes with gel were cooled on ice and mixed well following addition of 50 µl Turk solution (Wako, Osaka, Japan). The suspension obtained in this way was set on the C-Chip (Disposable haemocytometer, Neubauer improved, DHC-No.1, Digital Bio, Seoul, Korea), and the migratory cell number was counted microscopically. Migrating neutrophils were calculated by a 20 times multiplication of the counted cell number (28).

Oxidative stress markers

To analyze the plasma levels of reactive oxygen metabolites, and antioxidant capacity, diacron reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and total antioxidant capacity (OXY-absorbent test) were performed respectively using the Free Radical Analytical System (Diacron, Grosseto, Italy) according to the manufacturer's instructions. The d-ROMs test provides a measure for the oxidative stress of blood samples by evaluating the level of

reactive oxygen metabolites particular to hydroperoxides. This assay is based on the capability of N,N-dimethyl-para-phenylen-diamine (DMPD) to give a stable, colored solution when it is transformed into its radical cation ($DMPD^+$). The assay was performed in a 5 ml plastic tube by adding 20 µl of DMPD (final concentration 1mM) and 10 µl of plasma sample to 2 ml of 0.1 M acetate buffer, pH 4.8. The formation of the colored $DMPD^+$ was monitored by reading the absorbance at 505 nm. The amount of the colored $DMPD^+$ is related to the oxidative stress of the plasma and can be expressed in terms of hydrogen peroxide equivalents, with 1 U. CARR (Carratelli unit) corresponding to 0.8 mg/l hydrogen peroxide (11,74). The BAP assay is a photometric test that determines the serum concentration of antioxidants capable of reducing the iron from the ferric to the ferrous form. A plasma aliquot (10 µl) was dissolved in 1 ml of colored solution obtained by mixing 50 µl of ferric ions ($FeCl_3$; ferric chloride) with a chromogenic substrate (a sulfur-derived compound). Following 5 min incubation, the intensity of the color change was assessed spectrophotometrically at 505 nm. The amount of reduced ferric ions was calculated and the BAP unit was expressed as µM (71). The OXY-absorbent test allows assessment of the antioxidant power of plasma by measuring the ability of such barrier to oppose the large oxidant action of hypochlorous acid (HOCl). HOCl is used as an indicator because it is one of the strongest ROS produced by leukocytes. In the OXY-absorbent test, 1 ml of R1 reagent (HOCl solution) was put into an empty cuvette, to which 10 µl of previously diluted sample (plasma or serum) was added and mixed. The solution was incubated at 37°C for 10 min, before addition of 10 µl of reagent R2 (chromogen). The cuvette contents were mixed and the absorbance measured spectrophotometrically at 546 nm. The results were expressed as µM of HOCl adsorbed by 1 ml of sample (µM HOCl/ml).

Statistical analyses

Data were presented as mean ± standard deviation (SD). Statistical validation was assessed using Friedman's test. If significance was detected, the Scheffe method was used for multiple comparisons. Associations among measured variables were determined by Spearman's rank correlation coefficient analysis. Statistical findings were deemed to be significant where the probability of events occurring at random was less than 5% ($p < 0.05$).

RESULTS

Markers of renal function in urine and plasma lactate

The degree of renal damage, as measured by creatinine clearance, urinary excretion rates of ALB and NAG, was higher in the damaged group than those in the minor-damage group (59). As shown in Figure 2, plasma lactate concentrations increased significantly after the race compared with the pre-race values in both groups, but were higher in the damaged group (3.4-fold) than those in the minor-damage group (2.7-fold).

Oxidative stress and renal function parameters in the circulation and ex vivo

As shown in Table 1 and Figure 2, many biochemical variables were affected by the exercise intervention and varied between the two groups. Serum OXY increased significantly immediately after exercise compared with pre exercise in the damaged group only (0 h: 1.2-fold).

Urinary TRX and C5a

As shown in Figure 1, the excretion rate of TRX in the minor-damage group did not significantly change, while in the damaged group, TRX was significantly increased at 1.5 h (20.1-fold) after the race and then decreased 3 h (4.9-fold) post-exercise. There was no significant change in the excretion rate of C5a for the minor-damage group. The excretion rate of C5a increased significantly 3 h (31.8-fold) after exercise in the damaged group when compared to pre-exercise values.

Associations between urinary NAG, TRX, ALB and MCP-1

As shown in Table 2, the urinary excretion rate of NAG, as a marker of renal tubular epithelial cell injury, was positively correlated with that of TRX, ALB and MCP-1 in the damaged group. In the minor-damage group, the urinary excretion rate of NAG 0 h after the race was positively correlated with that of ALB immediately after exercise only.

Relations among urinary TRX, C5a, renal function makers and cytokines

As shown in Table 3, the area under the curve (AUC) for pre-, 0 h, 1.5 h and 3 h of urinary excretion rate of TRX was positively correlated with that of ALB in the damaged group. Furthermore, there was a trend for a positive correlation with serum Cr concentrations, whereas there was a trend for TRX to be negatively correlated with AUC of urinary excretion rate of C5a, IL-2 and negatively correlated with AUC of urinary excretion rate of IL-4, IL-8, IL-10 and IFN-γ. There was a trend for the AUC of urinary excretion rate of C5a to be nega-

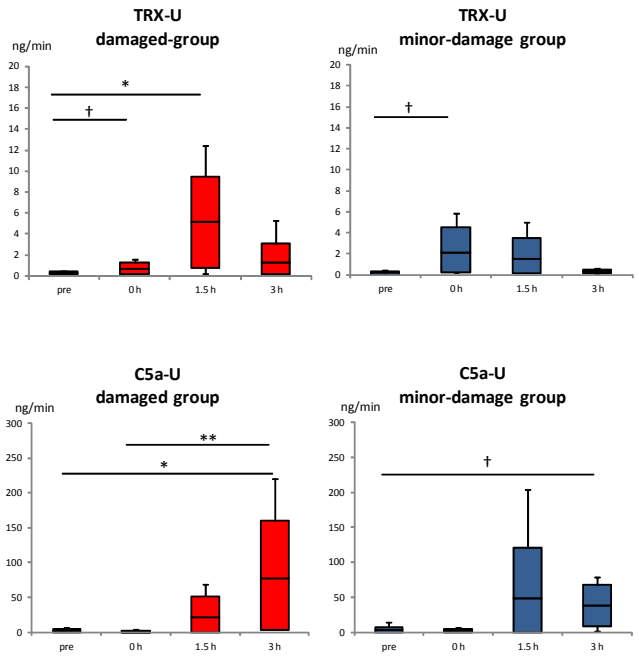

Figure 1. Changes of urinary excretion rates of TRX and C5a.
Statistics: $^{**}p < 0.01$, $^{*}p < 0.05$, †$p < 0.1$.
Box plot: (minimum values)-(means - SD)-means-(means + SD)-(maximum values), N=7
-U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).
pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.
(-): minor-damage group; renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: thioredoxin (TRX), complement 5a(C5a).

Table 1. Changes of leukocytes, cytokines and biochemical variables following the duathlon race.

	Renal tubular epithelial cells	Unit	Pre	0 h	1.5 h	3 h	Fried -man test	Scheffe test
Blood leukocytes	(+) n=7	$\times 10^2/\mu l$	41.9±8.9	127.1±32.6	128.9±32.5	112.6±25.3	**	Pre-0 h** Pre-1.5 h*
	(-) n=7		52.9±15.8	168.7±31.2	143.6±39.6	137.3±43.5	**	Pre-0 h** Pre-1.5 h*
UA-U	(+)	μg/min	11.2±5.2	1.0±0.9	3.4±1.7	4.4±1.3	**	Pre-0 h**
	(-)		10.8±5.1	2.1±1.1	5.5±2.4	4.9±1.9	**	Pre-0 h*
Mb-U	(+)	ng/min	20.0±9.6	3.2±2.6	11.0±3.2	15.2±9.2	**	Pre-0 h** 0 h-1.5 h*
	(-)		21.0±10.9	6.6±4.5	13.8±9.6	29.7±31.5	*	Pre-0 h†
ALB-U#	(+)	μg/min	5.0±2.0	115.5±69.0	198.1±154.8	41.4±36.8	*	Pre-1.5 h*
	(-)		4.6±2.3	129.7±111.2	44.9±47.4	19.9±27.3	**	Pre-0 h** 0 h-3 h*
ALB-P	(+)	g/dl	4.5±0.3	5.2±0.4	4.9±0.2	4.8±0.3	**	Pre-0 h**
	(-)		4.5±0.2	4.9±0.2	4.7±0.3	4.7±0.3	**	Pre-0 h**
NAG-U#	(+)	mU/min	6.6±2.4	2.5±1.8	8.7±3.6	8.1±2.7	**	0 h-1.5 h* 0 h-3 h†
	(-)		5.9±3.6	5.1±1.8	6.8±2.6	4.9±1.2	NS	NS
MCP-1-U#	(+)	pg/min	1.7±0.8	0.5±0.3	2.5±1.2	12.2±15.7	**	0 h-3 h**
	(-)		2.7±1.9	2.4±3.2	2.8±2.3	2.6±1.5	NS	NS
LDH-S	(+)	IU/l	188.0±36.8	266.6±31.9	250.3±35.2	243.1±45.6	**	Pre-0 h** Pre-1.5 h*
	(-)		174.9±38.6	249.7±38.0	235.0±41.5	231.7±45.9	**	Pre-0 h** Pre-3 h†
AST-S	(+)	IU/l	32.3±11.8	41.0±14.9	38.9±13.6	38.9±12.0	**	Pre-0 h** Pre-3 h*
	(-)		26.9±8.7	33.4±10.3	33.1±10.1	35.6±11.6	**	Pre-0 h† Pre-3 h**
ALT-S	(+)	IU/l	21.9±10.6	26.0±12.3	24.4±11.3	24.1±10.6	**	Pre-0 h**
	(-)		20.1±4.9	22.6±5.0	21.6±5.1	22.1±5.3	*	Pre-0 h* Pre-3 h†
IL-8-P#	(+)	pg/ml	17.2±11.1	49.8±23.1	35.6±11.3	24.3±11.5	**	Pre-0 h** Pre-1.5 h* 0 h-3 h*
	(-)		16.1±10.4	40.1±17.3	30.1±14.1	19.9±11.3	**	Pre-0 h* 0 h-3 h*
IL-10-P#	(+)	pg/ml	5.1±11.6	25.6±42.2	21.8±54.4	20.6±52.3	**	Pre-0 h*
	(-)		1.0±0.3	6.6±11.6	1.2±0.4	0.8±0.4	**	0 h-3 h**

Values: means ± SD (n=7). Statistics: ** $p < 0.01$, * $p < 0.05$, † $p < 0.1$ ≦ not significance (NS).

-P: Data are plasma concentrations.

-S: Data are serum concentrations.

-U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).

The pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.

(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.

(-): minor-damage group; renal tubular epithelial cells did not exist in the urinary sediments.

#: Data modified from Figure 1 of the reference No. 59.

Abbreviations: uric acid (UA), myoglobin (Mb), albumin. (ALB), β-*N*-acetyl-D-glucosaminidase (NAG), monocyte chemotactic protein (MCP)-1, lactate dehydrogenese (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin (IL).

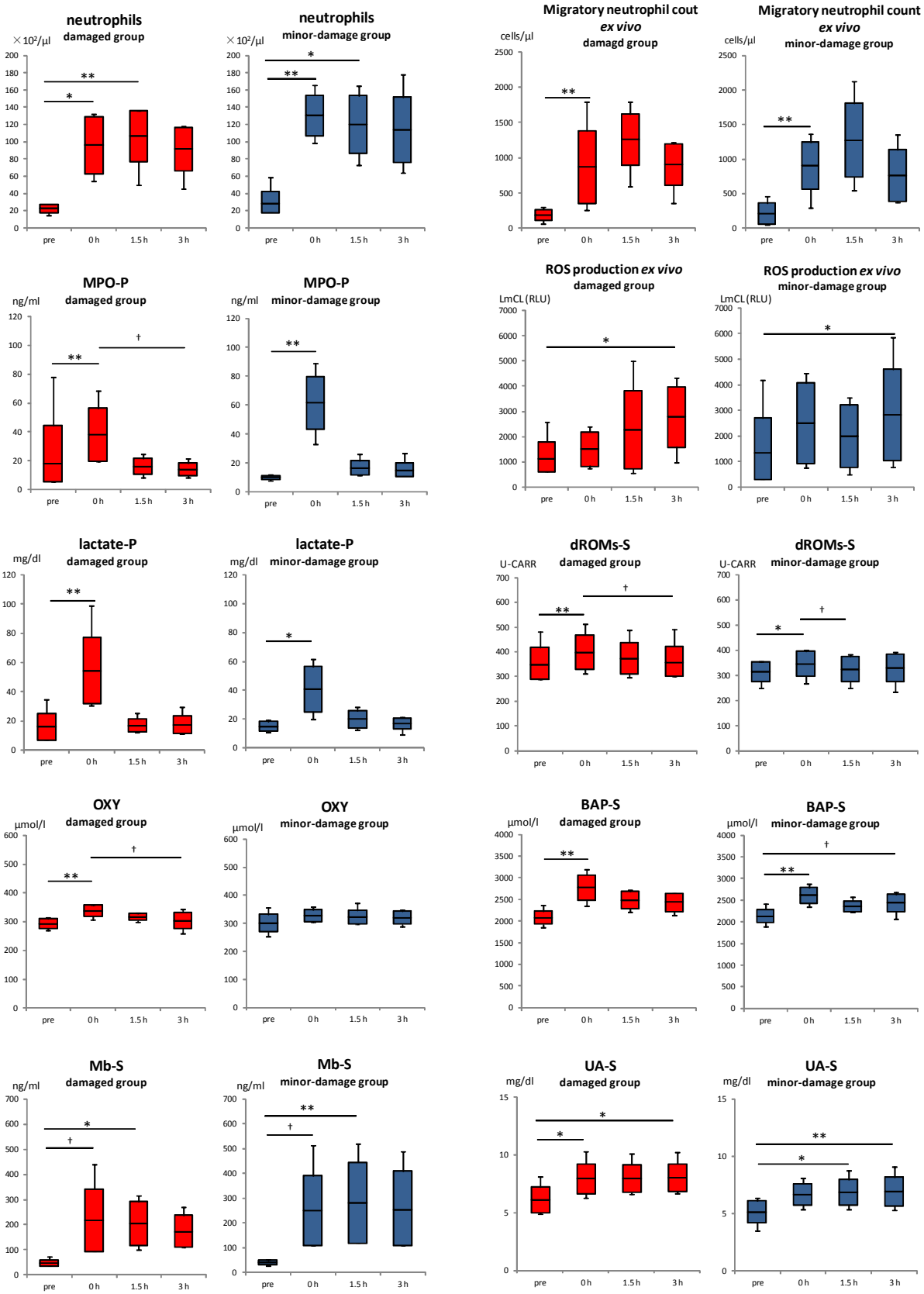

Figure 2. Changes in neutrophil activity and other variables following the duathlon race.

Statistics: **p < 0.01, *p < 0.05, †p < 0.1.
Box plot: (minimum values)-(means ‗ SD)-means-(means ₊ SD)-(maximum values), N=7
-P: Data is plasma concentration.
-S: Data is serum concentration.
pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
damaged group: renal tubular epithelial cells existed in the urinary sediments.
minor-damage group: renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: uric acid (UA), diacron reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), antioxidant capacity (OXY), myeloperoxidase (MPO), reactive oxygen species (ROS), myoglobin (Mb), luminol-dependent chemiluminescence (LmCL), relative light unit (RLU). Carratelli unit (U-CARR).

Table 2. Spearman's rank correlation coefficient matrix of urinary excretion rates of NAG, TRX, ALB and MCP-1 in the damaged group and the minor-damaged group.

damaged group

	NAG-pre	NAG-0 h	NAG-1.5 h	NAG-3 h	TRX-pre	TRX-0 h	TRX-1.5 h	TRX-3 h	ALB-pre	ALB-0 h	ALB-1.5 h	ALB-3 h	MCP-1-pre	MCP-1-0 h	MCP-1-1.5 h	MCP-1-3 h
NAG-pre	1.000	-0.607	0.143	0.500	0.500	-0.964**	0.429	0.393	0.500	-0.679†	0.071	0.286	0.179	-0.429	0.000	0.286
NAG-0 h	-0.607	1.000	-0.643	-0.964**	0.357	0.536	-0.893**	-0.857*	-0.143	0.714†	-0.786*	-0.857*	0.571	0.893**	-0.714†	-0.607
NAG-1.5 h	0.143	-0.643	1.000	0.786*	-0.643	-0.036	0.500	0.464	-0.500	-0.250	0.821*	0.679†	-0.607	-0.536	0.750†	0.643
NAG-3 h	0.500	-0.964**	0.786*	1.000	-0.500	-0.429	0.857*	0.786*	0.000	-0.607	0.893**	0.821*	-0.643	-0.857*	0.750†	0.571
TRX-pre	0.500	0.357	-0.643	-0.500	1.000	-0.536	-0.429	-0.393	0.500	-0.071	-0.821*	-0.536	0.821*	0.429	-0.750†	-0.286
TRX-0 h	-0.964**	0.536	-0.036	-0.429	-0.536	1.000	-0.464	-0.429	-0.643	0.750†	-0.036	-0.250	-0.143	0.393	0.036	-0.143
TRX-1.5 h	0.429	-0.893**	0.500	0.857*	-0.429	-0.464	1.000	0.964**	0.357	-0.750†	0.786*	0.857*	-0.679†	-0.929**	0.714†	0.357
TRX-3 h	0.393	-0.857*	0.464	0.786*	-0.393	-0.429	0.964**	1.000	0.286	-0.821*	0.714†	0.929**	-0.714†	-0.857*	0.786*	0.500
ALB-pre	0.500	-0.143	-0.500	0.000	0.500	-0.643	0.357	0.286	1.000	-0.393	-0.214	-0.0714	0.286	-0.321	-0.357	-0.500
ALB-0 h	-0.679†	0.714†	-0.250	-0.607	-0.071	0.750†	-0.750†	-0.821*	-0.393	1.000	-0.393	-0.714†	0.464	0.536	-0.536	-0.500
ALB-1.5 h	0.071	-0.786*	0.821*	0.893**	-0.821*	-0.036	0.786*	0.714†	-0.214	-0.393	1.000	0.786*	-0.857*	-0.750†	0.857*	0.464
ALB-3 h	0.286	-0.857*	0.679†	0.821*	-0.536	-0.250	0.857*	0.929**	-0.071	-0.714†	0.786*	1.000	-0.786*	-0.786*	0.929**	0.750†
MCP-1-pre	0.179	0.571	-0.607	-0.643	0.821*	-0.143	-0.679†	-0.714†	0.286	0.464	-0.857*	-0.786*	1.000	0.500	-0.929**	-0.500
MCP-1-0 h	-0.429	0.893**	-0.536	-0.857*	0.429	0.393	-0.929**	-0.857*	-0.321	0.536	-0.750†	-0.786*	0.500	1.000	-0.607	-0.321
MCP-1-1.5 h	0.000	-0.714†	0.750†	0.750†	-0.750†	0.036	0.714†	0.786*	-0.357	-0.536	0.857*	0.929**	-0.929**	-0.607	1.000	0.750†
MCP-1-3 h	0.286	-0.607	0.643	0.571	-0.286	-0.143	0.357	0.500	-0.500	-0.500	0.464	0.750†	-0.500	-0.321	0.750†	1.000

minor-damage group

	NAG-pre	NAG-0 h	NAG-1.5 h	NAG-3 h	TRX-pre	TRX-0 h	TRX-1.5 h	TRX-3 h	ALB-pre	ALB-0 h	ALB-1.5 h	ALB-3 h	MCP-1-pre	MCP-1-0 h	MCP-1-1.5 h	MCP-1-3 h
NAG-pre	1.000	-0.214	0.714†	-0.143	0.893**	-0.143	-0.071	-0.250	0.893**	-0.214	0.571	0.071	0.679†	-0.036	-0.036	-0.786*
NAG-0 h	-0.214	1.000	-0.250	-0.429	-0.036	0.429	0.321	0.571	-0.036	0.929**	0.286	0.536	-0.143	0.036	0.036	0.360
NAG-1.5 h	0.714†	-0.250	1.000	-0.250	0.750†	-0.143	-0.429	-0.321	0.750†	-0.321	0.250	-0.429	0.179	-0.571	-0.571	-0.571
NAG-3 h	-0.143	-0.429	-0.250	1.000	-0.071	0.357	0.250	0.214	-0.071	-0.143	0.036	-0.321	0.321	0.071	0.071	0.107
TRX-pre	0.893**	-0.036	0.750†	-0.071	1.000	-0.071	-0.250	-0.214	1.000	-0.071	0.464	0.000	0.500	-0.357	-0.357	-0.857*
TRX-0 h	-0.143	0.429	-0.143	0.357	-0.071	1.000	0.786*	0.964**	-0.071	0.714†	0.679†	0.214	0.429	0.286	0.286	0.536
TRX-1.5 h	-0.071	0.321	-0.429	0.250	-0.250	0.786*	1.000	0.857*	-0.250	0.607	0.714†	0.500	0.607	0.786*	0.786*	0.643
TRX-3 h	-0.250	0.571	-0.321	0.214	-0.214	0.964**	0.857*	1.000	-0.214	0.821*	0.643	0.393	0.357	0.429	0.429	0.679†
ALB-pre	0.893**	-0.036	0.750†	-0.071	1.000	-0.071	-0.250	-0.214	1.000	-0.071	0.464	0.000	0.500	-0.357	-0.357	-0.857*
ALB-0 h	-0.214	0.929**	-0.321	-0.143	-0.071	0.714†	0.607	0.821*	-0.071	1.000	0.500	0.536	0.107	0.214	0.214	0.500
ALB-1.5 h	0.571	0.286	0.250	0.036	0.464	0.679†	0.714†	0.643	0.464	0.500	1.000	0.429	0.857*	0.429	0.429	0.000
ALB-3 h	0.071	0.536	-0.429	-0.321	0.000	0.214	0.500	0.393	0.000	0.536	0.429	1.000	0.357	0.679†	0.679†	0.286
MCP-1-pre	0.679†	-0.143	0.179	0.321	0.500	0.429	0.607	0.357	0.500	0.107	0.857*	0.357	1.000	0.536	0.536	-0.179
MCP-1-0 h	-0.036	0.036	-0.571	0.071	-0.357	0.286	0.786*	0.429	-0.357	0.214	0.429	0.679†	0.536	1.000	1.000	0.536
MCP-1-1.5 h	-0.036	0.036	-0.571	0.071	-0.357	0.286	0.786*	0.429	-0.357	0.214	0.429	0.679†	0.536	1.000	1.000	0.536
MCP-1-3 h	-0.786*	0.360	-0.571	0.107	-0.857*	0.536	0.643	0.679†	-0.857*	0.500	0.000	0.286	-0.179	0.536	0.536	1.000

Values: N=7. Statistics: **p < 0.01, *p < 0.05, †p < 0.1.
Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).
pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
damaged group: renal tubular epithelial cells existed in the urinary sediments.
minor-damage group: renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: ß-*N*-acetyl-D-glucosaminidase (NAG), thioredoxin (TRX), albumin (ALB), monocyte chemotactic protein (MCP)-1.

tively correlated with that of urinary excretion rate of ALB. C5a excretion was negatively correlated with serum Cr concentrations, whereas that of urinary excretion rate of C5a was positively correlated with IL-2, IL-4, IL-8, IL-10 and IFN-γ.

In the minor-damage group, AUC of urinary excretion rate of C5a was positively correlated with IL-2, IL-4, IL-8 and IFN-γ. There was a trend for the AUC of urinary excretion rate of C5a to be positively correlated with IL-10. On the other hand, AUC of urinary excretion rate of TRX was not significantly correlated with any variables.

Associations among variables in the circulation

As shown in Table 4, in the damaged group the AUC for pre-, 0 h, 1.5 h and 3 h of plasma lactate concentrations was positively associated with leukocyte count, neutrophil count, chemokines and tended to be positively associated with oxidative stress markers. AUC of plasma MPO concentrations was positively correlated with antioxidant capacity markers. AUC of neutrophil ROS production *ex vivo* tended to be positively associated with oxidative stress markers and was associated with IL-1ra, an anti-inflammatory cytokine. Moreover, migratory neutrophil count *ex vivo* was correlated with IL-1ra. AUC of serum d-ROMs was positively associated with leukocyte count, neutrophil count, IL-1ra and Mb, and tended to be positively correlated with OXY.

In the minor-damage group AUC of plasma concentration of lactate was associated with migratory neutrophil count *ex vivo* and d-ROMs, and tended to be positively correlated with MPO. Plasma concentration of MPO was correlated with leukocyte count and neutrophil count, and tended to be corre-

Table 3. Spearman's rank correlation coefficient matrix of urinary excretion rates of TRX, C5a and variables of renal damage following the duathlon race.

	group	TRX-U	C5a-U	ALB-U	Cr-S	IL-2-U	IL-4-U	IL-8-U	IL-10-U	IFN-γ-U
TRX-U	(+)	1000	-0.714†	0.786*	0.679†	-0.750†	-0.786*	-0.857*	-0.857*	-0.857*
	(-)	1000	-0.464	0.607	-0.286	-0.536	-0.536	-0.464	-0.607	-0.286
C5a-U	(+)	-0.714†	1000	-0.679†	-0.821*	0.964**	0.929**	0.893**	0.893**	0.893**
	(-)	-0.464	1000	-0.536	-0.393	0.857*	0.857*	0.786*	0.714†	0.821*

All data were calculated as area under the curve (AUC)
AUC: total value of pre, 0 h, 1.5 h and 3 h.
Values: N=7. Statistics: **p < 0.01, *p < 0.05, †p < 0.1.
-S: Data are serum concentration.
-U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).
pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.
(-): minor-damage group; renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: thioredoxin (TRX), complement 5a (C5a), albumin (ALB), creatinine (Cr), interleukin (IL)-2, 4, 8, 10, interferon (IFN)-γ.

Table 4. Spearman's rank correlation coefficient matrix of circulating lactate, variables of oxidative stress, chemokines and anti-inflammatry cytokines following the duathlon race.

	Groups	lactate-P	MPO-P	Migratory neutrophil count *ex vivo*	ROS production *ex vivo*	d-ROMs-S	BAP-S	OXY-S	IL-8-P	IL-10-P	IL-1ra-P
lactate-P	(+)	1.000	0.536	0.357	0.321	0.714†	0.143	0.750†	0.929**	0.607	0.464
	(-)	1.000	0.750†	0.857*	0.643	0.857*	0.107	0.393	-0.143	0.143	-0.429
Blood leukocytes	(+)	0.847*	0.324	0.487	0.595	0.847*	0.036	0.559	0.613	0.577	0.631
	(-)	0.607	0.857*	0.286	0.25	0.536	0.786*	0.679†	0.000	0.179	-0.321
Blood neutrophils	(+)	0.821*	0.357	0.536	0.571	0.821*	0.071	0.536	0.571	0.500	0.607
	(-)	0.571	0.821*	0.357	0.179	0.429	0.714†	0.643	-0.071	0.286	-0.429
MPO-P	(+)	0.536	1.000	0.643	0.357	0.536	0.821*	0.786*	0.643	0.000	0.536
	(-)	0.750†	1.000	0.571	0.679†	0.607	0.643	0.393	-0.036	0.464	-0.179
Migratory neutrophil count *ex vivo*	(+)	0.357	0.643	1.000	0.750†	0.500	0.429	0.500	0.286	-0.143	0.786*
	(-)	0.857*	0.571	1.000	0.679†	0.571	-0.179	0.107	-0.214	0.429	-0.429
ROS production *ex vivo*	(+)	0.321	0.357	0.750†	1.000	0.750†	0.286	0.464	0.214	0.214	0.964**
	(-)	0.643	0.679†	0.679†	1.000	0.429	0.179	0.000	-0.036	0.571	0.071
d-ROMs-S	(+)	0.714†	0.536	0.500	0.750†	1.000	0.357	0.750†	0.571	0.464	0.821*
	(-)	0.857*	0.607	0.571	0.429	1.000	0.036	0.179	0.071	-0.214	-0.143
BAP-S	(+)	0.143	0.821*	0.429	0.286	0.357	1.000	0.393	0.286	0.036	0.357
	(-)	0.107	0.643	-0.179	0.179	0.036	1.000	0.571	0.143	0.321	0.036
OXY-S	(+)	0.750†	0.786*	0.500	0.464	0.750†	0.393	1.000	0.786*	0.143	0.679†
	(-)	0.393	0.393	0.107	0.000	0.179	0.571	1.000	-0.357	-0.107	-0.679†
IL-8-P	(+)	0.929**	0.643	0.286	0.214	0.571	0.286	0.786*	1.000	0.571	0.393
	(-)	-0.143	-0.036	-0.214	-0.036	0.071	0.143	-0.357	1.000	0.179	0.786*
IL-10-P	(+)	0.607	0.000	-0.142	0.214	0.464	0.036	0.143	0.571	1.000	0.179
	(-)	0.143	0.464	0.429	0.571	-0.214	0.321	-0.107	0.179	1.000	0.143
IL-1ra-P	(+)	0.464	0.536	0.786*	0.964**	0.821*	0.357	0.679†	0.393	0.179	1.000
	(-)	-0.429	-0.179	-0.429	0.071	-0.143	0.036	-0.679†	0.786*	0.143	1.000
UA-P	(+)	0.000	0.536	0.786*	0.536	0.071	0.429	0.321	0.143	-0.321	0.571
	(-)	-0.643	-0.036	-0.679†	-0.429	-0.500	0.536	-0.143	0.321	0.179	0.429
MCP-1-P	(+)	0.964**	0.500	0.179	0.17	0.607	0.179	0.679†	0.964**	0.714†	0.321
	(-)	0.179	-0.250	0.000	-0.143	0.321	-0.214	0.107	0.571	-0.357	0.179
IL-6-P	(+)	0.250	0.179	0.286	0.607	0.321	0.214	0.250	0.393	0.536	0.571
	(-)	0.393	0.500	0.286	0.429	0.464	0.321	-0.143	0.821*	0.429	0.536
Mb-S	(+)	0.571	0.429	0.214	0.536	0.929**	0.357	0.643	0.429	0.429	0.607
	(-)	-0.143	0.107	0.143	0.500	-0.429	0.179	-0.250	0.357	0.857*	0.429

All data were calculated as area under the curve (AUC)
AUC: total value of pre, 0 h, 1.5 h and 3 h.
Values: N=7. Statistics: ** $p < 0.01$, * $p < 0.05$, † $p < 0.1$.
-S: Data are serum concentrations.
-P: Data are plasma concentrations.
The pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
(+): damaged group; renal tubular epithelial cells existed in the urinary sediments.
(-): minor-damage group; renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: interleukin (IL)-6, 8, 10, 1ra, uric acid (UA), monocyte chemotactic protein (MCP)-1, diacron reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), antioxidant capacity (OXY), myeloperoxidase (MPO), reactive oxygen species (ROS), myoglobin (Mb).

lated with ROS production *ex vivo*. AUC of ROS production *ex vivo* tended to be associated with migratory neutrophil count *ex vivo*. Migratory neutrophil count *ex vivo* was associated with UA. Plasma IL-10 concentrations were correlated with Mb.

DISCUSSION

It is known that blood flow is redistributed during endurance exercise. In this study, we provide evidence that the presence of renal epithelial cells in urine may be induced by ischemia/reperfusion caused by a reduction in renal blood flow. We have already investigated AKI caused by endurance exercise and the possible associations between AKI and the increases in urinary levels of IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 (59). This study further analyzed the associations among AKI, cytokines, inflammation and oxidative stress with a special focus on TRX and C5a. In particular, the excretion rate of TRX increased significantly at 1.5 h (20.1-fold) from pre-exercise in the damaged group only (Figure 1), and the excretion rates of NAG (3 h) were positively correlated with those of TRX (3 h) (Table 2). These findings suggested that TRX was related to renal tubular injury. Therefore, it might be possible that the excretion rate of TRX increased in response to oxidative stress as a result of renal tubular injury following intensive endurance exercise.

In a previous study on murine kidney, transgenic hTRX was predominantly observed in the outer medulla after renal ischemia/reperfusion. Thereafter, the immunoreactivity for hTRX was revealed in the intraluminal region of the renal tubule, coinciding with a decrease in TRX protein in the kidneys and an increase in urine. Interestingly, TRX protein concentration did not change in the blood, and expression of TRX mRNA did not reveal localization or change in abundance after renal ischemia/reperfusion. Therefore, it is suggested that urinary TRX protein is derived from proximal tubule cortical region of the kidney (30). In this study, however, it might

be possible that blood-derived TRX was mixed with TRX from proximal tubule cortical region, because the excretion rates of TRX at the same time points after the race were positively correlated with the excretion rates of ALB at pre, 0 h, 1.5 h and 3 h after exercise and TRX is small protein of 12 kDa. Whereas urinary excretion rates of ALB at the same time points after the race tended to be positively correlated with the excretion rates of NAG (as a generally accepted marker of renal tubular injury) at pre, 0 h, 1.5 h and 3 h after the race, the correlations between urinary excretion rates of TRX and NAG at 3 h after exercise were significant (Table 2). Serum NAG protein is not excreted into urine because its molecular weight is 130-140 kDa. Hence, it might be possible that urinary excretion of TRX was derived from both blood and kidney. In this study, renal ischemia/reperfusion of subjects was induced by endurance exercise, but in the previous work, bilateral renal arteries were clipped for 30 min and then released (30). The inconsistency of the results might be derived from the difference of the above induction methods for ischemia and exercise. It is reported that chemokines such as MCP-1 are key modulators in renal ischemia/reperfusion injury, and urinary chemokines are good markers of clinical diseases and AKI (78, 79). In the damaged group, urinary excretion rates of MCP-1 significantly increased (Table 1), and tended to be positively correlated with the excretion rates of TRX and NAG (Table 2). This suggests that TRX, MCP-1 and NAG may be associated with renal tubular injury.

In the present study, the excretion rates of C5a in the damaged group and the minor-damage group significantly increased following intensive endurance exercise (Figure 1). Urinary excretion rate of C5a was positively correlated with that of IL-2, IL-4, IL-8, IL-10 and IFN-γ after intensive endurance exercise (Table 3). It is suggested that the chemotactic factors C5a and IL-8 increased after reperfusion, making inflammatory cells infiltrate into tubular epithelium or glomerular capillary (13, 14, 15, 35, 39, 42, 76, 77 84). On the other hand, it may also be suggested that IL-10 increased to suppress progressive inflammation, and/or to repair damaged tissues (58). Therefore, it might be possible the excretion rates of C5a, IL-8 and IL-10 reflect inflammatory levels in the renal tubular injury. In contrast, in the damaged group, the excretion rates of TRX after intensive exercise were negatively correlated with the excretion rates of C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ following intensive exercise (Table 3). In the damaged group, it may be possible that levels of TRX were influenced by levels of IL-10 as an anti-inflammatory cytokine, and TRX regulates chemotactic activity C5a and IL-8, or TRX controls inflammatory progress by C5a and IL-8, because TRX functions as an antioxidant, as a chemotaxis inhibitor and as a redox-regulating protein in the signal transduction (12, 21, 22, 57).

We examined systemic oxidative stress and inflammation induced by endurance exercise within two groups (damaged group and minor-damage group) based on urinary measures of AKI. When evaluating the circulating oxidative stress and inflammatory state in these same groups, lactate levels were increased significantly in both (0 h: damaged group 3.4-fold; minor-damage group 2.7-fold). Lactate-related factors such as lactate threshold (LT) and onset of plasma lactate accumulation (OPLA) are critical for setting exercise intensity (16, 72). Since this study was carried out in an actual competition race, we could not examine LT and OPLA, but the athletes' lactate levels suggested a difference of exercise intensity between the two participant groups.

Endurance exercise increases the circulating number of leukocytes, especially neutrophils, which exhibit the greatest change in cell count and function (7, 41, 49, 50 62-65, 82). Moreover, IL-6, IL-8 and M-CSF responses are positively correlated with the delayed-onset neutrophil mobilization from the bone marrow reserve after exercise (64, 82), particularly when the duration is over 2 h. In this study, leukocytes and neutrophil counts in the minor-damage group (whose race time was over 2 h) tended to be greater than the damaged group (Figure 1 and Table 1). MPO catalyses the conversion of hydrogen peroxide into hypochlorous acid in neutrophils and macrophages (75). MPO is located in the primary (azurophilic) granules (5) and is a marker of neutrophil activation after exercise (7, 50). MPO produces a large amount of ROS and induces oxidative damage to proteins, lipids and DNA (43). MPO increases depending on exercise intensity (49, 53). These findings suggest that the intensity was also higher in the minor-damage group, but MPO increased depending on exercise duration rather than intensity in case of such a long-duration exercise. In the damaged group we found serum concentrations of d-ROMs as an oxidative stress marker, and BAP and OXY as antioxidant capacities tended to be higher than those in the minor-damage group immediately after the race. It was suggested that acute endurance exercise-induced oxygen consumption in many organs in the damaged group was greater and produced ROS, because the intensity of the damaged group was higher compared with that in the minor-damage group. Previous studies showed that scavengers such as enzymatic activities of plasma SOD and catalase (a scavenger for H_2O_2) and plasma concentration of vitamin C (ascorbate: a scavenger for O_2^-, $\cdot OH$, $^1O_2$ and other oxidants) for toxic ROS might be induced in response to intensive exercise (67). Moreover, it was reported that free radical scavengers prevent not only oxidation of molecules in the body but also adhesion of neutrophils to the endothelial lining and inhibiting neutrophil infiltration (19, 57). It was also reported that anti-inflammatory cytokines prevent inflammatory tissue damage (31, 66, 73). In particular, the anti-inflammatory cytokine IL-10 is an immunosuppressive cytokine that inhibits both proinflammatory cytokine production and ROS production by activated neutrophils (31). Furthermore, anti-inflammatory cytokines and free radical scavengers work to counteract oxidative tissue damage by ROS (9, 24, 56). In this study, the plasma concentration of IL-10 significantly increased only in the damaged group (Table 1). Plasma IL-1ra concentrations increased significantly after exercise in both groups (59) and were significantly correlated with ex vivo neutrophil migratory activity and ROS-production in the damaged group only. Accordingly, increased antioxidant capacity and anti-inflammatory cytokines in the damaged group might inhibit neutrophil activation as compared with those in the minor-damage group. We found also that serum Mb concentrations in the minor-damage group (0 h: 6.2-fold, 1.5 h: 7.0-fold) were higher than those in the damaged group (0 h: 4.8-fold, 1.5 h: 4.2-fold) after the race. These results might suggest that muscle damage increased due to ROS from activated neutrophils in the minor-damage group, whereas in the damaged group, muscle damage was prevent-

ed by elevated antioxidant capacity and anti-inflammatory cytokines.

Kanda *et al*. reported enhanced neutrophil migration and ROS production after one-leg calf-raise exercise through the use of a newly-developed *ex vivo* methodology in imitation of tissue damage (28). In this study, we assessed ROS production by neutrophils that migrated into the hydrogel. We found that both neutrophil migration and ROS production increased after exercise in both groups. The ROS production immediately after the race in the damaged group was lower than that in the minor-damage group, but serum concentration of OXY and plasma concentration of IL-10 were higher than those in the minor-damage group. These results suggest that neutrophil activation was suppressed by antioxidant and anti-inflammatory cytokines immediately following intensive endurance exercise.

In conclusion, we infer that the excretion rates of TRX, MCP-1 and NAG were associated with renal tubular injury. It might be possible that the excretion rates of C5a, IL-8 and IL-10 reflect inflammatory levels in renal tubular injury, where the excretion rate of C5a was strongly associated with that of IL-2, IL-4, IL-8, IL-10 and IFN-γ. In the damaged group, the excretion rates of TRX after exercise were negatively correlated with the excretion rates of C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ following exercise. Therefore, in the damaged group, it could be inferred that levels of TRX were influenced by levels of IL-10 as an anti-inflammatory cytokine, and that TRX regulates chemotactic activity of C5a and IL-8, or that TRX controls inflammatory progress by C5a and IL-8, because TRX functions as an antioxidant, as a chemotaxis inhibitor and as a redox-regulating protein in the signal transduction. Clarification of these pathways might be valuable in the assessment of AKI risk following intensive endurance exercise.

In the circulation of the damaged group ROS production was found to be higher than the minor damage group, while antioxidant capacity and anti-inflammatory cytokines increased immediately after intensive endurance exercise. From these data it might be inferred that neutrophil activation and efflux of MPO were inhibited. Therefore, we suggest that damage to muscle and other tissues are likely to be lower in this group. On the other hand, results from the longer duration group (minor-damage group) showed neutrophil count and efflux of MPO in the circulation were higher when compared to the damaged group. Furthermore, both variables were significantly correlated with neutrophil count and plasma concentration of MPO immediately following intensive endurance exercise. In combination, these results suggest that muscle is likely to be damaged by activated neutrophils to a greater extent than in the damaged group. This inference was further supported by the results from our application of the newly-developed ex vivo method that estimated the functional impact of activated neutrophils.

In the present study, we confirmed that intensive endurance exercise caused “Risk” or “stage I” in the AKI diagnosis criteria such as RIFLE and AKIN, suggesting that not only blood but also urine analyses are important for estimating tissue damage. The relationships among the variables in the urine and circulation, and further delineation of their clinical significance must be revealed in future research.

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A single bout of dynamic exercise enhances the expansion of MAGE-A4 and PRAME-specific cytotoxic T-cells from healthy adults

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ABSTRACT

The ex vivo expansion of tumor-associated-antigen (TAA)-specific cytotoxic T-cells (CTLs) from healthy donors for adoptive transfer to cancer patients is now providing additional treatment options for patients. Many studies have shown that adoptive transfer of expanded CTLs can reduce the risk of relapse in cancer patients following hematopoietic stem cell transplantation (HSCT). However, the procedure can be limited by difficulties in priming and expanding sufficient numbers of TAA-specific-CTLs. Because acute dynamic exercise mobilizes large numbers of T-cells to peripheral blood, we hypothesized that a single bout of exercise would augment the ex vivo expansion of TAA-specific-CTLs.We therefore collected lymphocytes from blood donated by healthy adults at rest and after brief maximal dynamic exercise.TAA-specific CTLs were expanded using autologous monocyte-derived-dendritic cells pulsed with melanoma-associated antigen 4 (MAGE-A4), with preferentially expressed antigen in melanoma (PRAME), and with Wilms' tumor protein (WT-1). Post exercise, 84% of the participants had a greater number of CTLs specific for at least one of the three TAA.Cells expanded from post exercise blood yielded a greater number of MAGE-A4 and PRAME-specific-cells in 70% and 61% of participants, respectively. In the 'exercise-responsive' participants (defined as participants with at least a 10% increase in TAA-specific-CTLs post-exercise), MAGE-A4- and PRAME-specific-CTLs increased 3.4-fold and 6.2-fold respectively. Moreover, expanded TAA-specific CTLs retained their antigen-specific cytotoxic activity. No phenotype differences were observed between expanded cells donated at rest and postexercise. We conclude that exercise can enhance the ex vivo expansion of TAA-specific-CTLs from healthy adults without compromising cytotoxic function. Hence, this study has implications for immunotherapy using adoptive T-cell transfer of donor-derived T-cells after allogeneic HSCT.

Key words: Immunotherapy, CTLs, physical activity, adoptive transfer, tumor-associated-antigens

INTRODUCTION

Immunotherapy-based cancer treatments are providing a new wave of treatment options in conjugation with more traditional approaches, including chemotherapy and hematopoietic stem cell transplantation (3). The adoptive transfer of *ex vivo* expanded tumor-associated-antigen (TAA)-specific cytotoxic T-cells (CTLs) has been shown to be a potentially potent approach for the treatment of various types of cancer, including metastatic melanoma (26), lymphoma (5), neuroblastoma (29), and lymphocytic and myeloid leukemia (12, 17). However, there are challenges that must be overcome before the treatment can be used more broadly in cancer patients with various malignancies. Techniques for manufacturing antigen-specific CTLs using GMP compliant methodologies for patients after allogeneic HSCT have arisen from studies using virus-specific T-cells, where products can now be rapidly manufactured in less than 10 days (16, 31, 40). A key factor of this technique is the selection of target antigens where a natural occurrence of T-cells specific for that antigen can be found. However, non-viral TAA are often self-antigens, which in healthy donors are infrequent and usually also of low avidity, as most self-reactive T-cells are anergized (13). Therefore, extensive cell stimulationsare required to expand sufficient numbers of TAA-specific CTLs for adoptive transfer. Further, results are often marred by the concomitant expansion of CTLs that are reactive to healthy cells (13, 23, 25, 42). Despite recent advances that have allowed researchers to overcome difficulties in adoptive transfer immunotherapy, such as avoiding mechanisms of tumor escape (7, 13, 23), the limitation of expanding sufficient numbers of tumor-reactive CTLs from healthy donors remains, even if an apheresis procedure is performed.

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A single bout of dynamic physical exercise (e.g., running or cycling) markedly increases the numbers of leukocyte subtypes in peripheral blood and alters their composition. This occurs due to mechanisms such as shear stress and increases in catecholamines, glucocorticoids, and other hormones that have direct impact on blood leukocytes (41). A single bout of exercise causes a 2-4 fold increase in the number of circulating blood monocytes and lymphocytes, the magnitude of which is related to the intensity and duration of the exercise (1, 22, 39, 41). Exercise specifically mobilizes CTLs with a phenotype consistent with tissue migration and enhanced cytotoxicity, and also increases the expression of molecules associated with T-cell activation (15, 19). The redeployment of these activated cells by exercise may provide a means to overcome the difficulty in obtaining sufficient numbers of functional TAA-specific CTLs from healthy donors.

The aim of this study was to determine if a single bout of dynamic exercise would enhance the *ex vivo* expansion of TAA-specific CTLs. Three leukemia- and lymphoma-associated antigens were selected as representative targets: the cancer-testis antigen melanoma-associated antigen 4 (MAGE-A4), and the antigens overexpressed by malignant cells preferentially-expressed antigen of melanoma (PRAME) and Wilm's Tumor 1 (WT-1).The robust expansion of MAGE-A4-specific CTLs for clinical use would improve treatment options for Hodgkin's Lymphoma patients with EBV-negative tumors (32),while enhancing the expansion of PRAME (38)- and WT-1 (8)-specific CTLs would offer an important T-cell therapeutic to patients with acute leukemias after allogeneic HSCT. We demonstrate here that a single bout of dynamic exercise increases the yield of MAGE-A4 and PRAME-specific CTLs in the majority of the healthy adult participants. These findings indicate that exercise is a simple yet effective- and economical approach to enhance the expansion of TAA-specific CTLs for adoptive T-cell therapy post allogeneic HSCT.

METHODS

Experimental Design

This was a within-subjects repeated measure study of 19 healthy adults (10 women) designed to assess the effects of brief dynamic exercise on the expansion of TAA-specific T-cells. Standard sample size calculations using an estimated effect size (d) of 0.6 and α=0.05 indicated that 19 participants were expected to yield a power of 0.8 to detect differences between resting and post exercise cells. Unstimulated and expanded cells donated at rest and post exercise were analyzed using flow cytometry, IFN-γ ELISPOT assays, and ^{51}CR release cytotoxicity assay (expanded cells only).

Participants

Healthy adult volunteers were recruited in Houston, TX for this study. Written informed consent and medical history were obtained from each participant after the procedures, benefits, and risks were explained verbally and provided in writing. Institutional review boards at the University of Houston and at Baylor College of Medicine granted ethical approval for the study. Participants were instructed to avoid alcohol, nonprescription drugs, and strenuous exercise 24h prior to each laboratory visit.

Exercise trials and blood sampling

Participants visited the laboratory between 7am and 10am on two occasions separated by 7 days. During Visit 1, participants were asked to ascend 260 stairs (10 floors) as quickly as possible. Completion time, maximal heart rate (Polar Electro, USA), and circulating lactate values were recorded in 11 participants. Earlobe capillary blood samples were drawn using heparin-lined microcapillary tubes at rest and post exercise, and analyzed in duplicate for lactate concentration using an automated lactate analyzer (Analox P-GM7 Micro Stat, Analox, UK). A 3ml venous blood sample was collected at rest and immediately post exercise in vacutainer tubes treated with ethylene-diamine-tetra-acetic acid (EDTA) (Becton-Dickenson, USA) for differential leukocyte cell counts (BC3200, Mindray North America, Mahwah, NJ). A 40ml venous blood sample was collected at rest and post exercise in vacutainer tubes treated with sodium heparin (Becton-Dickenson) for monocyte-derived (mo)-dendritic cell (DC) generation and CTL expansion. An additional blood sample was taken at rest and post exercise into a 6ml serum gel tube (Becton-Dickenson). Blood was processed within 4 hours of being drawn. Blood serum was frozen at -80°C until analysis. During Visit 2, participants donated a resting 40ml blood sample collected in sodium heparin vacutainer tubes. At the second visit, 13 participants also completed the Bruce Maximal Exercise Test (9) on a treadmill (Woodway Desmo, WoodwayUSA Inc, Waukesha, WI) until volitional exhaustion. The speed and incline increased at three-minute intervals to increase the intensity. Heart rate, ventilation, and oxygen consumption were measured throughout the test using an automated metabolic cart (Cosmed Quark CPET, Rome, Italy).

Generation of DCs from blood

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (Lymphoprep; Nycomed) density gradient separation. Mo-DCs were generated by plate adherence of PBMCs. PBMCs were incubated for 2 hours in DC medium (CellGenix media with 2mM L-glutamine (GlutaMAX; Invitogen)) at a concentration of 10 x 10^6 cells/well in a 6-well plate (Costar). Nonadherent cells were removed by gentle washing with PBS (Sigma), and cryopreserved at -80°C for later stimulation with mature mo-DCs. Adherent cells were cultured in DC media with 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 400U/ml interleukin-4 (IL-4) (both R&D Systems) for 5 days at 37° C in a humidified CO_2 incubator. On day 5, immature mo-DCs were harvested by gentle rinsing and resuspended at 0.5 x 10^6 cells/ml in DC medium and matured in a cytokine cocktail of GM-CSF (800 U/ml), IL-4 (400 U/ml), IL-1β (10ng/ml), IL-6 (10ng/ml), tumor necrosis factor-α (TNF-α; 10ng/ml, all R&D), and prostaglandin E2 (PGE2; 1μg/mL; Sigma-Aldrich). On day 7, mature mo-DCs were harvested and used as antigen presenting cells.

Expansion of tumor-associated-antigen-specific T-cells

Mature mo-DC were incubated with each TAA, usingthe peptide libraries of MAGE-A4, PRAME, and WT-1 (100 ng/PepMix; JPT Peptide Technologies), in 10 μl PBS for 1 hour at

37° C. Autologous, cryopreserved, non-adherent PBMCs were thawed and stimulated with the TAA-pulsed mo-DCs at a stimulator-to-effector ratio of 1:10. Non-adherent PBMCs isolated from resting blood were stimulated with mo-DCs generated from resting blood, and non-adherent PBMCs isolated from post exercise blood were stimulated with mo-DCs generated from post exercise blood. Cells were cultured 7 days at 1 x 10^6 cells/ml in 24 well plates (Costar) in 2ml RPMI-1640 supplemented with 45% Clicks media (Irvine Scientific), 5% human AB serum, and 2mmol/L GlutaMax, and recombinant human IL-7 (10ng/ml), IL-12 (10ng/ml), and IL-15 (5ng/ml)(all Peprotech). After 7 days, cells were harvested and restimulated with autologous TAA-pulsed mo-DCs at a ratio of 1 to 4 for a further 7 days. All mo-DCs for the second stimulation were generated from resting blood. Cells were split and fed with fresh media containing IL-7, IL-12, and IL-15 on days 5 and 12. Due to limitations in the number of available cells, all three TAA-specific cell lines were not expanded for each participant; MAGE-A4 specific cells were expanded from 17 participants, PRAME specific cells were expanded from 18 participants, and WT-1 specific cells were expanded from 13 participants.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) analysis was conducted on PBMCs and twice-stimulated (expanded) cells using IFN-γ ELISPOT multiscreen IP plates (Millipore, USA). Cells were stimulated overnight with MAGE-A4, PRAME, and WT-1 PepMixes (1 μg/ml) at a concentration of 150000 cells/well (PBMCs) or 100000 cells/well (expanded cells) in a 37° C humidified CO_2 incubator. Cells stimulated with staphylococcal enterotoxin B (1μg/ml; Sigma-Aldrich) served as a positive control, and cells stimulated with Ad-penton (JPT Peptide Technologies) or incubated in media only served as negative controls. Each condition was measured in triplicate. Plates were evaluated by Zellnet Consulting. Spot-forming cells (SFCs) were enumerated to obtain the frequency of TAA-reactive cells.

Cytotoxicity assay

The cytotoxic specificity of TAA-specific T-cells expanded from resting and post exercise blood were analyzed in a standard 4h ^{51}Cr release assay using effector:target ratios of 40:1, 20:1, 10:1, and 5:1. Target cells were autologous Phytohemagglutinin(PHA)-blasts pulsed with each TAA pepmix, and were generated from non-adherent cells cultured for one week in the presence of IL-2 (100U/mL; Chiron) and PHA (Sigma, 5μg/mL) and fed every other day. A portion of the resultant PHA-blasts were pulsed for 1h with TAA-PepMix (0.2μg) and used as autologous target cells. The remaining PHA-blasts were pulsed with irrelevant (Ad-penton) peptide and used as controls to measure non-specific T-cell cytotoxicity. All PHA-blasts were pulsed with 10μl ^{51}Cr for 1 h. ^{51}Cr – labeled target cells were mixed with effector cells at doubling dilutions to produce the desired ratios. Target cells incubated in complete medium or 5% Triton X-100 (Sigma Aldrich) were used to determine spontaneous and maximum ^{51}Cr release respectively. Supernatants were collected after 4h and radioactivity was measured on a gamma counter. The mean percentage of specific lysis was calculated as: 100 x (experimental release – spontaneous release)/(maximal release – spontaneous release. Each condition was measured in triplicate.

Flow cytometry

To document exercise-induced shifts in leukocyte subsets in peripheral blood, PBMCs from rest and post exercise were labeled with FITC-conjugated anti-CD45RA (IgG2b, clone HI100), anti-CD28 (clone CD28.2) or Alexa488-conjugated anti-KLRG1 (clone 13D12F2), PE-conjugated anti-CD57 (clone TB01), anti-CD62L(clone DREG-56), or anti-CD27 (IgG1, clone O323), PerCP-Cy5.5-conjugated anti-CD4 (IgG2b, clone OKT-4), anti-CD8 (IgG1, clone RPA-T8), or anti-CD56 (IgG1 Clone CMSSB), and APC-conjugated anti-CD3 (IgG1, Clone UCHT1) in a four-color direct immunofluorescence procedure. All monoclonal antibodies were previously titrated to determine optimal dilutions. Cells were incubated with 50 μl of each pre-diluted mAb for 30 minutes in the dark at room temperature. Antibodies were purchased from eBioscience Inc (San Diego CA, USA), except anti-CD57 (Abcam, Cambridge, UK) and the anti-KLRG1, which was kindly provided by Hans Peter Pircher of the University of Freiburg, Germany (21). To document the phenotypes of the expanded cells, aliquots of twice-stimulated cells were labeled with the above antibody panel in a four-color direct immunofluorescence procedure.

Cell phenotypes were assessed on a BD Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI, USA) equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wave length of 640 nm. Lymphocytes were identified by forward and side scatter characteristics and gated electronically using Accuri C6 (CFlow software v1). Single color tubes were used for compensation. A minimum of 20,000 events in the lymphocyte gate were collected. Naïve $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-cells were identified as $CD45RA^+CD62L^+$, central memory (CM) cells were identified as $CD45RA^-CD62L^+$, effector memory (EM) cells were identified as $CD45RA^-CD62L^-$ and the $CD45RA^+$ highly differentiated effector memory (EMRA) cells as $CD45RA^+CD62L^-$(30).

Measurement of blood hormones and determination of viral serostatus

ELISA kits were used to determine serum levels of epinephrine and norepinephrine (2-CAT ELISA, LDN, Nordhorn, Germany), cortisol (Abcam, Cambridge, MA), and neopterin (IBL International, Toronto ON, Canada), and to detect IgG antibodies against CMV (Genway Biotech, San Diego, CA) and EBV (EBV-VCA, IBL International, Toronto ON, Canada). Assays were performed according to manufacturers' directions using a 96 well microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

Data were screened for normality and transformed (logarithm or square root) when required. Paired T-tests were used to compare the number and phenotype of PBMCs and CTLs and the number of SFCs from blood donated at rest and post exercise. Independent T-tests were used to compare participant characteristics between exercise-responders and exercise-non-responders; Fisher's exact test was used to compare categori-

cal variables and group. Restricted maximum likelihood linear mixed models were used to compare physiological responses to exercise, as well as the number and phenotype of PBMCs and CTLs from rest and post exercise, among the exercise-responders and exercise non-responders. The models included main effects of exercise-time (resting and post exercise) and group assignments (MAGE-A4- and PRAME- exercise-responders and exercise-non-responders), and interaction effects between exercise-time and each group. Analyses were performed using Statistical Package for the Social Sciences version 17 software (SPSS, Chicago, IL, USA). Statistical significance was set at $p<0.05$.

RESULTS

Exercise increases leukocyte number for expansion

We first evaluated the effect of exercise on absolute white cell numbers. As shown in **Table 1**, brief exercise (i.e. rapidly ascending 260 steps, led to a significant increase in the number of leukocytes, lymphocytes, and monocytes in whole blood, and increased the numbers of T-cells in the peripheral blood mononuclear cell (PBMC) fraction. To determine whether CTLs could be expanded from both resting and post exercise blood obtained from healthy donors, PBMCs isolated from blood at rest and post exercise were stimulated with autologous TAA-pulsed mo-DCs in the presence of interleukin- (IL-)7, IL-12, and IL-15. While a greater number of PBMCs were stimulated post exercise **(Table 2)**, the numbers of T-cells expanded from resting versus post exercise blood samples following the second week of stimulation were the same **(Table 2)**. Further, the fold- expansion of the CTLs calculated from the total number of input PBMCs did not differ with exercise **(Fig. 1A)**; however, when the fold-expansion was calculated relative to the number of input naïve ($CD45RA^+CD62L^+$) T-cells, the rate of expansion was significantly greater post exercise (mean ±SD: day 7 resting: 9.90 ±4.58, post exercise: 17.07 ±9.84; t(10)=-3.793, p=0.004;day 14 resting: 71.35 ±55.36, post exercise: 115.0 ±101.41; t(10)=-2.683, p=0.023) **(Fig. 1B)**.

Table 1. Cell numbers at rest and post exercise. Data are displayed as mean ±SEM (range). Significant differences from resting are indicated by ** (p<0.01) and * (p<0.05). PBMCs: Peripheral blood mononuclear cells.

Source	Cell type	Resting	Post exercise
Whole blood	Leukocytes ($x10^3$ cells/µl	5.26 ±2.0 (3.6-10.7)	9.28 ±2.8 ** (6.8-17)
	Lymphocytes ($x10^3$ cells/µl)	1.54 ±0.29 (1.1-2.1)	3.56 ±0.72 ** (2.6-5.3)
	Monocytes ($x10^3$ cells/µl)	0.39 ±0.12 (0.25-0.60)	1.04 ±0.31 ** (0.50-1.70)
	Granulocytes ($x10^3$ cells/µl)	3.33 ±1.69 (1.8-8.0)	4.67 ±1.99 ** (2.8-10.0)
PBMCs	CD3+T-cells ($x10^3$ cells/µl)	1.10 ±0.29 (0.66-1.63)	1.80 ±0. 51** (1.07-2.91)
	CD45RA+CD62L+ T-cell ($x10^3$ cells/µl)	0.53 ±0.18 (0.27-0.81)	0.65 ±0.22** (0.30-1.01)
	CD45RA-CD62L+ T-cell ($x10^3$ cells/µl)	0.31 ±0.10 (0.18-0.46)	0.44 ±0.16** (0.23-0.80)
	CD45RA-CD62L- T-cell ($x10^3$ cells/µl)	0.19 ±0.06 (0.08-0.34)	0.44 ±0.23** (0.16-0.98)
	CD45RA+CD62L- T-cell ($x10^3$ cells/µl)	0.04 ±0.03 (0.01-0.12)	0.16 ±0.12** (0.04-0.37)

Table 2. The numbers of monocyte-derived dendritic cells (mo-DCs), PBMCs, and once and twice stimulated CTLs in resting and post exercise blood. Data are displayed as mean ±SEM (range). Significant differences from resting are indicated by ** (p<0.01) and * (p<0.05).

Cell type	Resting	Post exercise
Mo-DCs (Day 0) ($x10^6$ cells)	0.94 ±0.48 (0.4-1.8)	1.56 ±0.78* (0.6-2.9)
PBMCs (Day 0) ($x10^6$ cells)	10.25 ±5.08 (4-18)	15.38 ±7.31 * (6-24)
Once-stimulated CTLs (Day 7) ($x10^6$ cells)	16.74 ±11.79 (4.78-48.90)	22.16 ±12.70* (6.27-49.05)
Twice-stimulated CTLs (Day 14) ($x10^6$ cells)	80.44 ±75.13 (5.04-329.58)	90.61 ±69.03 (11.57-262.08)

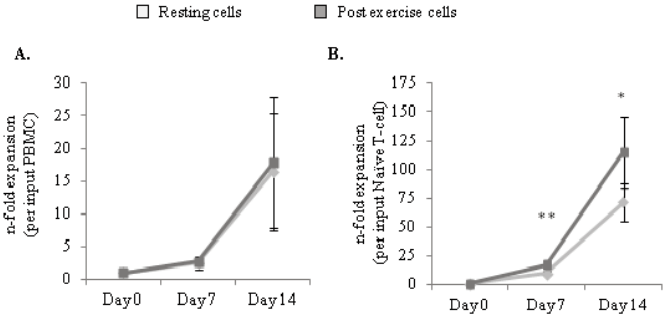

Figure 1. The expansion of the CTL lines. **A**) *n*-fold expansion of CTLs per input PBMC and **B**) *n*-fold expansion of CTLs per input naïve (CD45RA+CD62L+) T-cell. Cell counts were assessed at end of each stimulation cycle on days 7 and 14. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by ** (p<0.01) and * (p<0.05). Mean ±S.D. is shown, data are from 12 (**A**) and 11 (**B**) participants.

CTLs expanded from rest and post exercise have similar phenotypes

Although exercise increased the proportion of natural killer (NK) cells and later-differentiated T-cells in blood (data not shown), resting and post exercise CTLs no longer differed in the proportions of NK-cells, T-cells, and most T-cell subsets after two stimulations. CTLs expanded post exercise contained a greater proportion of low differentiated ($CD45RA^+CD62L^+$) $CD4^+$ T-cells (resting: 18.2±7.5 %, post exercise: 21.9± 8.1%; t(7)=-2.613, p=0.035) and a smaller proportion of later differentiated ($CD45RA^-CD62L^-$) $CD4^+$ T-cells (resting: 29.1 ±7.4 %, postexercise: 24.8 ± 7.6%; t(7)=3.903, p=0.006) **(Fig. 2A)**. The T-cell subsets that exhibited the greatest increase in cell number in both resting and post exercise cells during expansion were the central memory ($CD45RA^-CD62L^+$) $CD4^+$ and $CD8^+$ T-cells **(Fig. 2B)**. Similar results were obtained when T-cell subsets were identified based on combinations of CD27 and CD28 surface molecules (data not shown).

Most participants are 'exercise-responders' for one or more TAA

To determine whether the twice stimulated T-cells expanded from rest and post exercise were specific for each TAA, we measured interferon-γ (IFN-γ) secretion after stimulating CTLs overnight with MAGE-A4, PRAME, and WT-1 pepmixes in an ELISPOT assay. All of the *ex vivo* expanded T-

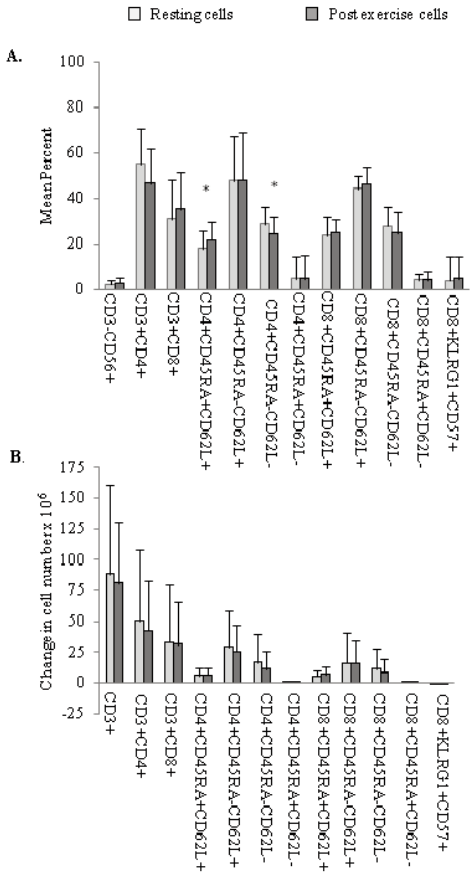

Figure 2. The phenotype of the CTL lines. **A**)The percentage of the indicated T-cell subsets within CTLs expanded by two weeks of stimulation with autologous TAA-presenting mo-DC. **B**) The absolute change in number of the expanded CTL subsets from the number of T-cells in each subset at day 0. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by * ($p<0.05$). Mean ±S.D. is shown, data are from 8 participants.

cells showed TAA recognition; however we observed a large variation among the participants with respect to the difference in the number of IFN-γ-secreting cells (spot-forming cells; SFCs) between TAA-specific T-cells expanded from rest versus post exercise. **(Table 3)**. We therefore classified participants as 'exercise-responders' (greater than 10% increase in SFC post exercise) or 'exercise-non-responders' (less than 10% increase in SFCs post exercise) for each TAA-specific CTL line expanded. 84% of the participants (16 of 19) were exercise-responders for at least one TAA-specific CTL line **(Fig. 3A)**. For MAGE-A4-specific CTLs, 70% of participants were exercise-responsive, for PRAME-specific CTLs, 61% of participants were exercise-responsive, and for WT-1-specific CTLs, 38% of participants were exercise-responsive **(Fig. 3B).**

Table 3. The total number of CTLs (x 10^3) recognizing the indicated TAA expanded from rest and post exercise by two stimulations with autologous mo-DC. CTLs were enumerated by IFN-γ ELISPOT. Results for each participant are shown.

	MAGE-A4		PRAME		WT-1	
Participant	Rest	Post exercise	Rest	Post exercise	Rest	Post exercise
1	2.8	10.8	10.9	15.4	6.4	17.8
2	5.9	12.9	85.6	0.7	209.1	8
3	45.2	12.8	25.5	8.8	28.7	10.7
4	2.1	5	0.9	23.1		
5	0.7	2.8	5.2	4.6		
6	33	37.7	16.8	20		
7	5.2	1.2	9.3	3.8	2.9	1.2
8	0.4	2.2	3.8	3.3	0.8	0.2
9	11.5	10.6	93.9	37.5	24.1	12.8
10	1.1	6.1	2.2	4.7	7.7	4.7
11	22.1	44.6	84.5	137.2	100.1	90
12	10.8	18.6	11.3	1.0	3.6	41.2
13					13.1	122.8
14	4	10	2.1	12.6	1.4	7.9
15	2.5	6.5	0.8	5.2	0.6	0.5
16	0.2	1.6	1.7	2		
17	0.4	0.3	0.5	8.1		
18	19.4	10.9	25.5	31.1	15.5	16.9
19			0.2	4.0		

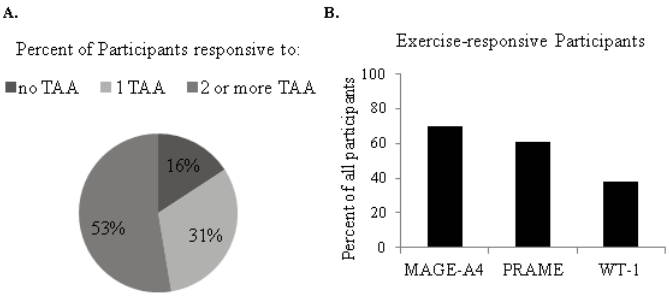

Figure 3. Exercise increases the number of TAA-specific CTLs in some, but not all, participants. **A**) The percent of participants that were exercise-responders for none of the TAA, for 1of the TAA, or for 2 or more TAA. **B**) The percent of participants who had at least a 10% increase in TAA-specific CTLs post exercise (exercise-responders) for the MAGE-A4, PRAME, and WT-1 specific-cell lines.

Exercise increases the number of TAA-specific CTLs among 'exercise-responders'

Exercise-responders had a significantly greater number of MAGE-A4, PRAME- and/or WT-1-specific CTLs post exercise ($p<0.05$) **(Fig. 4A)**. The 12 (of 17) participants who were MAGE-A4-exercise-responders had a mean increase of 6118 (SD: 5629) MAGE-A4-specific CTLs post exercise, compared to resting cells (median: 4825, range: 1489 to 22492; $t(11)=-6.307$, $p=0.000$) (corresponding to a mean percent increase of 254% in SFCs among the 12 participants). The 11 (of 18 participants) PRAME-exercise-responders had a mean increase of 10671 (SD: 15124) PRAME-specific CTLs post exercise (median: 4590, range: 220 to 52690; $t(10)=-2.902$, $p=0.016$) (mean increase of 523%). The 5 (of 13 participants) WT-1-exercise-responders had a mean increase of 33332 (SD: 44927) WT-1-specific CTLs post exercise (median: 11426, range: 1419 to 109728; $t(4)=-3.497$ $p=0.025$) (a mean increase of 511%). Exercise-responders also had a marked increase in SFCs without stimulation (CTL alone) following exercise; however the number of SFCs resulting from TAA-stimulation was significantly greater than without stimulation at rest and post exercise ($p<0.05$).

Exercise-non-responders had no significant difference between blood donated at rest versus post exercise with respect to the number of T-cells recognizing MAGE-A4 and PRAME ($p>0.05$). However, there was a trend for a decreased frequency of MAGE-A4- and PRAME-specific T-cells expanded from post exercise samples versus samples obtained at rest (mean decrease±SD: 45%±30% and 57%± 34%, respectively). WT-1-exercise-non-responders had significantly fewer WT-1-specific T-cells expanded from post

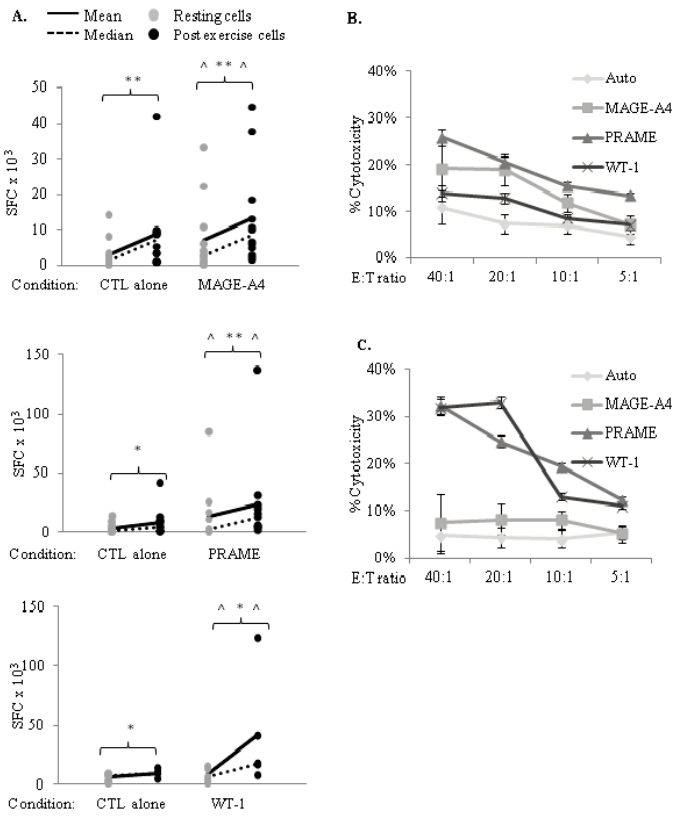

Figure 4. TAA-specific responses after two weeks of stimulation among exercise-responders. **A**) The total number of IFN-γ-secreting cells (SFCs) among cells expanded from blood donated at rest (grey) and postexercise (black) following overnight stimulation with the indicated TAA, or without stimulation (CTL alone). Results for the exercise-responsive participants are shown; each spot represents an individual participant. Significant difference between cells expanded from blood donated at rest and post exercise is indicated by ** ($p<0.01$) and * ($p<0.05$), ^indicates significant difference from CTL alone condition; $p<0.05$. ^{51}Cr release at 4 hours after coincubation of representative CTL lines expanded from resting cells (**B**) and post exercise cells (**C**) with autologous PHA blasts pulsed with MAGE-A4, PRAME, WT-1, or unpulsed PHA blasts (auto). The data are the mean ±SD percentage lysis at effector: target ratios of 40:1, 20:1, 10:1, and 5:1. Each condition was measured in triplicate.

exercise blood, with a mean decrease of 30721 (SD: 69130) WT-1-specific CTLs post exercise (median: -6514, range: -87 to -201093;$t(7)=2.742$, $p=0.029$) (data not shown).

The cytolytic activity of the CTL lines was measured using a 4h Cr^{51} release assay. Data from the cytotoxicity assays confirmed that the TAA-specific T-cells were functional, as both resting and post exercise CTLs exhibited antigen-specific killing of TAApeptide-pulsed autologous target cells **(Fig. 4B and C)**.

PRAME exercise-responders and non-responders differ in T-cell phenotype

We sought to identify other parameters in which exercise-responders and exercise-non-responders differed. Although exercise-responders appeared to have fewer MAGE-A4-, PRAME-, and WT-1-specific CTLs at rest compared to non-responders, this did not reach statistical significance **(Table 4)**. Exercise-responders and non-responders did not differ in the number of SFCs in the CTL alone condition or in response to SEB (data not shown). Further, using data collected in 11 of the 19 participants, we compared demographic characteristics and physiological responses to exercise between the exercise-responders and non-responders to the MAGE-A4- and PRAME antigens **(Table 5)**. WT-1-specific-CTL lines were not examined in this context as only two of these 11 participants were exercise-responders. While

Table 4. The total number of CTLs recognizing the indicated TAA from blood donated at rest and post exercise and expanded by two stimulations with autologous mo-DCs in exercise-responsive and exercise-non-responsive participants. CTLs were enumerated as spot-forming cells (SFCs) by IFN-γ ELISPOT. Groups did not differ in the number of SFCs within each time point. Mean ±S.D. (range) is shown. Significant difference from cells expanded from rest is indicated by ** ($p<0.01$) and * ($p<0.05$).

	MAGE-A4 cell line		PRAME cell line		WT-1 cell line	
	responders N=12 (7 female)	non-responders N=5 (2 female)	responders N=11 (7 female)	non-responders N=7 (2 female)	responders N=5 (4 female)	non-responders N=8 (3 female)
Resting SFC (x 10^3)	7.1 ±10.3 (0.2-33)	16.3 ±17.3 (0.4-45)	13.3 ±25.0 (0.4-84.4)	33.5 ±39.1 (3.8-93.9)	8.0 ±6.1 (1.4-15.5)	46.7 ±73.4 (0.6-209.1)
Post exercise SFC (x 10^3)	13.2 ±14.0 (1.6-44.6)**	7.2 ±5.9 (0.3-12.8)	23.9 ±38.6 (2.0-137.1)**	8.5 ±13 (0.7-37.5)	41.3±47.2 (7.9-122.8)*	16.0 ±30.2 (0.2-90.0)*

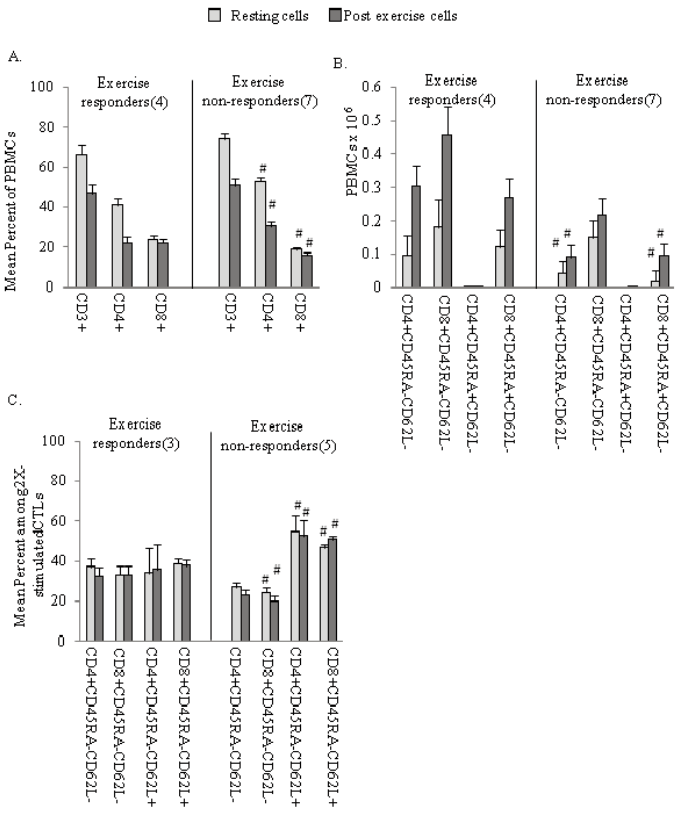

Figure 5. PRAME exercise responders and exercise non-responders differ in the phenotype of T-cell subsets at rest and post exercise in unstimulated and expanded cells. **A**) The percent of total T-cells and $CD4^+$ and $CD8^+$ T-cells in PBMCs (day 0) at rest and post exercise. **B**) The number of medium and late differentiated $CD4^+$ and $CD8^+$ T-cells in PBMCs at rest and post exercise. **C**) The percent of medium and late differentiated $CD4^+$ and $CD8^+$ T-cells among CTLs (day 14) at rest and post exercise. Data are displayed as means ± SEM of 11 or 8 participants, # indicates significant difference between PRAME exercise-responders and exercise-non-responders ($p<0.05$).

Table 5. Characteristics and exercise performance measures of representative participants from MAGE-A4 and PRAME exercise-responders and exercise-non-responders. Mean ±SD (range) from 11 participants (5 female) are shown. BMI: body mass index, PA: self-reported physical activity level (1=infrequent, 7=>3h/week of vigorous activity) #indicates significant difference from non-responder ($p<0.05$),* indicates significant difference from resting ($p<0.05$).

		MAGE-A4 cell line		PRAME cell line	
		responders N=8 (4 female)	non-responders N=3 (1 female)	responders N=4 (3 female)	non-responders N=7 (2 female)
Age (yrs)		27.4 ±1.8 (25-30)#	24.3 ±2.5 (22-27)	27.0 ±2.4 (25-30)	26.3 ±2.4 (22-29)
BMI (kg/m^2)		23.6 ±3.3 (18.0-28.3)	23.3 ±2.9 (19.9-25.1)	21.5 ±3.1 (18.0-25.5)	24.6 ±2.6 (19.9-28.3)
PA rating (1-7)		6.3 ±0.7 (5-7)	5.3 ±0.6 (5-6)	6.5 ±0.6 (5-7)	5.7 ±0.8 (5-7)
VO_2max (ml/kg/min)		50.5 ±6.3 (38-59)	50.0 ±2.6 (47-52)	50.7 ±3.6 (48-56)	50.2 ±6.5 (38-59)
CMV serostatus (% seropositive)		25%	33%	50%	14%
CMV IgG titer of seropositive (IU/ml)		16.15 ±6.7 (5.1-18.4)	24.07	16.15 ±6.7 (5.1-18.4)	24.07
EBV serostatus (% seropositive)		75%	66%	100%	57%
EBV IgG titer of seropositive (IU/ml)		140.4 ±73.4 (22-199)	154.19±64.8 (108-200)	178.37±70.9 (22-199)	168.27±40.9 (108-200)
Time to completion (sec)		105±19 (73-133)	111±16 (96-128)	106±17 (93-133)	107±20 (73-131)
Maximum heart rate obtained (bpm)		168±14 (142-185)	182±5 (177-187)	174±10 (164-185)	171±15.5 (142-187)
Blood lactate (mmol)	Resting	1.3±0.5 (0.75-2.1)	1.4±0.4 (1-1.9)	1.5±0.5 (1-2.1)	1.2±0.4 (0.75-1.9)
	Post exercise	8.5±2.3 (5-11.7)*	9.5±2.5 (7-12)*	8.8±2.3 (6.9-11.7)*	8.8±2.5 (5-12)*
Serum Neopterin (nmol/L)	Resting	6.15±3.9 (1.7-11.8)	9.3±3.9 (5.7-13.5)	4.9 ±3.9 (1.7-10.4)	8.2±3.8 (2.4-13.5)
	Post exercise	7.4±4.2 (1.8-12.4)*	11.5±5.8 (6.8-18.0)*	6.6±4.1 (1.8-10.9)	9.7±5.1 (2.0-18.0)
Serum Cortisol (ng/ml)	Resting	193±163 (52-506)	105±16 (87-118)	76 ±20.9 (52-90)	205±153 (87-506)
	Post exercise	202±190 (54-582)	89.6±9.4 (79-97)	77±26 (54-106)	208±185 (79-582)
Serum epinephrine (pg/ml)	Resting	61.8±19.3 (40.5-90.3)	46.9±3.26 (44.8-50.7)	55.2±14.4 (40.6-74.9)	59.2 ±20.2 (40.7-74.9)
	Post exercise	320±323 (55.3-1022)*	115±59.6 (80.9-184)*	190±152 (55.3-383)*	307±347 (80.9-1022)*
Serum norepinephrine (pg/ml)	Resting	511±261 (208-1036)	1192±372 (908-1613)	641±280 (384-1036)	728±502 (208-1613)
	Post exercise	1960±1858 (482-5553)*	3396±1248 (2189-4680)*	2018±2374 (482-5553)*	2543±1536 (749-4680)*
Lymphocytes (x10^3 cells/µl)	Resting	1.54±0.21 (1.2-1.9)	1.53±0.51 (1.1-2.1)	1.45±0.21 (1.2-1.7)	1.59±0.33 (1.1-2.1)
	Post exercise	3.49±0.42 (2.3-4.9)*	3.77±1.39 (2.6-5.3)*	3.52±0.58 (2.9-4.3)*	3.58±0.84 (2.6-5.3)*
Monocytes (x10^3 cells/µl)	Resting	0.36±0.98 (0.25-0.50)	0.47±0.15 (0.30-0.60)	0.34±0.11 (0.25-0.50)	0.41±0.12 (0.30-0.60)
	Post exercise	0.98±0.26 (0.5-1.3)*	1.23±0.42 (0.9-1.7)*	0.94±0.15 (0.75-1.1)*	1.11±0.37 (0.50-1.7)*

there were more females in the exercise-responder groups than in the non-responder groups, this did not reach statistical significance ($p>0.05$). Compared to exercise-non-responders, exercise-responders tended to be slightly older (MAGE-A4 exercise-responders: 27.4 ±2.4 yrs, MAGE-A4 exercise-non-responders: 24.3 ±2.5 yrs; $F=1.73$,$t(10)=-2.479$, $p=0.033$) **(Table 5)**. No other differences in participant demographics, such as fitness or viral serostatus, or in physiologic responses to exercise, including maximum heart rate and circulating levels of stress hormones, were found between the groups; all participants demonstrated a significant increase in blood lactate, hormone levels, and cell counts following exercise **(Table 5)**.

In the unstimulated cells (pre-expansion), PRAME exercise responders had a smaller proportion of $CD4^+$T-cells and a greater proportion of $CD8^+$T-cells compared to PRAME exercise non-responders,as well as a greater number of late-differentiated cells ($CD45RA^-CD62L^-$ $CD4^+$ T-cells and $CD45RA^+CD62L^-$ $CD8^+$ T-cells) **(Fig. 5A and 5B)**. The two groups did not differ in the exercise-induced mobilization of these cell subsets. Following two weeks of stimulation, the PRAME exercise responders had a greater proportion of late differentiated cells($CD45RA^-CD62L^-$ $CD4^+$ and $CD8^+$ T-cells) and a smaller proportion of central memory cells compared to PRAME exercise non-responders **(Fig. 5C)**. T-cell subsets did not differ between the MAGE-A4 exercise-responders and non-responders either before or after CTL expansion.

DISCUSSION

While the *ex vivo* expansion of virus specific T-cells from healthy seropositive donors is becoming increasingly routine (28), expansion of TAA-specific T-cells has proven more difficult, partially due to low numbers of naturally occurring TAA-specific T-cells among healthy individuals. As acute dynamic exercise elicits a profound and almost instantaneous leukocytosis (41), we hypothesized that a single bout of exercise could serve as a non-invasive and economical approach to increase the number of activated mononuclear cells in the blood. This strategy could thus augment the manufacture of TAA-specific T-cells. In agreement with this hypothesis, brief maximal exercise increased the expansion of CTLs specific to at least one of three TAAs in 84% of the healthy adults sampled, with exercise increasing the number of CTLs specific for MAGE-A4 and PRAME in 70% and 61% of all participants respectively. Both resting and post exercise expanded CTL lines were functional, as evidenced by their ability to secrete IFN-y in response to peptide stimulation and to kill autologous peptide-pulsed target cells in an antigen-specific manner.The CTLs expanded post exercise included both CD4+ and CD8+ T-cells with central and effector memory phenotypes, which is associated with persistence after infusion of antigen-specific CTLs (20).

Compared to CTLs expanded from resting blood, the exercise-responders demonstrated a 3.4- and 6.2- fold increase post exercise in the numbers of MAGE-A4- and PRAME-specific CTLs, respectively. Although only 38% of participants exhibited an exercise-enhancement for the expansion of WT-1-sepcific CTLs, the WT-1 exercise-responders showed a marked (6.1-fold) increase in the number of WT-1-specific CTLs post exercise. As very large numbers of TAA-specific CTLs are required for adoptive transfer (patient doses range from 4×10^7 to 3.3×10^9CTL/m^2 per transfer, 2-8 transfers) (6, 43), any increase in the number of TAA-specific CTLs is desirable. Exercise used in conjunction with apheresis, a clinical technique used to increase the number of leukocytes collected from a donor, could lessen the volume of blood

processed, thereby decreasing the potential risks and discomforts associated with this procedure.Because exercise increases the number of TAA-specific CTLs, it is possible that a sufficient number of these cells could be manufactured and transferred to the patients more quickly than with traditional protocols using resting blood. Current protocols typically require 4 to 12 weeks to stimulate and expand TAA-specific CTLs, limiting the applicability of this therapy to many patients (4, 40). Although we did not perform a time course experiment in the present study, it will be important to determine if exercise can reduce the time needed to manufacture clinically sufficient numbers of TAA-specific CTLs. The rapid generation of TAA-specific CTLs from healthy exercising donors could improve the efficacy of TAA-specific CTL transfer as both a prophylactic and early stage relapse treatment for a range of hematologic malignancies.

Although exercise did not increase TAA-specific CTL expansion from all participants in this study, it is important to note that exercise did not appear to substantially impair the expansion of TAA-specific CTLs either. That is, in the exercise-non-responders, the numbers of TAA-specific CTLs expanded post exercise were often similar to the numbers generated from resting blood. It is not known why exercise was less effective at expanding WT-1-specific CTLs compared to MAGE-A4- and PRAME-specific CTLs. Differences in antigen size seem unlikely, as the number of amino acids in the WT-1 peptide pool is greater than PRAME but less than-MAGE-A4. It would be interesting for future studies to broaden the tumor antigen repertoire examined post exercise, including tumor antigens involved in a variety of both solid organ and hematological cancers such as Aurora kinase (27), BMI-1 (33), and survivin (2).

It would be advantageous to identify individuals in whom exercise is most likely to benefit TAA-specific CTL expansions. We therefore attempted to identify demographic and physiological predictors of the exercise-responders in the current study. Although persistent herpes viruses such as CMV are believed to impair T-cell responses to novel antigens (34), CMV and EBV serostatus did not differ between the exercise-responders and non-responders. Moreover, although the physiological responses to exercise were not controlled, we did not find any differences in peak heart rate, exercise duration, blood lactate concentration, leukocyte mobilization, or serum hormones between the groups. It is therefore unlikely that differences between the groups were due to variations in demographic characteristics or the physiological responses to exercise. We also considered if exercise-induced changes in the composition of lymphocyte subsets differed between groups. Although PRAME-exercise-responders had a greater proportion of $CD8^+$ T-cells in initial cell cultures, and thus a greater proportion of T-cells with potential cytotoxic effector function, the groups no longer differed in the number or proportions of these cells following two weeks of stimulation. And while PRAME-exercise-responders had a greater proportion of EM $CD8^+$ cells among the expanded cells than non-responders, there was no exercise effect on the proportions of these cells in either group. The fact that exercise did not impact cell redeployment differently in the two groups suggests that shifts in cell proportions due to exercise can not fully explain differences between exercise-responders and non-responders. However, we acknowledge that this analysis may be limited by the small sample size and it remains possible that certain demographic characteristics and/or physiological responses to exercise may serve as good predictors of those donors likely to have an increased TAA-specific CTL response after exercise. Moreover, because the exercise protocol used in this study was rather rudimentary, future studies should make better attempts to optimize the intensity and/or duration of exercise for augmenting the manufacture of TAA-specific CTL and also include a non-exercise control condition to account for potential variability in CTL generation between blood draws.

The mechanisms that underpin the effects of exercise on TAA-specific CTL expansion are unknown. Shear stress, due to increases in cardiac output and blood pressure, and the actions of catecholamines binding to adrenergic receptors on leukocytes are thought to underlie many of the exercise-induced changes in immune cells (11, 14, 41). However, in the present study, epinephrine and norepinephrine levels increased to a similar extent in both the exercise-responders and non-responders, and the groups did not differ in maximum heart rate. This indicates that these physiological responses to exercise cannot alone explain the effect of exercise on TAA-specific CTL expansion. As has been documented elsewhere (10, 18, 19, 36), we observed a preferential mobilization of NK-cells and late differentiated subsets of T-cells, which in turn decreased the proportion of naïve cells within the PBMCs post exercise.We did not account for this increase in cells which are unlikely to respond to TAA (such as T-cells specific to alternate antigens), as a fixed ratio of mo-DC: PBMCs was used in all cultures. Despite beginning with a smaller proportion of naïve T-cells post-exercise, the two-week expansion using mo-DC stimulation yielded similar numbers of cells. This could suggest that the naïve T-cells mobilized by exercise were more susceptible to the stimulatory signals provided by the mo-DCs. Although not measured in these experiments, other studies have shown that T-cells mobilized with exercise express activation markers such as HLA-DR (15). Future work should account for exercise-induced changes in the composition of T-cell subsets and culture equal numbers of naïve cells from rest and post exercise.

The idea of using exercise as a simple adjuvant to improve immune-based treatments is quite new (35). Millard et al recently found that exercise increases peripheral blood NK-cells without causing substantial changes in their function, thus rendering them useful for some *in vitro* experiments requiring large numbers of NK-cells (24). We have also shown that many of the T-cells mobilized into the blood by exercise are specific to viruses such as CMV (37), indicating that a single bout of exercise could augment the manufacture of virus-specific CTLs as a means to prevent or treat viral infections after HSCT. The current study adds to this literature, demonstrating that exercise can also increase the expansion of TAA-specific CTLs. Although the current results are limited to donors capable of completing a maximal exercise bout, future studies able to identify the mechanism(s) by which exercise improves the expansion of TAA-specific CTLs may highlight pathways that could be manipulated by pharmacological

means to mobilize desirable cell populations to the peripheral blood compartment. This would expand the results of the present study to allogeneic donors lacking the fitness level necessary to complete an exercise bout, or to cancer patients requiring an autologous transfer but too ill to exercise.

In conclusion, we have shown for the first time that a short bout of maximal exercise in healthy adults yields greater numbers of functional CTLs specific for the tumor-associated-antigens MAGE-A4 and PRAME, and in some cases WT-1. Therefore, due to its simplicity, cost-effectiveness, likelihood of success and low risk of impairing the response, a single bout of exercise immediately prior to blood donation should be considered a worthwhile means to augment the manufacture of TAA-specific CTLs in healthy allogeneic donors. Future research should aim to optimize the intensity and duration of the exercise protocol that will best elicit this response, and determine if exercise could be used to minimize the time required to manufacture clinically sufficient numbers of TAA-specific CTLs for adoptive transfer immunotherapy.

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Influence of age and physical fitness on miRNA-21, TGF-β and its receptors in leukocytes of healthy women

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ABSTRACT

Rationale: *The TGF-β superfamily has been shown to play an important role in a wide range of physiological as well as pathological processes including ageing, immune modulation, atherosclerosis and cancer development. The aim of the current study was to investigate (i) whether TGF-β signalling in peripheral blood mononuclear cells (PBMCs) would differ between young and old females and (ii) whether physical performance parameters of elderly women would be related to the expression of TGF-β or its receptors.*

Methods: *Sixteen healthy young (22-28 years; YF) and 90 healthy older (65-92 years; OF) females participated in the study. In addition to several components of health-related physical fitness, circulating CRP and TGF-β levels were determined together with the mRNA expression of TGF-β, TGF-βRI, TGF-βRII, and miRNA-21 (known to interfere with TGF-β signalling) in PBMCs.*

Results: *Physical fitness as determined by 6-minutes walking test (YF:median 932 (range 573-1254) m; OF:360 (114-558) m), handgrip strength (YF: 32 (24-39) kg; OF:18(10-30) kg), relative isokinetic peak torque of knee extensors (YF:1.9 (1.2-2.3) Nm/kg; OF:1.0 (0.2-1.9) Nm/kg and flexors (YF: 1.1 (0.7-1.5) Nm/kg; OF: 0.5 (0.2-1.0) Nm/kg was substantially lower in older women ($p<0.001$ for all comparisons). These changes were paralleled by an increase in hs-CRP (YF: 0.9 (0.1-4.3)mg/L; OF: 2.3 (0.3-56.7)mg/L,$p<0.001$). Serum levels of TGF-β and TGF-β mRNA levels from PBMCs did not differ between young and old women whereas, both TGF-βRI/GAPDH (YF: 4.07 (1.38-14.60); OF: 2.08 (0.14-28.81); $p=0.020$) and TGF-βRII/GAPDH levels (YF: 3.16 (1.14-10.25); OF: 1.71 (0.51-14.86); $p=0.020$) were lower with respect to old age. In elderly women, only TGF-βRI expression correlated negatively with miRNA-21 expression in PBMCs ($\rho=-0.315$; $p=0.004$). Interestingly, hs-CRP and miRNA correlated positively with handgrip strength ($\rho=0.237$ and $\rho=243$, $p<0.05$), while none of the TGF-β-related parameters were related to physical performance.*

Conclusion: *The results suggest that age affects TGF-β signalling in leukocytes by altering the expression levels of its receptors. These changes seem to occur independently of physical fitness of old women.*

Key Words: Inflamm-ageing, TGF-β Pathway, TGF-β receptors, microRNA-21, physical performance, Vienna Active Ageing Study

INTRODUCTION

Although the causes of human ageing are multifaceted, the molecular inflammation hypothesis of ageing implies that increased oxidative stress will lead to the activation of redox-sensitive transcription factors which in turn enhance the expression of pro-inflammatory genes in a variety of different cell types (8). As a consequence ageing is associated with a chronic inflammatory state, where pro-inflammatory factors such as tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) or C-reactive protein (CRP) are continuously present (over years) at levels higher than baseline but much lower than those found during acute inflammation (11, 64). Chronically elevated levels of IL-6 (> 2.0 ng/L) of middle-aged persons reduce the chance of successful ageing and increase the risk of future cardiovascular events or non-cardiovascular death later in life (1). Up to now it is unclear whether the main source of these pro-inflammatory factors is the chronically activated immune system (inflamm-ageing) or the senescence of cells with their senescence-associated secretory phenotype (SASP) leading to an enhanced secretion of pro-inflammatory mediators (15, 16, 18, 27).

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Besides being associated with age-related diseases such as diabetes mellitus type 2, cardiac illnesses or neurological diseases, chronic subclinical inflammation may also contribute to impaired physical function in older adults. Several large scale studies have revealed that higher levels of circulating CRP or IL-6 are associated with lower physical performance such as handgrip strength or gait speed (4, 7, 12, 51). While alterations in TNF-α, IL-6 or CRP in response to ageing, physical inactivity as well as acute and chronic exercise have been studied extensively (14, 38, 42), much less is known about the involvement of transforming growth factor-β (TGF-β) in this context.

TGF-β is known as a potent regulatory cytokine with diverse effects on haemopoietic cells. It has pivotal function in the immune system through keeping tolerance via the regulation of lymphocyte proliferation, differentiation and survival (28). Furthermore, TGF-β controls the initiation and resolution of inflammatory responses through the regulation of chemotaxis, activation and survival of lymphocytes, natural killer cells, dendritic cells, macrophages, mast cells and granulocytes (31). It has been suggested that the age-associated dysregulation of the immune system might be caused by a decreased expression and functioning of receptors or signalling rafts and/or defects in signalling pathways finally leading to altered function of immune cells and immunosenescence (25). TGF-β mediates its biological function via binding to type II and forming a complex with type I transmembrane serine/threonine kinase receptors (TGF-βRI, TGF-βRII). Ligand binding assembles a complex consisting of two type I receptor components and two type II components, whereby the type I components are phosphorylated further activating intracellular Smad proteins whose nuclear localization is required for the transcriptional regulation of target genes (35).

Interestingly, TGF-β signalling is intimately involved in biogenesis and control of microRNAs (miRNAs) as Smad proteins play a regulatory role in the processing of miRNAs in the nucleus (2, 21). MiRNAs are small non-coding RNAs that affect gene expression either by inhibiting translation of their target proteins or by degrading the respective mRNA (67). They have been shown to regulate many biological functions including ageing, immune function and the response to exercise (33, 36, 53, 63). It also has been suggested that senescent donor cells contribute to SASP by secreting not only soluble proteins but also microvesicles containing miRNAs which are taken up by recipient cells and may cause or contribute to age related pathologies like osteoporosis, atherosclerosis, Alzheimer's disease or diabetes mellitus, type 2 (62). Several miRNAs such as miRNA-155, -146a and -21 are involved in the regulation of inflammation by controlling Toll-like receptor or nuclear factor-κB (NF-κB) signalling (45, 48). MiRNA-21, despite being well established as an 'onco-miR' due to its aberrant expression in numerous cancers, seems to be of particular importance as it links inflamm-ageing to cellular senescence (44). Intracellular as well as circulating miRNA-21 levels are higher in elderly in comparison to young subjects or centenarians. This is associated with a lower TGF-βRII expression in leukocytes (46), whereby TGF-βRII is a validated target of miRNA-21 in adipocytes (24) and colon cancer cells (66). Mice lacking the TGF-βRII develop an autoimmune biliary ductular disease similar to human primary biliary cirrhosis. Using this experimental model it has been shown that the lack of TGF-β signalling leads to a down-regulation of several miRNAs in T cells but interestingly to an up-regulation of miR-21 concomitant with an increased production of TNF-α and interferon-γ (2). It is hypothesized that the up-regulation of miRNA-21 is caused by a dysregulated gene expression normally controlled by TGF-β. This would result in the activation of inflammatory pathways such as NF-κB which directly or indirectly (via induction of miRNA-21) leads to an up-regulation of inflammation. Another possibility is that a global down-regulation of miRNA expression induces multiple genes causing inflammation; this in turn could lead to elevated levels of miR-21 with a feedback up-regulation of inflammation (2).

However, TGF-β signalling seems to play an important role in relation to several diseases associated with chronic-low grade inflammation. We hypothesized that both, age and physical fitness could affect TGF-β and its receptors in peripheral blood mononuclear cells of healthy women. Therefore, the aim of the current study was to investigate (i) whether circulating TGF-β, as well as intracellular TGF-β, TGF-β receptors and miRNA-21 would differ between young and elderly women and (ii) whether these markers would be associated with parameters of physical performance.

METHODS

Subjects

Ninety elderly women (aged 65-92 years) who were recruited in 5 different senior residencies in the area of Vienna (Curatorship of Viennese Retirement Homes (KWP)) participated in the study. For the current study we used baseline characteristics of study participants intended to take part in a prospective training study. In addition 16 young women (aged 18-28 years) responded to flyers at the University of Vienna. Young as well as elderly women were sedentary (less than 1h of sports activities per week) and free of severe diseases that would contra-indicate medical training therapy or measurement of physical performance, serious cardiovascular diseases, diabetic retinopathy and regular use of cortisone-containing drugs. Written informed consent was obtained from all participants before entry into the study in accordance with the Declaration of Helsinki and after approval by the ethics committee of the City of Vienna (EK-11-151-0811).

Anthropometric Measurements

Using a commercial stadiometer (Seca, Hamburg, Germany), standing height was measured without shoes to the nearest 0.5 cm. Shoulders kept in a relaxed position and arms allowed to hang freely. Body mass was evaluated with a digital scale (BWB 700, Tanita, Amsterdam, Netherlands) to the nearest 0.1 kg with subjects lightly dressed and barefoot. Body mass index (BMI) was calculated by dividing body mass in kilograms by height in meters squared. For determining body composition (muscle and fat mass) we used bioelectric impedance analyses, due to successful validation against data obtained by magnetic resonance imaging (50). Bioelectric Impedance Analyses (BIA) were performed in the morning

after an overnight fast using a BIA Analyzer 2000-S (Data-Input GmbH, Darmstadt, Germany). Participants were asked not to perform any exercise or strenuous physical activity the day before the tests.

Determination of physical performance

To evaluate each participant's aerobic endurance a *6-minutes walking test* was conducted. Therefore, participants walked for 6 minutes as fast as possible on a 30 metre shuttle track. They were allowed to reduce their speed or to rest if the selected speed was too high to be sustained. The completed distance within 6 minutes was recorded (55).

To measure *handgrip strength* participants performed two trials of an isometric handgrip strength test (kg) using a dynamometer in a sitting position with an angle of 45° in the elbow and the lower arm on the armrest. The participant was instructed to squeeze the handle as hard as possible for 4-5 seconds and the maximum isometric contraction was recorded (SAEHAN Corporation, Masan, Korea). The two trials were separated by one minute of passive recovery (37). Out of the two trials on each arm, the best result regardless of side was used for further calculation.

The *isokinetic peak torque of knee extension and flexion* consisted of concentric isokinetic torque measurements (Lido Loredan Biomedical, Inc., Davis, USA; Range of Motion 30°-80°, speed 60°/s or 120°/s). The left leg was tested in all participants except for 3 older women with acute injuries of the left leg making it necessary to test the right leg. The best result of two trials separated by a rest period of two minutes between the attempts was documented. Absolute values were divided by body mass to obtain relative values.

Blood sampling and analyses

Routine blood analyses

Between 06:30 and 08:00 in the morning venous blood samples were taken after an overnight fast. Venous blood was collected in Z Serum Clot Activator collection tubes (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria) for cytokine analyses and in EDTA tubes for the determination of leukocyte subpopulation numbers. For the isolation of peripheral blood mononuclear cells (PBMCs) from whole-blood, BD Vacutainer® CPT™ Tubes containing ~130 IU Na-Heparin and 2 ml Ficoll™ (Becton, Dickenson and Company, Schwechat, Austria) were used.

After at least 30 min and at most 60 min after blood collection, the serum tubes were centrifuged (10 min, 3,000 x g). An aliquot of 1 ml was used for immediate determination of glucose, insulin and hs-CRP. The remaining serum was stored in aliquots at -80°C until further analysis. Glucose was analyzed by hexokinase method and insulin was estimated using a solid-phase, enzyme-labeled chemiluminescentimmunometric assay (IMMULITE 2000, Siemens Healthcare Diagnostics Inc., Llanberis, UK). Cholesterol, HDL cholesterol, LDL cholesterol, triglyceride and hs-CRP were routinely quantified on a Cobas 8000 (Roche Diagnostics, Vienna, Austria). Leukocytes, lymphocytes, monocytes and granulocytes were quantified by flow cytometry on a Sysmex XE-2100™ Automated Hematology System (Sysmex Austria GmbH, Vienna, Austria).

Serum levels of TGF-β

TGF-β was determined using a commercially available DuoSet development kit for performing enzyme-linked immunosorbent assays (DY240, R&D Systems; Abingdon, UK) consisting of a capture antibody (2 µg/ml of mouse anti-TGF-β1), a detection antibody (300 ng/ml of biotinylated chicken anti-human TGF-β1), and recombinant human TGF-β1 to prepare a standard curve (31-2,000pg/ml). Twenty µl of each serum sample were activated by adding 10 µl of 1N HCl, incubated at room temperature for 10 min and neutralized with 10 µl of 1.2N NaOH/0.5 M Hepes. The activated sample was diluted 20-fold with reagent diluent (0.05% Tween® 20 in PBS) and used in the assay following the instructions of the manufacturer. Spectrophotometric measurements were performedon a Victor^3 1420 Multilabel Counter (Perkin Elmer, MA, US).

Isolation of total RNA from PBMCs

PBMCs were separated from red blood cells and neutrophils by centrifugation of BD Vacutainer® CPT Tubes at 1,650 x g for 20 min at room temperature. After removing 2 ml of the plasma supernatant, the cells comprising PBMCs were resuspended in the remaining plasma and transferred to another tube. PBMCs were washed twice with PBS without Ca and Mg according to the protocol provided by the manufacturer. Finally, the pellet was carefully resuspended in 700 µl of QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and stored on -80° until analysis.

Total RNA including small RNAs was isolated after thawing and incubating the samples for 5 min at room temperature using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and following the instructions of the manufacturer. In order to prepare a miRNA-enriched fraction separated from the larger RNAs (>200nt) the RNeasy Min Elute Cleanup Kit (Qiagen, Hilden, Germany) was used. Reverse transcription was for the miRNA-enriched fraction was performed using the miScript II RT Kit (Qiagen, Hilden, Germany) while larger RNAs were reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Quantitative real-time RT-PCR

TGF-β, TGF-βRI and TGF-βRI mRNA were determined usingthe respective primer assays (Hs_TGFB1_1 (QT00000728), Hs_TGFBR1_1 (QT00083412), Hs_TGFBR2_1 (QT00014350), Qiagen, Hilden, Germany) in conjunction with the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). A standard curve was prepared by pooling equal amounts of cDNA from PBMCs of 7 young and 23 old subjects which were randomly selected from the study population. In addition, GAPDH (Hs_GAPDH_2 (QT01192646)) served as endogenous control and was used to normalize the data. Quantification was performed on an Applied Biosystems® 7500 Real-Time PCR System.

MiRNA-21 expression levels were detected using a miScript Primer Assay specific for miRNA-21 (hs_miR-21_2 (MS00009079), Qiagen, Hilden, Germany). A standard curve was prepared by using a commercially available totalRNA of peripheral blood leukocyte cells of a 24 year old female donor (Total RNA (R1234148-10), BioChain, Newark, USA). Quan-

tification was performed on an Applied Biosystems® 7500 Real-Time PCR System.

Statistical analyses

The data acquisition and data processing took place using commercial software (IBM SPSS for Windows, Version 20). Shapiro-Wilk test was used to determine if data sets were normally distributed. As most of the variables did not meet the criteria for normal distribution, Mann-Whitney U test was used in order to compare young and elderly women. Associations between variables were analysed using Spearman´s ρ correlation coefficient. Data are shown as median (minimum-maximum), statistical significance was set at $p<0.05$.

RESULTS

Subject characteristics

Old females with a median age of 84 years had a higher body mass (+23%, $p<0.001$) as well as BMI (+36%, $p<0.001$) compared to young females (median age: 25 years). Bioelectric impedance analyses revealed a higher percentage of body fat mass (+37%, $p<0.001$) and a lower muscle mass (-12%, $p<0.001$). The age-related changes in body composition were accompanied by substantial worsening of health-related parameters such as circulating glucose levels (+10%, $p<0.001$), cholesterol (+22%, $p=0.002$), LDL cholesterol (+49%, $p<0.001$), HDL cholesterol (-16%, $p=0.006$), and triglyceride (+41%, $p=0.006$) (Table 1).

Physical fitness

Aerobic fitness was determined by 6-minutes walking test (6MWT). Old females reached a significant lower distance within a time frame of 6 minutes (-61%, $p<0.001$) than younger females (Figure 1A). Strength was determined by handgrip dynamometer as well as isokinetic peak torque measurements. Isometric handgrip strength was significantly lower in old women (-43%, $p<0.001$, Figure 1B). Similarly, relative peak torque knee extension (PTE) as well as relative peak torque knee flexion (PTF) differed significantly between groups at both tested velocities (Fig. 1C-F) reflecting strength loss of M. quadriceps (-48% at 60°/s and -51% at 120°/s, $p<0.001$) and hamstrings (-53% at 60°/s as well as at 120°/s, $p<0.001$).

Inflammatory parameters

Differences in inflammatory parameters between young and old females are summarized in Table 2. The number of leukocytes in whole blood did not differ between young and old women. However, the subpopulation analysis revealed a significant higher percentage of monocytes in elderly (+24%, $p=0.007$), with no changes in lymphocytes and granulocytes. As expected, hs-CRP was significantly higher in old women in comparison to young females (+156%, $p<0.001$). Serum levels of TGF-β did not differ between young and elderly women ($p=0.290$). Similarly, TGF-β mRNA levels from PBMCs did not vary between groups ($p=0.290$). Interestingly, both TGF-βRI (-49%, $p=0.020$) and TGF-βRII mRNA levels (-46%, $p=0.020$) were lower with respect to old age.

Table 1: Subject Characteristics

Parameter	Young (n=16)	Old (n=90)	*p-value*
Age [years]	24.9 (21.7-28.4)	83.8 (65.0-92.2)	***<0.001***
Body mass [kg]	58.1 (51.0-65.2)	71.7 (46.2-112.4)	***<0.001***
Height [m]	1.65 (1.57-1.71)	1.57 (1.40-1.72)	***<0.001***
BMI [kg/m²]	21.7 (18.9-23.6)	29.6 (18.1-50.0)	***<0.001***
Body fat mass [%]	26.3 (21.2-33.2)	36.1 (14.0-50.4)	***<0.001***
Muscle mass [kg]	22.2 (20.6-23.1)	19.5 (12.9-26.4)	***0.019***
Glucose [mg/dl]	87 (67-106)	96 (79-196)	***<0.001***
Insulin [µIU/ml]	6.40 (2.50-13.50)	8.05 (1.32-41.57)	*0.068*
Cholesterol [mg/dl]	171 (136-264)	209 (144-336)	***0.002***
HDL-Cholesterol [mg/dl]	74 (54-99)	62 (33-120)	***0.006***
LDL-Cholesterol [mg/dl]	81 (47-146)	121 (43-238)	***<0.001***
Triglyceride [mg/dl]	79 (41-191)	111 (43-275)	***0.006***

Data are expressed as medians (min-max); Differences were detected using Mann-Whitney U test;
BMI (body mass index); HDL (high density lipoprotein); LDL (low density lipoprotein)

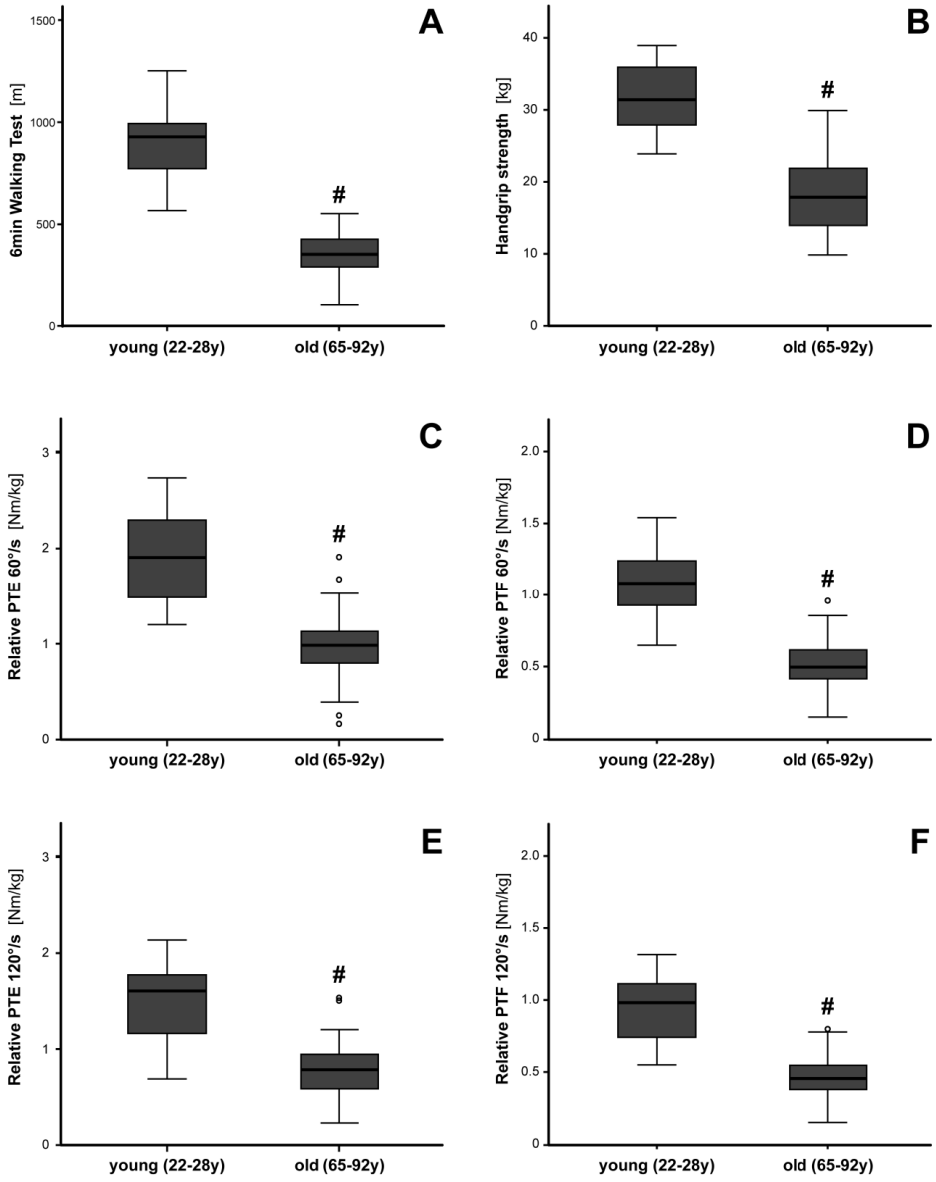

Figure 1. Age-related differences in physical fitness: (A) 6-minutes Walking Test, (B) Handgrip strength, (C) Relative Peak Torque Knee Extension (PTE) 60°/s, (D) Relative Peak Torque Knee Flexion (PTF) 60°/s, (E) Relative Peak Torque Knee Extension (PTE) 120°/s, (F) Relative Peak Torque Knee Flexion (PTF) 120°/s. # denotes a significant differences between young and old women ($p<0.001$).

To investigate whether the change in TGF-β receptor mRNA expression could be influenced by miRNA-21, its level was measured in PBMCs. While we did not detect any differences in intracellular miRNA-21 levels between young and old women ($p=0.190$), miRNA-21 in PBMCs of old women correlated negatively with TGF-βRI expression ($\rho=-0.315$; $p=0.004$) but not with TGF-βRII or TGF-β.

Correlations between fitness and inflammation in elderly women

Next we were interested whether higher fitness levels within the cohort of older women would be associated with lower pro-inflammatory states (Table 3). Indeed, hs-CRP levels and relative peak torque measurements revealed negative correlation; however, significance was reached only for relative peak torque of knee extension at 120°/s ($\rho=-0.276$; $p=0.013$). Surprisingly, hs-CRP was not associated with performance in 6MWT and even positively correlated to handgrip strength ($\rho=0.237$; $p=0.035$). Serum TGF-β, its expression level in PBMCs, and the expression of its receptors TGF-βRI and TGF-βRII were not related to any performance measure.

Body composition influences inflammatory and fitness-related parameters in elderly

As the subgroup of older women showed a substantial variation in body composition, its influence on inflammatory, fitness- and health-related variables was investigated. It has to be mentioned that age in this subgroup was even negatively associated with muscle mass ($\rho=-0.546$; $p<0.001$) but surprisingly also with BMI ($\rho=-0.219$; $p=0.039$) and body fat mass ($\rho=-0.316$; $p=0.003$).

Hs-CRP was positively correlated to BMI ($\rho=0.326$; $p=0.002$), body fat ($\rho=0.331$; $p=0.002$) and muscle mass ($\rho=0.291$; $p=0.007$), but not age. Furthermore, total leukocyte counts were positively associated with BMI ($\rho=0.342$; $p=0.001$) and body fat ($\rho=0.212$; $p=0.050$). In contrast, TGF-

Table 2: Age-related changes in immune parameters

Parameter	Young (n=16)	Old (n=90)	*p-value*
Circulating			
Leukocytes [10^9/l]	6.0 (4.6-8.0)	6.5 (3.4-13-3)	*0.208*
Lymphocytes [%]	33.8 (23.5-46.6)	31.6 (15.3-48.1)	*0.375*
Monocytes [%]	6.7 (3.7-11.5)	8.3 (1.0-14.1)	***0.007***
Granulocytes [%]	59.0 (43.8-67.2)	56.3 (38.4-75.0)	*0.584*
hs-CRP [mg/l]	0.9 (0.1-4.3)	2.3 (0.3-56.7)	***0.001***
TGF-β [pg/ml]	33,321 (23,793-42,543)	34,851 (16,667-73,681)	*0.290*
PBMCs (intracellular)			
TGF-β / GAPDH [-]	0.65 (0.30-1.59)	0.53 (0.06-3.36)	*0.387*
TGF-βRI / GAPDH [-]	4.07 (1.38-14.60)	2.08 (0.14-28.81)	***0.022***
TGF-βRII / GAPDH [-]	3.16 (1.14-10.25)	1.71 (0.51-14.86)	***0.022***
hsa-miRNA-21[copies /pg RNA]	1,821 (380-3,824)	2,452 (57-5,481)	*0.190*

Data are expressed as medians (min-max); Differences were detected using Mann-Whitney U test; hs-CRP (high sensitive C-reactive protein), PBMC (peripheral blood mononuclear cells), TGF-β (transforming growth factor-β), TGF-βRI (transforming growth factor-β receptor type I), TGF-βRII (transforming growth factor-β receptor type II), GAPDH (glyceraldehyde-3-phosphate dehydrogenase); hsa-miRNA-21 (human microRNA-21)

Table 3: Correlation between fitness and immune parameters in old women

	6MWT	Handgrip	PTE_{rel}60°/s	PTF_{rel}60°/s	PTE_{rel}120°/s	PTF_{rel}120°/s
Leukocytes	-0.100	-0.102	-0.017	0.062	-0.020	-0.039
hs-CRP	0.049	**0.237***	-0.206	-0.127	**-0.276***	-0.175
TGF-β (circulating)	-0.086	-0.082	-0.060	-0.019	-0.046	-0.120
TGF-β / GAPDH (intracellular)	-0.117	-0.043	0.033	-0.101	0.138	0.033
TGF-βRI / GAPDH (intracellular)	-0.070	-0.092	-0.124	-0.180	-0.013	-0.089
TGF-βRII / GAPDH (intracellular)	-0.175	-0.045	-0.002	-0.116	0.092	0.038
hsa-miRNA-21 (intracellular)	-0.011	**0.243***	0.101	0.034	0.075	0.015

Data indicate Spearman-Rho correlation coefficients. * $p < 0.05$, $n \geq 80$; 6MWT (6 Minutes Walking Test), PTE_{rel} (relative peak torque of knee extension), PTF_{rel} (relative peak torque of knee flexion), hs-CRP (high sensitive C-reactive protein), TGF-β (transforming growth factor-β), TGF-βRI (transforming growth factor-β receptor type I), TGF-βRII (transforming growth factor-β receptor type II), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), hsa-miRNA-21 (human microRNA-21)

βRII mRNA was negatively associated with body fat (ρ=-0.263, p=0.018) but not with BMI (ρ=-0.185; p=0.091). None of the other inflammatory variables correlated with body composition.

With respect to fitness parameters BMI and body fat correlated negatively with relative peak torque of knee extension at 120°/s (BMI: ρ=-0.282; p=0.011, body fat: ρ=-0.237; p=0.038) and partly with relative peak torque of knee flexion

at 120°/s (BMI: ρ=-0.292; p=0.009, body fat: ρ=-0.209; p=0.068). BMI and body mass were not associated with handgrip strength or 6MWT. Muscle mass correlated positively with handgrip strength (ρ=0.662; p<0.001), but not with other performance parameters.

DISCUSSION

The aim of the current study was to investigate the expression of TGF-β, its receptors and its potential modulator miRNA-21 in PBMCs in the context of age and fitness status. Young and old females differed substantially in fitness as measured by 6MWT, handgrip strength, isokinetic peak torque of knee extensors and flexors as well as serum hs-CRP levels. While serum levels of TGF-β as well as TGF-β and miRNA-21 expression levels in PBMCs did not differ between young and old females, TGF-βRI and TGFβ-RII mRNA were significantly lower in the elderly. However, within the cohort of elderly women neither TGF-β nor its receptors were associated with performance characteristics.

Initially, TGF-β was purified from human platelets (3). In mammals three different isoforms (TGF-β1, -β2, -β3) have been described, whereby TGF-β1 is the predominant form in immune cells. TGF-β is synthesized and secreted by most cell types as an inactive precursor complex, termed latent TGF-β, where TGF-β is bound non-covalently to the latency associated peptide (LAP). To be activated TGF-β has to be cleaved from the LAP using one of physiological mechanisms such as proteolytic cleavage by plasmin, cathepsin, and other enzymes, oxidation by free radicals or the interaction with thrombospondin (41). Circulating TGF-β has been detected in a variety of studies with plasma values ranging from below 0.1 ng/ml up to more than 25 ng/ml. Of course the study population differed between these studies ranging from healthy individuals of mixed gender and age-groups to different patient groups. However, methodological issues concerning plasma processing and assay system seem to be the most critical factors in determining TGF-β levels (20). In this respect it has been shown that platelet degranulation, haemolysis of erythrocytes and contamination with leukocytes can lead to an over-estimation of TGF-β protein levels in plasma, however different activation protocols to dissociate TGF-β from its complexes are in use. For this purpose, we and many other groups used acidification of serum samples prior to assessing TGF-β by ELISA (20). Furthermore, both serum from young and old females were treated in the same way, therefore minimizing the risk of bias within the study.

Regarding lifestyle-related diseases TGF-β seems to play conflicting roles as shown for cancer, where it can either act as tumour suppressor by inhibiting cell proliferation and inflammation in the early stage of cancer development or as tumour promoter by inducing metastasis or angiogenesis in later stages of the disease (52). Similarly, higher levels of TGF-β are measured in hypertensive humans (57), but increased serum levels of TGF-β may also protect patients with coronary artery disease against cardiovascular events and coronary interventions (59). With respect to age decreased levels have been reported for adults (21-67 years) in comparison to children (1-14 years) (43). Another study has revealed that TGF-β levels are higher in males than in females, but decrease with age and increase with obesity in both genders (32). However, in centenarians, serum TGF-β concentration seems to be higher than in younger adults, suggesting that high concentrations of TGF-β might be beneficial during extreme old age (6). These data are in conflict with our results as we did not detect any differences in TGF-β between young and old women. However, the broad range of age as well as other lifestyle related factors such as obesity or diabetes which were characteristic for our study could have influenced the results.

Conflicting data have also been reported with regard to TGF-β and acute exercise, whereby intensity and type of exercise seem to play an important role for data interpretation. While a graded cycling exercise to exhaustion of about 18 min duration (10) and 1 h of treadmill running at about 70-80% of VO_{2max} (22) are able to increase the concentration of circulating TGF-β, 1 h of cycling exercise at ~70% of VO_{2max} does not alter circulating TGF-β (17). However, salivary TGF-β is increased as late as 24 h after a moderate exercise bout (49). Long-term training for 6 weeks in healthy students resulted in a biphasic response of TGF-β with increased levels after 2 weeks of training and lower levels at the end of the training period (23). This is in contrast to another study in diabetic patients showing that 8 weeks of strength and aerobic training results in increased TGF-β levels which are accompanied by lower hs-CRP levels (61). Furthermore, 6 months of exercise (2.5 h per week) were able to increase TGF-β production of unstimulated as well as phytohaemaglutinin-stimulated peripheral blood mononuclear cells of persons at risk of developing ischemic heart disease (54). Taken together, it seems that chronic exercise lowers TGF-β in young and healthy persons but it might up-regulate TGF-β in patients suffering from lifestyle-related diseases.

In addition to circulating levels of TGF-β we were especially interested in expression of TGF-β and its receptors in PBMCs of young and old women. While intracellular TGF-β mRNA was not different between these two groups, lowered TGF-βRI and TGF-βRII have been detected in elderly. These results are partly in accordance with a previous study in young (20-30 years), old (75-85 years) and very old (>98 years) subjects, where leukocyte TGF-βRII mRNA were lowest in the 75-85 year old group in comparison to both, the young and the centenarians. In contrast to our study the decrease in TGF-βRII expression is accompanied by an increase in miRNA-21 levels. However, neither intracellular nor circulating TGF-β nor physical performance has been assessed in this study (46). The importance of signalling via TGF-βRII has been shown in an animal model where the induction of a TGF-βRII gene disruption results in a lethal inflammatory disease (29). On the one hand TGF-βRII is important in T-cell mediated immunity (30) but on the other hand macrophages lacking TGF-βRII have defects in expression of a set of genes that form the hallmark of the M2 polarizing program in macrophages which is important to induce the anti-inflammatory effects of M2 macrophages such as phagocytosis of apoptotic cells, resolution of inflammation and tissue repair (19, 34, 40).

Data support the hypothesis that exercise can reduce low grade inflammation in elderly (61) and provide long-term benefits with regard to cardiovascular, cognitive, psychosocial and other aspects in elderly (11). Hs-CRP has been shown to be consistently higher in elderly (26, 60), a fact that was confirmed in the current study. Moreover, hs-CRP correlated positively with BMI and body fat but negatively with relative peak torque measurements. This partly confirms several studies which revealed associations between a higher inflammatory state and lower physical performance (47, 56). Besides originating in the liver, the acute phase protein hs-CRP is produced and released from adipose tissue thereby linking obesity to a chronic inflammatory state (13, 65).

Although we detected a negative correlation between hs-CRP and isokinetic knee extension strength which is in line with many studies linking chronic inflammation to low physical performance in elderly (7, 9, 12, 58), this picture was not consistent for other performance parameters such as aerobic fitness or strength. The reason for this finding could be a complex interaction between several factors which has been suggested by Morrisette-Thomas et al. who applied principal component analysis in order to understand why inflamm-ageing does not simply reflect increases in pro-inflammatory markers (39). One especially interesting aspect in our study was that general fitness status of elderly women was not related to TGF-β, TGF-βRI or TGF-βRII expression in PBMCs. Furthermore, higher hs-CRP and miRNA-21 levels in older women were even associated with a higher handgrip strength. According to our hypothesis we would have expected higher levels of the inflammatory miRNA-21 in subjects with low physical performance as suggested by Bye et al. who showed that miRNA-21 was increased in male participants with low aerobic capacity as assessed by VO2max (5). However, these studies are sparely comparable as miRNA-21 was detected in serum, participants were male and younger and there might be a difference between performance indicators for strength or endurance. Furthermore, it would have been interesting to measure general physical activity levels by an objective method such as accelerometry as both current physical activity practice and performance are associated with inflammatory biomarkers (12).

In summary, it has been shown that TGF-β is involved in a variety of physiological as well as pathological process exerting positive but in some cases also negative effects on health. We have demonstrated that in older women TGF-β signalling in PBMCs might be impaired as reflected by a lower gene expression of its receptors TGF-βRI and TGF-βRII independent of physical fitness. However, further studies are needed to test whether a reduced expression of the TGF-β receptors indeed would reduce TGF-β signalling in PBMCs in order to get mechanistic insight as well as to reveal its functional consequences, more precisely.

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Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage

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ABSTRACT

The role of cell free DNA (cfDNA) has been intensively discussed under various pathological conditions and after acute bouts of exercise. To date, there is still no conclusive evidence concerning the cellular origin of cfDNA and the entire mechanism leading to elevated cfDNA concentrations in human plasma and serum. Here, we investigated the cellular origin of cfDNA in sex-mismatched haematopoietic stem cell transplantation (HSCT) and liver transplantation (LT) patients by determining the relative proportion of Y-chromosomal to total nuclear cfDNA. Total nuclear cfDNA and Y-chromosomal cfDNA concentrations were determined in blood plasma before and after an incremental exercise test via quantitative real-time PCR (qPCR). Female HSCT patients showed high proportions of Y-chromosomal cfDNA. Both total nuclear and Y-chromosomal cfDNA increased significantly and in a highly correlated fashion due to exercise. In male HSCT patients with female donors less than 10% of the cfDNA was of Y-chromosomal origin at any point in time and even though the total amount of cfDNA increased during exercise, no increases in Y-chromosomal DNA could be detected. The percentage of Y-chromosomal cfDNA in female LT patients with male donors was very low and levels remained unchanged during exercise. This indicates that cells not derived from the bone marrow, in this case transplanted liver cells, represented only a minor fraction of cfDNA in blood plasma and were not released during acute physical exercise. Even though many physiological conditions may be altered in transplant patients versus healthy people, our results strongly suggest that cells from the haematopoietic lineage are the main source of cfDNA released during acute bouts of exercise.

Keywords: cell free DNA, sex-mismatched transplantation, Y-chromosomal PCR, exercise, graft rejection.

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INTRODUCTION

The potential of cell free DNA (cfDNA) as a biological marker has attracted much interest in various biomedical disciplines. Under physiological conditions, the concentration of cfDNA is low whereas levels increase under chronic and acute pathological conditions like cancer, autoimmune diseases, sepsis and stroke (20, 37, 41, 49). Elevated concentrations of cfDNA have also been reported after acute bouts of exercise (4, 6). For all of these conditions, there is so far no conclusive evidence concerning the cellular origin and the precise mechanisms involved in cfDNA release into serum or plasma. Continuously occurring apoptosis in normal tissues could lead to the presence of cfDNA in healthy subjects (35) whereas elevated levels of cfDNA in cancer patients could result from both apoptosis and necrosis of tumour tissue and surrounding normal cells, autophagy or mitotic catastrophe (34, 40). Apart from cellular damage, strenuous physical exercise can also induce cfDNA levels (2). Since the appearance of DNA fragments in the circulation in response to cell death-stimuli would require several hours (18, 23, 25, 30), these mechanisms seem to be unlikely to account for the immediate increases of circulating cfDNA levels in settings applying short bouts of exercise (10). Several studies have suggested that the spontaneous DNA release from living cells could contribute to the cfDNA pool in blood plasma (39, 42). In a recent study, the rapidly increasing cfDNA concentrations observed during physical exercise were at least partially attributed to the release of neutrophil extracellular DNA traps (7).

In this pilot study we investigated the cellular origin of cfDNA induced by acute bouts of physical exercise. We used a sex-mismatched transplantation model to distinguish donor- from host-derived DNA by quantitative real-time PCR (qPCR), therefore being able to determine whether cfDNA release can be attributed to the transplanted organ. Since it was already shown that baseline cfDNA originates predominantly from the haematopoietic cell lineage (27) we decided to primarily focus on measuring cfDNA release during exercise in sex-mismatched haematopoietic stem cell transplantation (HSCT) patients. Considering the role of the liver in the clearance of DNA from the circulation (16, 44), we also analysed cfDNA release in sex-mismatched liver transplanta-

tion patients (LT). To track the donor-derived DNA in the patients we selectively amplified male DNA by using PCR primers targeting Y-chromosomal sequences. We compared the proportion of male DNA to the amount of total nuclear cfDNA in blood plasma of the patients, quantified by qPCR targeting long terminal repeat (LTR) loci. Whole blood was collected before and after exercise. We show that the majority of cfDNA released during physical exercise is derived from the haematopoietic system. Furthermore, liver cells only contribute to a low extent to the cfDNA pool and it seemed that this cell type does not liberate DNA in response to acute exercise.

MATERIAL AND METHODS

Subjects

We recruited seven HSCT patients at the Department of Internal Medicine III, University Medical Center, Mainz. Five were females with male donors and two male patients received HSCT from a female donor. In addition, five LT patients, who received an orthotropic liver transplant from a deceased male donor, were recruited at the Department of Internal Medicine I, University Medical Center, Mainz. Sex and diagnosis of the patients are shown in Table 1.

We also recruited three healthy male and three healthy female volunteers, serving as controls for the Y-chromosomal qPCR assay, from the Department of Sports Medicine in Mainz.

All experimental procedures were approved by the Human Ethics Committee of Rhineland-Palatine and were in line with the standards of the *Declaration of Helsinki of the World Medical Association.* All subjects were informed orally and in writing about the procedures and the aim of the study and gave written consent to participate.

Exercise protocol

All subjects performed an incremental treadmill test. The exercise protocol started for the patients at a velocity of 2 or 4 km/h and speed was increased 1 km/h every 3 min until volitional exhaustion. To allow for comparability of the protocol duration, the healthy controls started at a speed of 6 km/h with a step-wise increase of 2 km/h every 3 min. Capillary blood samples for lactate measurement were taken from the earlobe before the exercise test, after each incremental step, immediately after and 3, 5 and 10 min post-exercise. Respiratory gas exchange data and heart rate were continuously recorded during the test by spiroergometry and electrocardiogram monitoring, respectively. Borg scale values to monitor the self-reported level of exertion were assessed at the end of each speed step. The subjects were requested to avoid any exercise training for the 24 h before the test.

Table 1. Sex and diagnosis of the transplantation patients.

Patient	Sex of donor	Sex of recipient	Age (years)	BMI	Diagnosis	Months after transplantation	Infection	hsCRP (mg/l)	GVHD
HSCT1	M	F	23	25.8	sAA	13	No	53.8	No
HSCT2	M	F	41	29.1	AML	5	Sinusitis *	1.7	No
HSCT3	M	F	48	20.8	c-B-ALL	3	No	45.0	No
HSCT4	M	F	53	26.9	MM	13	parvovirus B19	2.4	Yes
HSCT5	M	F	58	24.3	Pre-B-ALL	5	No	0.4	Yes
HSCT6	F	M	24	21.5	Pro-T-ALL	11	No	8.9	No
HSCT7	F	M	59	22.6	MDS	28	Chronic respiratory infection	9.4	Yes
LT1	M	F	56	22.8	Hepatitis B/D liver cirrhosis	79	No	1.1	No
LT2	M	F	43	26.4	Acute drug induced liver failure (non-paracetamol)	96	No	1.9	No
LT3	M	F	57	22.1	Hepatitis C Liver cirrhosis	98	No	0.2	No
LT4	M	F	63	22.1	Hepatocellular carcinoma Alcoholic liver cirrhosis	29	No	1.1	No
LT5	M	F	56	20.9	Primary sclerosing cholangitis	19	No	0.2	No

HSCT: Haematopoietic stem cell transplantation; LT: Liver transplantation; M: Male; F: Female; BMI: Body Mass Index; ALL: Acute lymphoblastic leukaemia; pre-B-ALL: pre-B cell acute lymphoblastic leukaemia; c-B-ALL: common B cell acute lymphoblastic leukaemia; Pro-T-ALL: pro-T cell acute lymphoblastic leukaemia; AML: Acute myeloid leukaemia; MDS: Myelodysplastic syndrome; MM: Multiple myeloma; sAA: Severe aplastic anaemia; BM: Bone marrow; PB: Peripheral blood; hsCRP: high-sensitivity C-reactive protein; GVHD: graft-versus-host disease * Partial lung resection with pneumonia before transplantation.

Blood sampling and processing

10 ml of EDTA-anticoagulated blood were taken from the antecubital vein before, immediately after and 90 min after the treadmill exercise. The blood samples were centrifuged at 4°C, 1,600**g* for 10 min. In a second high-speed step the plasma was centrifuged at 4°C, 16,000**g* for 5 min to remove cellular debris. The plasma samples were stored at -20°C and cfDNA concentrations were measured within a maximum of 4 weeks. 2.5 ml of venous blood were sent to an external laboratory for the analysis of complete blood cell counts and other blood parameters. Capillary blood samples were measured with the lactate analyser Biosen 5130 (EKF Diagnostics, Magdeburg, Germany).

DNA extraction

Since column-based DNA extraction kits may not be as efficient as traditional methods for isolating low quantities of DNA from bodily fluids (48), we established a manual, non-column based phenol-chloroform method. 50 µl of plasma were diluted with 250 µl phosphate buffered saline (PBS, Life Technologies, Darmstadt, Germany) to a total volume of 300 µl. 1/100 Vol of Triton X-100 (Carl Roth, Karlsruhe, Germany) were added, samples were incubated at 98°C for 5 min and then cooled on ice for 5 min. Samples were mixed with 1 Vol Phenol:Chloroform:Isoamyl Alcohol, pH 8.0 (Sigma-Aldrich, Taufkirchen, Germany), vortexed for 30 s and centrifuged at 20°C, 16,000**g* for 10 min. The upper aqueous phase was pipetted off and DNA was precipitated with 2.5 Vol of 100% ethanol, 1/10 Vol 3 M sodium acetate, pH 5.2 and 20 µg Glycogen (Life Technologies, Darmstadt, Germany) overnight at -20°C. The next day, the precipitate mixture was centrifuged at 4°C, 16,000**g* for 30 min. DNA pellets were washed two times with 70% ethanol and a third time with 100% ethanol. After each washing step the samples were centrifuged at 4°C, 16,000**g* for 5 min. Pellets were dried for about 20 min at 55°C and eluted with 50 µl TE buffer, pH 8.0 (Life Technologies, Darmstadt, Germany). Samples were further incubated at 37°C for 30 min to completely dissolve the DNA.

Quantitative real-time PCR

Quantification of total nuclear cfDNA was based on the amplification of long terminal repeats (LTRs) of the human endogenous retrovirus K family (HERV-K LTR5 Hs). Since fragmented cfDNA is characterized by less intact target amplicons (21), we chose these multi-locus primers to enable a more precise and sensitive quantification of cfDNA. Sequence analysis revealed that LTR elements are represented in the cfDNA pool in an equal proportion as in genomic DNA (43), which minimizes a target-specific bias in quantification of cfDNA. Sequence information for primer design was obtained from the UCSC Genome Browser on the February 2009 human reference sequence (GRCh37) (33). The online software Primer3 was used to design multi-locus primers 5'-ACC GAG ACA TTC CAT TGC C -3' and 5'- GCC TCT TGC AGT TGA GAC AAG -3' targeting a 70 bp fragment of a LTR5 sequence with 195 matches in the human haploid genome. The binding frequency was assessed by aligning the primers using the *in-silico* PCR tool of the UCSC Genome Browser. For the quantification of Y-chromosomal cfDNA we used a set of primers targeting DYZ1 sequences (8): sense (5´-GTCCATTACACTACATTCCC -3´) and antisense (5´-AATGCAAGCGAAAGGAAAGG -3´) to amplify a 77 bp sequence.

The amplification of total nuclear cfDNA and Y-chromosomal cfDNA was performed on a CFX384 Touch™ Real-Time PCR detection system (Bio-Rad, München, Germany) under the following conditions for the LTR assay: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 61°C for 30 s, and extension at 80°C for 30 s and for the DYZ1 assay: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The experiments were performed in triplicates with a final volume of 5 µl per single reaction containing 1.6 µl of template DNA, 2.6 µl master mix containing 0.1 U/µl HotStar*Taq* Plus Polymerase (Qiagen, Hilden, Germany), 2 x PCR Buffer (Qiagen, Hilden, Germany), 1 µM $MgCl_2$ (Qiagen, Hilden, Germany), 0.4 mMdNTPs (Carl Roth, Karlsruhe, Germany), 0.28 x SYBR green (Sigma-Aldrich, Taufkirchen, Germany), 5 nM FITC (Sigma-Aldrich, Taufkirchen, Germany), and 0.8 µl primer-mix at a final concentration of 312 nM. Non-template controls (NTC) and positive controls for inter-plate calibration were also analysed in triplicate within each PCR run. Formation of the expected PCR product was confirmed by melting curve analysis.

The LTR assay was established and optimised with a genomic reference standard including the target sequences for the LTR5 primer set. The standard was generated from human genomic DNA (Novagen, Merck, Darmstadt, Germany) by PCR using primers 5´ TTC TCA AAG AGG GGG ATG TG 3´ and 5´ GTG GGA AGG GAA AGA CCT GA 3´ to amplify a 400 bp-fragment of a LTR-sequence. Sequence information was obtained from the UCSC Genome Browser on the February 2009 human reference sequence (GRCh37) (33). The amplification was performed on a Mini Thermal Cycler (Bio-Rad, München, Germany) with the following conditions: initial denaturation at 95°C for 5 min and 40 cycles of denaturation at 94°C for 15 s, annealing at 59°C for 30 s, and extension at 72°C for 30 s. The PCR product was TA-cloned in a pCR®2.1 vector (Invitrogen, Carlsbad, CA, USA) and the sequence was confirmed by Sanger sequencing (StarSEQ®, Mainz, Germany). For standard preparation the PCR product was cut out of the vector and the stock solution of genomic reference standard was determined fluorophotometrically (NanoDrop 3300, Thermo Fisher Scientific Inc., Waltham, MA). The DYZ1 assay was established with male genomic DNA (gDNA). Prior to amplification the high-molecular gDNA was treated for 1 h at 37°C with the restriction enzymes *DdeI* and *RsaI* (NEB, Frankfurt/M., Germany), followed by a heat-inactivation-step at 65°C for 20 min. We selected the restriction enzymes *DdeI* and *RsaI* for the fragmentation of high-molecular genomic DNA to mimic fragmented cfDNA. The coordinates of the restriction sites in the human genome were taken from an online database: http://tools.neb.com/~posfai/TheoFrag/grch38_site_counts.tools.html. The enzymes digest DNA into fragments of 200-600 bp (also confirmed by agarose gel electrophoresis) which corresponds to the typical cfDNA size profile of 180-200 bp and multiples of this (17, 22, 32). Furthermore, *DdeI* and *RsaI* were selected

since both enzymes can be combined in one reaction without activity loss.

DNA concentrations were converted into copy numbers by using the online program Finnzymes - DNA copy number calculation. Standards were prepared in defined copy numbers, ranging from 2 x 10^5 to 50 copies/µl for the LTR assay, and from 32 to 0.0125 copies/µl for the DYZ1 assay. The differences in the absolute copy numbers between both assays arise from the type of the PCR standards (specific sequence versus total genomic DNA) and, consequently, a different calculation basis for copy numbers. One copy of specific sequence is equivalent to one molecule of double-stranded DNA. One copy of genomic DNA corresponds to one human genome copy with a molecular weight of 3.3 pg. In subsequent qPCR analysis, these calibrators were used to construct standard curves by plotting the quantification cycle (Cq) value against the logarithm of calibrator copy number in each dilution. We also assessed the lower limit of quantification (LOQ), defined as the minimal concentration that could be quantified with 80% accuracy. The LOQ was determined with each reference standard measured in seven replicates. The LOQ of the LTR assay was set to 50 copies/µl, corresponding to a DNA concentration of 0.78 pg/µltemplate. The LOQ of the DYZ1 assay was set to 0.0125 copies/µl of genomic DNA which is equivalent to a DNA concentration of 0.09 pg/µl template.

Statistical analysis

The qPCR data were captured with the MyIQ5 Optical System Software, Version 2.4 (Bio-Rad, München, Germany). Microsoft® Excel 2007 was used for data analyses. We considered p values of p values less than 0.05 to be statistically significant ($p<0.001$***, $p<0.01$**, $p<0.05$*) and performed statistical analysis with JMP 11 (SAS Institute Inc., Cary, NC, USA). All data are presented as mean (±SD). Changes in cfDNA concentrations at the various points in time were compared by a nonparametric Wilcoxon-Test. Since the overall data were not normally distributed, a Spearman's rho test was calculated for nonparametric correlations.

RESULTS

Demographic data and exercise parameters

Seven HSCT patients (mean (±SD) age 44 (14) y, BMI 24.6 (2.8) kg/m^2) and five LT patients (mean (±SD) age 55 (7) y, BMI 22.9 (1.9) kg/m^2) performed an incremental treadmill test until volitional exhaustion. To evaluate the specificity of the Y-chromosomal qPCR assay, we monitored six healthy subjects (mean (±SD) age 28 (2) y, BMI (21.3 (1.6) kg/m^2, serving as positive (men) and negative (women) controls. To get an idea about the individual level of exhaustion, the values of peak oxygen consumption (VO_{2peak}), Borg scale rating of perceived exertion (RPE) and blood lactate concentration were assessed at maximal intensity (Table 2). The patients had RPE values of mean (±SD) 16.8 (1.7), VO_{2peak} of 22.4 (6.4) ml/kg/min) and lactate levels of 5.1 (2.7) mmol/l and the healthy controls had RPE values of 19.3 (0.5), VO_{2peak} of 44.4 (8.4) ml/kg/min) and lactate levels of 9.3 (1.5) mmol/l. Exercise parameters and physiological measures at the endpoint of the incremental treadmill test are shown in Table 2.

Table 2. Exercise parameters and physiological measures at the endpoint of the incremental treadmill test.

Subjects	Sex	Velocity (km/h)	Lactate (mmol/l)	VO_{2peak} (ml/kg/min)	Borg RPE value	Heart rate (beats/min)
HSCT	male	8.0 (1.6)	6.0 (2.3)	30.6 (6.4)	18.5 (1.5)	156.5 (31.5)
HSCT	female	6.3 (1.2)	6.4 (2.4)	21.3 (5.1)	16.4 (1.6)	164.6 (13.1)
LT	female	6.6 (0.6)	3.4 (1.9)	20.6 (4.1)	16.6 (1.5)	138.4 (21.7)
HC	male	16 (0.6)	10.3 (1.4)	51.3 (2.0)	19.7 (0.5)	194.7 (2.5)
HC	female	13.4 (2.5)	7.7 (0.7)	37.3 (6.6)	18.7 (0.5)	199 (6.5)

Values are given as mean (±SD); VO_{2peak}, peak oxygen consumption; RPE, rating of perceived exertion
HSCT: Haematopoietic stem cell transplantation; LT: Liver transplantation; HC: Healthy controls.

Quantification of total nuclear cfDNA and Y-chromosomal cfDNA

To evaluate qPCR performance in terms of sex specificity of the Y-chromosomal assay and for the agreement of DNA quantification by both methods we measured total nuclear (LTR sequences, amplified by the LTR 5 assay) and Y-chromosomal cfDNA (Y chromosomal DYZ sequences, amplified by the DYZ1 assay) concentrations in healthy males and females. The proportion of Y-chromosomal cfDNA amongst total nuclear cfDNA in male healthy controls was mean (±SD) 95.1 (25.2) % (Figure 1A). The amount of Y-chromosomal DNA at every point in time in female plasma was below the LOQ of the qPCR assay and therefore not quantifiable (Figure 1 B). One exception, however, was a post-exercise sample which gave weak, but positive signals when amplified with the Y-qPCR assay. In this case the relative proportion of Y-chromosomal cfDNA to total nuclear cfDNA was <0.1%. This discrepancy could be due to sequence homologies between the sex chromosomes, which can drive low level of unspecific amplification (19). Deviations from the theoretically expected proportion of 100% in healthy males could be explained by using two qPCR assays to quantify cfDNA which differ in terms of their target amplicons, PCR protocols, PCR calibrators and reaction efficiency. However, the fold-increase of cfDNA post-exercise compared to baseline levels is comparable between both assays (mean (±SD) 7.2 (0.3) for LTR 5 and 6.0 (1.56) for DYZ1, respectively) in the healthy male controls. There was a significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA in healthy male controls ($r= 0.95$, $p= 0.001$) and no correlation between both variables in the healthy female controls ($r= 0.29$, $p= 0.81$).

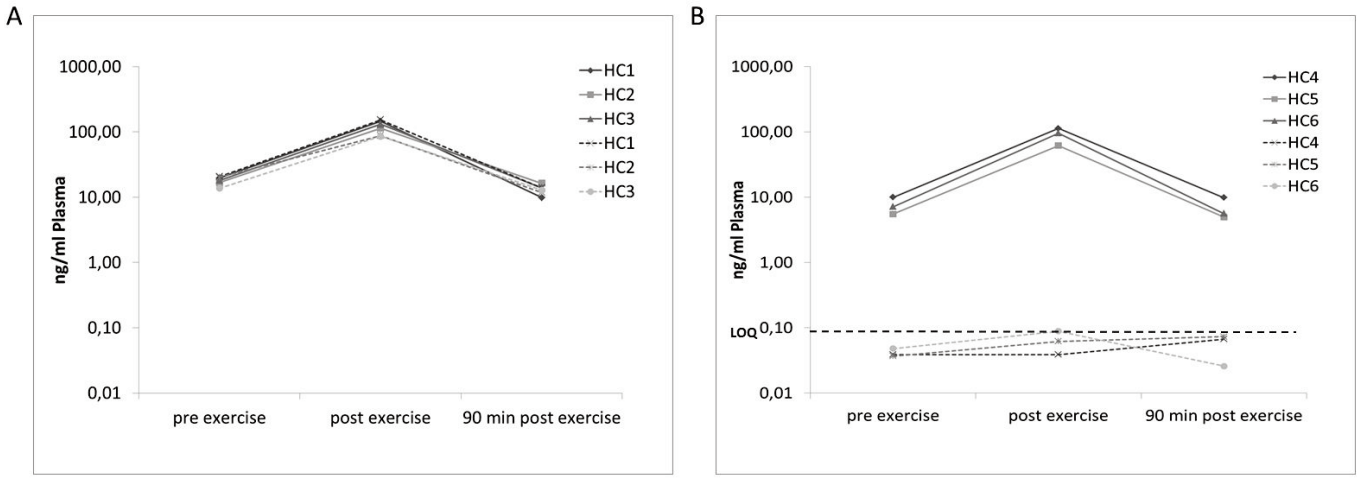

Figure 1: Mean total cfDNA and Y-chromosomal cfDNA concentrations in healthy male (A) and healthy female controls (B) (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA). The LOQ indicates the lower limit for the quantification of Y-chromosomal DNA concentrations.

To determine whether exercise-induced cfDNA levels originate from cells of the haematopoietic lineage, we measured the total nuclear cfDNA and the Y-chromosomal cfDNA concentrations in HSCT patients before and after exercise (Figures 2 and 3).

The percentage of donor DNA in female blood plasma was mean (±SD) 46.6 (12.2) % across all time points (Figure 2 A). Accordingly, the concentrations of total nuclear cfDNA (mean (±SD) before: 77.6 (97.9) ng/ml, after: 140.0 (136.4) ng/ml, 90 min after exercise: 75.7 (101.8) ng/ml) and Y-chromosomal cfDNA (before: 30.8 (39.8) ng/ml, after: 70.8 (81.5) ng/ml, 90 min after exercise: 33.5 (50.7) ng/ml) in plasma showed similar kinetics during and after the exercise test (Figure 2 B). Total nuclear cfDNA increased mean (±SD) 2.97 (1.99)-fold ($p = 0.17$) and Y-chromosomal cfDNA increased 3.23 (1.98)-fold ($p = 0.12$) compared to baseline (Figure 2 C).

We observed a significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA ($r = 0.98$, $p < 0.0001$) across all points in time. One female HSCT patient, who suffered from c-B-ALL, had a relapse (HSCT3) and showed increased total nuclear cfDNA levels at baseline with a lower proportion of Y-chromosomal cfDNA (32.2%) in comparison to the other relapse-free HSCT patients. This phenomenon could be expected since the host tumour cells are devoid of Y-chromosomal DNA.

The results obtained from the female patients were counterchecked with two male HSCT patients with a female donor. The percentage of Y-chromosomal cfDNA, representing host-derived DNA in this transplantation setting, in blood plasma across all time points was mean (±SD) 9.0 (2.8) % (Figure 3 A). Total nuclear cfDNA concentrations (mean (±SD) before: 76.0 (62.7) ng/ml, after: 179.7 (24.4) ng/ml, 90 min after exer-

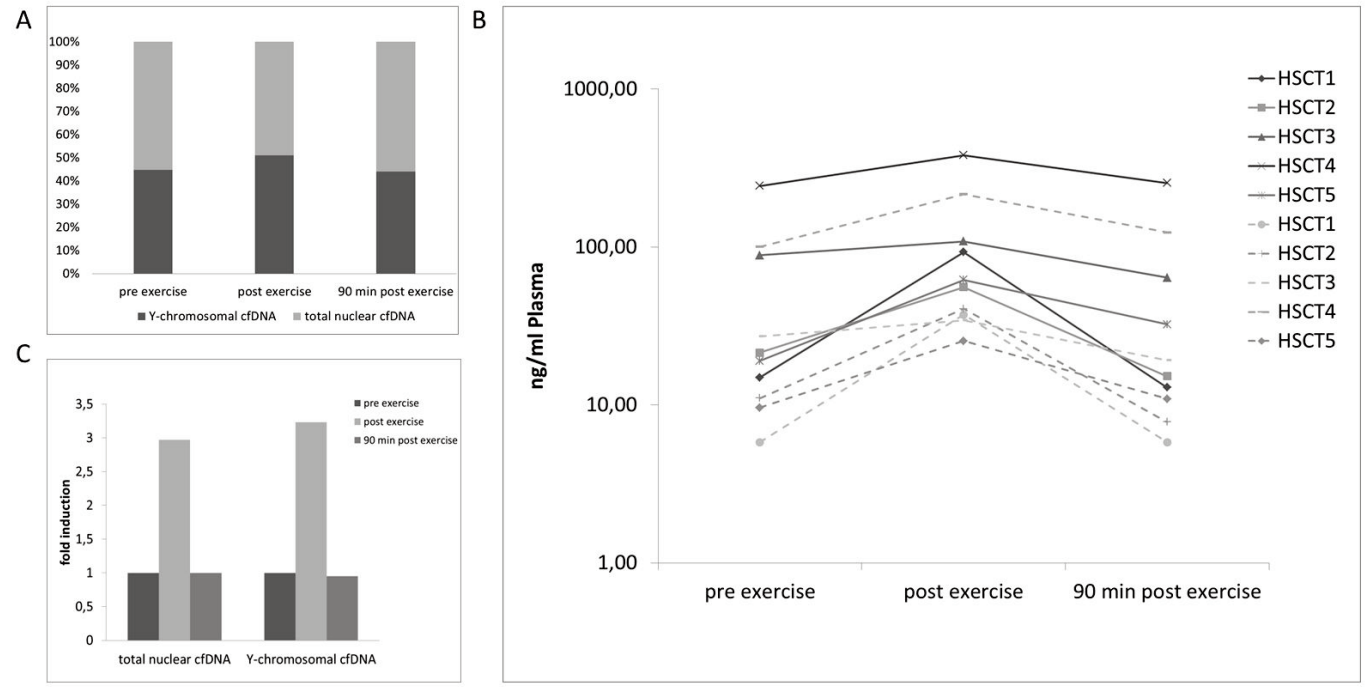

Figure 2: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of female HSCT patients with male donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).

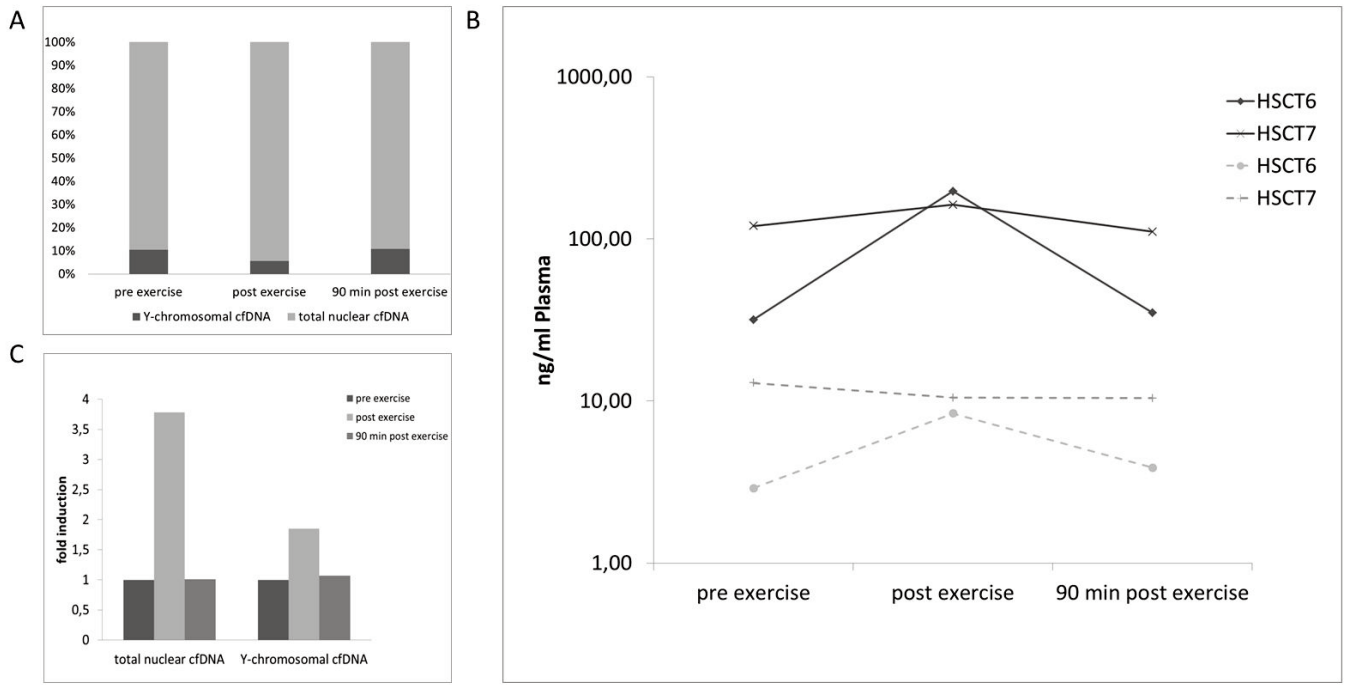

Figure 3: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of male HSCT patients with female donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).

cise: 73.0 (53.7) ng/ml) increased 3.78 (3.44)-fold (p = 0.12) in response to acute exercise (Figure 3B/C). Y-chromosomal cfDNA concentrations (mean (±SD) before: 7.9 (7.1) ng/ml, after: 9.4 (1.5) ng/ml, 90 min after exercise: 7.2 (4.6) ng/ml) was 1.85 (1.47)–fold (p =1.0) elevated compared to baseline levels (Figure 3B/C).

In order to investigate whether there is a contribution of non-bone marrow-derived cells to exercise-induced cfDNA levels, we analysed total nuclear cfDNA and the Y-chromosomal cfDNA concentrations in five female sex-mismatched LT patients. The percentage of liver-derived cfDNA in blood plasma across all time points was mean (±SD) 2.1 (1.4) % (Figure 4 A). Total nuclear cfDNA concentrations (mean (±SD) before: 59.3 (69.2) ng/ml, after: 160.6 (193.8) ng/ml, 90 min after exercise: 55.8 (87.4) ng/ml) increased 2.57 (0.42)-fold (p = 0.17) in response to acute exercise. In contrast, Y-chromosomal DNA concentrations (mean (±SD) before: 0.73 (0.50) ng/ml, after: 0.74 (0.57) ng/ml, 90 min after exercise: 0.53 (0.40) ng/ml) remained unchanged (0.99 (0.15)-fold) during and after the test (p = 0.60) (Figure 4 B/C). In one case (LT1) concentrations of Y-chromosomal cfDNA

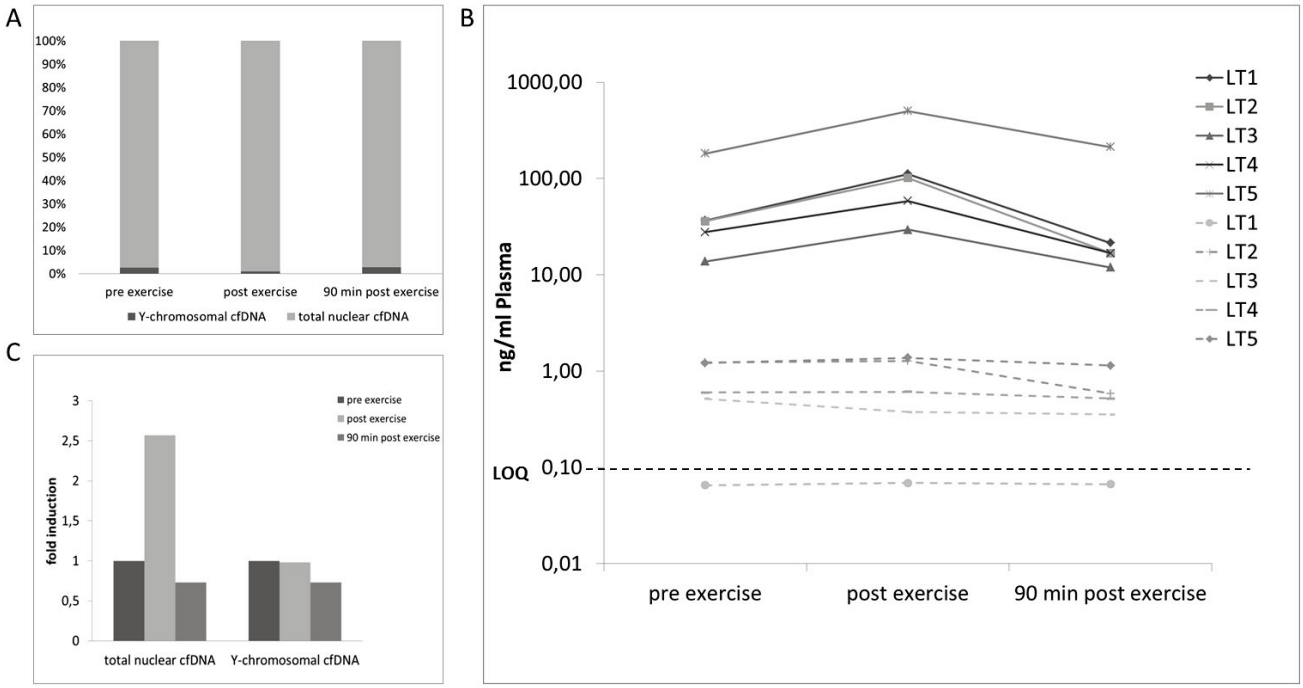

Figure 4: Percentage of Y-chromosomal cfDNA (A), absolute values (B), and fold-changes (C) of Y-chromosomal and total nuclear cfDNA in blood plasma of female HSCT patients with male donors (solid lines: total nuclear cfDNA, dashed lines: Y-chromosomal cfDNA).The LOQ indicates the lower limit for the quantification of Y-chromosomal DNA concentrations.

were below the LOQ of the qPCR assay. There was no significant correlation between total nuclear cfDNA and Y-chromosomal cfDNA (r=0.29, p = 0.31).

In summary, our results indicated that in response to exercise the majority of cfDNA is released by cells of the haematopoietic system. Liver cells only contribute to a low extent to the baseline cfDNA pool and this cell type would not release significant amounts of DNA in response to exercise. Table 3 summarises the individual results of the patients.

Table 3. Diagnosis and chimerism results of transplantation patients, increase of total and Y-chromosomal cfDNA levels after exercise and the percentage of Y-chromosomal cfDNA.

Patient	Sex of donor	Sex of recipient	Chimerism % (PB)	Chimerism % (BM)	Increase total nuclear cfDNA (pre-post) (fold change)	Increase Y-chromosomal cfDNA (pre-post) (fold-change)	% Y of total nuclear cfDNA (mean pre/post/90+)	Correlation total nuclear and Y-chromosomalcfDNA
HSCT1	M	F	87	---	6.22	6.41	43.5	0.98***
HSCT2	M	F	---	100	2.59	3.68	61.7	
HSCT3	M	F	---	73	1.22	1.26	32.4	
HSCT4	M	F	100	---	1.56	2.15	51.4	
HSCT5	M	F	---	100	3.26	2.65	44.0	
HSCT6	F	M	100	100	6.21	2.89	8.6	n.a
HSCT7	F	M	100	---	1.35	0.81	9.3	
LT1	M	F	---	---	3.02	1.05	0.19	0.29
LT2	M	F	---	---	2.81	1.03	3.6	
LT3	M	F	---	---	2.13	0.73	3.93	
LT4	M	F	---	---	2.12	1.02	2.21	
LT5	M	F	---	---	2.76	1.13	0.7	

p<0.001***, p<0.01**, p<0.05*
HSCT: Hematopoietic stem cell transplantation; LT: Liver transplantation; BM: Bone marrow; PB: peripheral blood. n.a: no analysis, no statistical analysis possible with 2 patients.

simple qPCR application, as previously described by others (15, 27, 28). The benefits offered by this qPCR-based approach compared to other methods like SNP-based sequence analysis (5, 38, 47) are time and cost efficiency. Furthermore, there is no need to analyse genomic DNA from the donor, which should also not be underestimated in terms of donor-anonymity. Finally, our approach allows a higher level of inter-individual comparability of the outcome, since we were able to measure all individuals with the same PCR assays.

DISCUSSION

In this pilot study we studied the cellular origin of cfDNA in human plasma released during exercise. In line with the findings of other studies (6, 7, 9), we showed that exhaustive short-term treadmill exercise led to increases of cfDNA concentrations. Increases of cfDNA in response to exercise have already been described for various exercise settings, e.g. endurance treadmill exercise (13), half- and ultra-marathon (2, 4), high-intensity cycling exercise (7), rowing (44) and weightlifting (3). However, the physiological or pathophysiological relevance of cfDNA increases in sports remains elusive (9), since essential questions regarding the cellular source and release mechanisms of cfDNA have not been answered yet. Here, we addressed the question of the cellular origin of cfDNA by exercising sex-mismatched HSCT and LT patients. This enabled us to study the relative contribution of bone marrow-and non-bone marrow-derived cells to the cfDNA pool in blood plasma. We distinguished donor-specific from recipient-specific DNA by targeting Y-chromosomal sequences in a

Our results indicate that cells of the haematopoietic lineage are the main source of DNA released by a short incremental exercise until volitional exhaustion. The most intriguing evidence comes from the data revealed from the two male sex-mismatched HSCT patients. Both showed considerable increases in total nuclear cfDNA of 42 ng/ml and 166 ng/ml post-exercise compared to pre-exercise.The respective increases of the Y-chromosomal cfDNA that reflects the DNA released from all other cells of the body except the HSCT cells from the female donor were 5.5 ng/ml increase and 2.6 ng/ml decrease, respectively. Accordingly, there does not seem to be any significant release of cfDNA due to exercise from other sources than the cells of the haematopoietic lineage. In line with this, sex-mismatched female HSCT patients showed a high (around 50%) and constant proportion of Y-chromosomal cfDNA relative to total nuclear cfDNA over the course of the experiment and the correlation of the respective absolute values was very high (r = 0.98; Table 3). In contrast to this, female sex-mismatched LT patients had very low proportions of Y-chromosomal cfDNA throughout all points in time with no correlation between Y-chromosomal and total nuclear cfDNA (r = 0.29;

Table 3) indicating that neither liver cells in general nor transplanted cells in particular contribute to cfDNA concentrations before exercise. Physical exercise exerts numerous effects on haematopoietic cells, as reflected by transient lymphocytosis, neutrophilia, monocytosis and activation of leukocytes and platelets (12, 45). Acute exercise and mitogen- and antigen-stimulation could activate T-cells (45). It has been reported that lymphocytes secrete DNA *in vitro* in response to mitogen-stimulation or in the presence of antigens (14). Interestingly, T-cell derived but no endothelial-cell specific DNA could be detected in the plasma of cancer patients (22). However, enucleation of erythroblasts during erythropoiesis could also contribute to the cfDNA pool in blood plasma (31). Concerning the release mechanisms, Breitbach *et al.* discussed that composite effects of different physiological stress parameters under exercise conditions could be responsible for increasing cfDNA levels (10). The authors suggest that, due to acute stress, cfDNA concentrations increase rapidly by a spontaneous unknown active or passive release mechanism (10). A further explanation could be the active release of cfDNA via neutrophil extracellular traps (NETs) (11). A current study has shown that post-exercise blood contains NET-like structures (7). They observed morphologic signs of NETosis in blood smear samples and found a striking correlation of cfDNA levels with the granule-derived enzyme myeloperoxidase in human plasma (7). Our results indicate that cells from the haematopoietic lineage respond to physical exercise by rapidly releasing DNA in the circulation, although the contribution of different cell subsets is currently unknown.

The results also permit some conclusions concerning the clinical use of cfDNA. Total baseline cfDNA concentrations are higher in the transplantation patients compared to healthy individuals. Increased levels of cfDNA concentrations in other pathological conditions were already shown in several studies (1, 20, 36, 37). Higher levels of cfDNA in the transplant could be due to infections or unspecific activation of the innate immune system, but may also occur due to acute transplant rejection or graft damage. Three of our patients (HSCT4, HSCT5 and HSCT7) suffered from graft-versus-host disease (GVHD), three from infections (HSCT2, HSCT4 and HSCT7), one from a relapse (HSCT3) and one HSCT patient (HSCT1) who had neither an infection, nor a GVHD, had very high plasma hsCRP values (> 40ng/ml). Unfortunately, given this situation it was not surprising that neither high total nuclear nor high Y-chromosomal cfDNA values could be clearly attributed to one of the three factors. HSCT3, who suffered a relapse, showed higher total cfDNA levels and lower proportions of donor-derived DNA than the relapse-free patients. This could be due to the fact that more host-derived blood cells were released from the bone marrow in the circulation with a concomitant reduction of donor-specific cells. The question whether elevated host- or donor-derived cfDNA concentrations in HSCT patients could serve as a biomarker was beyond the scope of this pilot study and should be analysed in larger patient populations. Higher levels of donor-specific DNA in three LT patients (LT2-4) could be a result of tissue damage in the transplanted organ and, possibly, of emerging graft rejection at a low level. However, none of the LT patients had clinical signs of acute or chronic rejection at the time of examination. Long-term monitoring would be necessary to elucidate if the concentration of liver-specific DNA in blood plasma rises prior to the first clinical signs of transplant rejection. However, given the high sensitivity and rapid dynamics, graft-derived cfDNA could indicate graft injury earlier compared to conventional markers (24). In a kinetic study using a renal allograft model in the rat, donor-derived DNA concentrations peaked shortly before acute rejection (29). The measurement of donor-specific cfDNA could therefore be used as a clinical marker for the detection and monitoring of rejection and the evaluation of relapse after transplantation (5, 26, 27, 38, 46).

Our study has several limitations. We only detected a mean of 46.6% of donor DNA in the female HSCT plasma instead of values close to 90% (assuming that 10% of cfDNA in plasma is derived from other tissues or organs). This could be due to technical reasons, such as the underestimation of DNA concentrations determined with the Y-qPCR assay. However, the percentage of donor-derived cfDNA could reflect the success of grafting. Therefore, the percentage of donor-derived cfDNA in blood plasma should be compared to the chimerism found in different compartments (e.g. bone marrow, full blood, different blood cell types). Unfortunately, the chimerism results presented here (see Table 3) are incomplete and retrospective. Since the percentage of donor-derived cells changes continuously, chimerism has to be assessed at the day of the exercise test, ideally at every point in time. Furthermore, the analysis of chimerism in specific subsets of blood cells, such as granulocytes, lymphocytes, monocytes or reticulocytes could decipher the cellular origin of cfDNA more precisely. A further limitation of our study is the fact that only sex-mismatched transplantation patients can be analysed with our qPCR system. When studying non sex-mismatched patients different methods, based on distinct individual sequence differences, must be implemented.

Taken together, our results suggest that cfDNA released during acute bouts of exercise mainly originated from cells of the haematopoietic linage. In future, questions like the use of host- or donor-derived cfDNA concentrations as a biomarker in transplant patients should be analysed in larger patient populations. In addition, various transplant patients could be analysed to determine a possible involvement of other organs to the cfDNA pool after physical exercise. Elucidating these mechanisms is important in terms of the physiological role and, consequently, evaluating the validity of cfDNA as a biomarker for exercise and clinical diagnostics.

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Inflammatory cytokine kinetics to single bouts of acute moderate and intense aerobic exercise in women with active and inactive systemic lupus erythematosus

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ABSTRACT

Objectives: *The aim of this study was to evaluate changes in the cytokines INF-γ, IL-10, IL-6, TNF-α and soluble TNF receptors (sTNFR1 and sTNFR2) in response to single bouts of acute moderate and intense exercise in systemic lupus erythematosus women with active (SLE_{ACTIVE}) and inactive ($SLE_{INACTIVE}$) disease.* ***Methods:*** *Twelve $SLE_{INACTIVE}$ women (age: 35.3 ± 5.7 yrs; BMI: 25.6±3.4 kg/m²), eleven SLE_{ACTIVE} women (age: 30.4 ± 4.5 yrs; BMI: 26.1±4.8 kg/m²), and 10 age- and BMI-matched healthy control women (HC) performed 30 minutes of acute moderate (~50% of VO_2peak) and intense (~70% of VO_2peak) exercise bout. Cytokines and soluble TNF receptors were assessed at baseline, immediately after, every 30 minutes up to three hours, and 24 hours after both acute exercise bouts.* ***Results:*** *In response to acute moderate exercise, cytokines and soluble TNF receptors levels remained unchanged in all groups (P>0.05), except for a reduction in IL-6 levels in the SLE_{ACTIVE} group at the 60th and 180th minutes of recovery (P<0.05), and a reduction in sTNFR1 levels in the HC group at the 90th, 120th, 150th, 180th minutes of recovery (P<0.05). The $SLE_{INACTIVE}$ group showed higher levels of TNF-α, sTNFR1, and sTNFR2 at all time points when compared with the HC group (P<0.05). Also, the SLE_{ACTIVE} group showed higher levels of IL-6 at the 60th minute of recovery (P<0.05) when compared with the HC group. After intense exercise, sTNFR1 levels were reduced at the 150th (P=0.041) and 180th (P=0.034) minutes of recovery in the $SLE_{INACTIVE}$ group, whereas the other cytokines and sTNFR2 levels remained unchanged (P>0.05). In the HC group, IL-10, TNF-α, sTNFR1, and sTNFR2 levels did not change, whilst INF-γ levels decreased (P=0.05) and IL-6 levels increased immediately after the exercise (P=0.028), returning to baseline levels 24 hours later (P > 0.05). When compared with the HC group, the $SLE_{INACTIVE}$ group showed higher levels of TNF-α and sTNFR2 in all time points, and higher levels of sTNFR1 at the end of exercise and at the 30th minute of recovery (P<0.05). The SLE_{ACTIVE} group also showed higher levels of TNF-α at all time points when compared with the HC group (P<0.05), (except after 90 min, 120 min and 24 hours of recovery) (P>0.05). Importantly, the levels of all cytokine and soluble TNF receptors returned to baseline 24 hours after the end of acute exercise, irrespective of its intensity, in all three groups (P>0.05).* ***Conclusion:*** *This study demonstrated that both the single bouts of acute moderate and intense exercise induced mild and transient changes in cytokine levels in both $SLE_{INACTIVE}$ and SLE_{ACTIVE} women, providing novel evidence that acute aerobic exercise does not trigger inflammation in patients with this disease.*

Key-words: exercise training, immune system, inflammation, rheumatic diseases, physical activity, non-active disease, active disease.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a rheumatic autoimmune disease characterized by chronic inflammation as evidenced by higher levels of interferon gamma (IFN-γ), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10), and soluble TNF receptors (sTNFR1 and sTNFR2) (1, 13, 24, 31, 52, 56). This chronic inflammation has been associated with disease-related co-morbidities, such as accelerated atherosclerosis (28), fatigue (66), and impaired cardiac autonomic control (17). As a result, SLE patients show a low aerobic capacity level (28, 33) and poor health-related quality of life (4). In this scenario, physical exercise has been considered as a promising therapeutic tool to partially offset these adverse outcomes.

There is, however, a concern that acute physical exercise in SLE patients could further increase the cytokine levels and, consequently, the inflammatory process, thereby aggravating the disease symptoms. Based on this premise, SLE patients (particularly those with disease flare-ups) have often been recommended to avoid physical activity, but the evidence to support this practice is scarce (16, 43, 60). In fact, physical low- to moderate-intensity exercise programs have been shown not

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to aggravate inflammation in rheumatoid arthritis (6, 19), or idiopathic inflammatory myopathies (30, 35). To date, however, the body of evidence on the safety of exercise in SLE is still lacking and, to our knowledge, restricted to non-active patients undergoing lower-intensity activities (11, 14, 33, 59).

The cytokine kinetics response to a single bout of acute exercise has emerged as an experimental model that provides relevant clues on the impact of exercise upon inflammatory status in patients and healthy populations (46, 57). In SLE patients, da Silva et al. (15) showed preliminary evidence that IL-6, IL-10, and TNF-α levels remained unchanged at the end of a graded exercise session. However, this study did not allow a definitive conclusion, as cytokine levels were measured only at baseline and immediately after the test, in spite of the well-known time-dependent pattern of cytokine response to an acute exercise session (46, 57). Moreover, exercise intensity, which is known to influence cytokine responses to exercise (21, 38, 53), was not explored in this study. Therefore, the time-course responses of cytokines and soluble TNF receptors (sTNFR1 and sTNFR2) to different intensities of aerobic exercise require further investigation in SLE in order to provide further evidence regarding the effects of acute exercise on cytokine kinetics in this disease.

The purpose of this study was to assess the time-course response of cytokines (*i.e.*, INF-y, IL-6, IL-10, TNF-α) and soluble TNF receptors (*i.e.*, sTNFR1 and sTNFR2) to different intensities (*i.e.*, moderate and intense) of acute aerobic exercise bouts in SLE women with active and inactive disease (SLE_{ACTIVE} and $SLE_{INACTIVE}$, respectively). Our hypothesis was that the acute exercise bouts would equally affect cytokine kinetics in the SLE women and healthy controls in an intensity-dependent manner. Furthermore, we speculated that in both SLE_{ACTIVE} and $SLE_{INACTIVE}$ women, cytokine levels would normalize after a 24-hour recovery period following both an acute moderate and intense exercise bout, suggesting no acute exacerbation of disease.

MATERIALS AND METHODS

Patients and healthy controls

From 287 SLE patients followed at our outpatient clinic (Clinical Hospital, School of Medicine, University of Sao Paulo, Brazil), twelve $SLE_{INACTIVE}$ women and eleven SLE_{ACTIVE} women were selected to participate in this study. Ten age- and body mass index (BMI)-matched women also took part in this study as a healthy control (HC) group.

The inclusion criteria for both SLE groups were the following: aged between 20 and 40 years and physically inactive for at least six months before selection. The exclusion criteria for the SLE women included: secondary rheumatic disease (*e.g.*, Sjögren syndrome, Antiphospholipid syndrome), BMI $\geq$ 30 kg/m^2, acute renal failure, cardiac and pulmonary involvement, fibromyalgia, and musculoskeletal and joint disorders which could preclude exercise testing. The particular inclusion criteria for $SLE_{INACTIVE}$ group were the following: Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score < 4 and not receiving glucocorticoid therapy for at least six months prior to the beginning of the study. The SLE_{ACTIVE} group had SLEDAI scores between 4 and 8 and received daily glucocorticoid treatment of $\leq$ 20 mg.

This study and was approved by the Local Ethical Committee and registered at clinicaltrials.gov as NCT01515163. All of the subjects signed an informed consent before entering in this trial.

Procedures

SLE diagnosis

All the women in both SLE groups fulfilled the American College of Rheumatology criteria for SLE diagnosis (25) and were regularly followed at the outpatient Lupus clinic of the Rheumatology Division of the School of Medicine at the University of Sao Paulo, Brazil. Disease activity was determined by SLEDAI scores (8). SLE manifestations were defined as follows: cutaneous disease, articular involvement, neuropsychiatry disease, renal disease, cardiopulmonary disease, and hematologic complications.

Study design

The three groups (*i.e.*, $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC) completed a maximal graded treadmill cardiopulmonary exercise test to determine the anaerobic ventilatory threshold (VAT), the respiratory compensation point (RCP), and the peak of oxygen uptake (VO_2peak). Thereafter, $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC performed two single bouts of acute aerobic exercise (*i.e.*, moderate and intense) for time-response assessments of cytokines and soluble TNF receptors.

Preliminary Testing

Cardiopulmonary exercise test

A maximal graded exercise test was performed on a treadmill (Centurion 200, Micromed, Brazil), with increments in velocity and grade at every minute until volitional exhaustion, as previously described elsewhere (49). Oxygen consumption (VO_2) and carbon dioxide output were obtained through breath-by-breath sampling and expressed as a 30-s average using an indirect calorimetric system (Cortex - model Metalyzer IIIB, Leipzig, Germany). Heart Rate (HR) was continuously recorded at rest, during exercise and at recovery, using a 12-lead electrocardiogram (Ergo PC Elite, InC. Micromed, Brazil). The cardiopulmonary exercise test was considered to be maximal when one of the following criteria was met: VO_2 plateau (*i.e.*, < 150 ml/min increase between two consecutive stages), HR no less than 10 beats below age-predicted maximal HR (58) and respiratory exchange ratio value above 1.10 (48). VO_2peak was considered as the average of the final 30 s of the test. Ventilatory threshold (VAT) was identified following previously described procedures (64). In brief, VAT was determined when ventilatory equivalent for VO_2 (VE/VO_2) increased without a concomitant increase in ventilatory equivalent for carbon dioxide (VE/VCO_2). Respiratory compensation point was determined when VE/VO_2 and VE/VCO_2 increased simultaneously.

Interventions

At least 72 hours after the cardiopulmonary exercise test, two single bouts of acute aerobic exercise were performed in a treadmill to assess the cytokines and soluble TNF receptors kinetics.

The exercise order was randomized for each group and the sessions were interspaced by at least 72 hours. The first acute exercise bout was performed after at least 72 hour of the cardiopulmonary exercise test. The room temperature was kept at 22°C during all of the experimental conditions. Each acute exercise bout (*i.e.*, moderate and intense) was comprised of 5 minutes of warm-up and 30 minutes of exercise at the predetermined exercise intensity.

Acute moderate exercise bout

The acute moderate exercise bout was performed at an intensity correspondent to 10% below the VAT ($SLE_{INACTIVE}$: 48.5 ± 7.7% of VO_2peak; SLE_{ACTIVE}: 51.5 ± 8.4% of VO_2peak HC: 47.5 ± 8.6% of VO_2peak).

Acute intense exercise bout

The acute intense exercise bout was set at an intensity correspondent to 50% of the delta difference (Δ) between the VAT and the RCP ($SLE_{INACTIVE}$: 67.2 ± 7.0% of VO_2peak; SLE_{ACTIVE}: 68.7 ± 6.4% of VO_2peak; HC: 66.8 ± 6.7% of VO_2peak).

Blood sampling

Before performing each of the acute aerobic exercise bouts, an antecubital vein was cannulated for blood sampling. Blood (5 mL) was sampled and drawn into a dry tube at baseline, at the end of exercise (End-ex), every 30 minutes during a 3-hour recovery period (Rec30, Rec60, Rec90, Rec120, Rec150 and Rec180), and 24 hours after the end of exercise (Rec24h) (36). Blood samples were centrifuged at 3000 rpm for 15 minutes at 4°C, and the serum aliquot was stored at −80°C for subsequent analyses. Baseline values were considered as the average between the baseline assessments obtained before both the acute moderate and intense exercise bouts.

Cytokines assessments

Cytokines (*i.e.*, IFN-γ, IL-10, IL-6 and TNF-α) and soluble TNF receptors (*i.e.*, sTNFR1 and sTNFR2) were measured using a multiplex human panel. The immunoassays were performed according to the manufacturer's procedures (Milliplex®). The reliability of cytokines and soluble TNF receptors measurements were tested using the baseline serum samples from the moderate and intense exercise sessions. The intraclass correlation coefficients [ICC (95% of confidence interval)] for each cytokine [IFN-γ: 0.93 (0.86-0.97); IL-10: 0.97 (0.95-0.98); IL-6: 0.98 (0.98-0.99); TNF-α: 0.84 (0.78-0.94)] and soluble TNF receptors [sTNFR1: 0.89 (0.78-0.94); sTNFR2: 0.93 (0.85-0.96)] suggest a high reliability of the assays.

Statistical analysis

Data are presented as mean ± standard error. The Gaussian distribution of the data was tested by Kolmogorov-Smirnov's test (with Lilliefor's correction). Demographic data of the three groups ($SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC) were compared using one way ANOVA followed by Bonferroni post hoc test. Drugs proportions of both SLE groups were compared with χ^2 test. Within-group serum cytokine levels were analyzed by using Friedman's ANOVA (repeated-measures) followed by Wilcoxon test, while between-group cytokine levels were compared with Kruskall-Wallis test followed by Mann-Whitney U-test. All data analysis was performed using the Statistical Package for Social Sciences (SPSS), version 17.0 for Windows. The level of significance was set at $P \leq 0.05$.

RESULTS

Patients and healthy controls

The main characteristics of the patients and healthy controls are presented in Table 1. Age, weight, height, and BMI were comparable between the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups ($P > 0.05$). The SLEDAI score was higher in the SLE_{ACTIVE} group when compared with the $SLE_{INACTIVE}$ group (5.8 ± 2.0 *vs.* 1.4 ± 1.0, $P = 0.037$), whereas the disease duration was higher in the $SLE_{INACTIVE}$ group when compared with the SLE_{ACTIVE} group (11.1 ± 6.0 *vs.* 6.1 ± 3.5 years, $P = 0.037$). Cardiopulmonary exercise test data are presented in Table 2. All the aerobic indexes were lower in the $SLE_{INATIVE}$ and SLE_{ACTIVE} groups when compared with their healthy counterparts ($P < 0.05$), while there were no significant differences between the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups ($P > 0.05$).

Baseline cytokine levels

$SLE_{INACTIVE}$ *vs.* HC

Baseline levels of TNF-α (16.4 ± 1.8 vs. 7.4 ± 1.0 pg/mL, $P < 0.001$), IL-10 (1.5 ± 0.4 vs. 0.4 ± 0.1 pg/mL, $P = 0.021$) and sTNFR2 (6864.3 ± 619.3 vs. 3311.6 ± 352.6 pg/mL, $P < 0.001$) were higher in the $SLE_{INACTIVE}$ than in the HC group, whereas IFN-γ, IL-6 and sTNFR1 baseline levels were not significantly different between these groups (IFN-γ: 17.6 ± 5.9 *vs*. 6.9 ± 1.1 pg/mL , $P = 0.307$; IL-6: 0.88 ± 0.21 *vs*. 0.5 ± 0.24 pg/mL, $P = 0.065$; sTNFR1: 1053.8 ± 72.9 *vs.* 749.5 ± 108.8 pg/mL, $P = 0.070$).

SLE_{ACTIVE} *vs.* HC

The SLE_{ACTIVE} group showed higher baseline levels of IL-6 and TNF-α than the HC group (7.4 ± 5.5 *vs.* 0.5 ± 0.2 pg/mL, $P = 0.043$ and 13.5 ± 2.0 *vs.* 7.4 ± 0.9 pg/mL; $P = 0.020$, respectively), whereas IFN-γ (18.8 ± 9.6 *vs.* 6.9 ± 1.1 pg/mL, $P = 0.944$), IL-10 (3.3 ± 2.0 *vs.* 0.4 ± 0.1 pg/mL, $P = 0.139$), sTNFR1 (864.2 ± 104.8 *vs.* 749.5 ± 108.8 pg/mL, $P = 0.379$), and sTNFR2 (4814.6 ± 770.5 *vs.* 3311.6 ± 352.6 pg/mL, $P = 0.139$) were similar between these groups.

$SLE_{INACTIVE}$ *vs.* SLE_{ACTIVE}

sTNFR2 levels were significantly higher in the $SLE_{INACTIVE}$ when compared with the SLE_{ACTIVE} group (6864.2 ± 604.9 *vs*. 4814.6 ± 703.4 pg/mL, $P = 0.016$). The remaining cytokines and sTNFR1 levels did not differ between these groups ($P > 0.05$).

Effects of acute moderate aerobic exercise on cytokines and soluble TNF receptors kinetics

Cytokines and soluble TNF receptors responses to a single bout of moderate exercise bout in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups are showed in Figure 1.

IFN-γ

Serum IFN-γ levels did not change in response to acute moderate aerobic exercise bout in any of the three groups ($P > 0.05$). Additionally, no between-group differences were noticed ($P > 0.05$).

IL-6

Serum IL-6 levels remained unchanged in response to a single bout of moderate exercise in the HC and $SLE_{INACTIVE}$ groups ($P > 0.05$), whereas it was reduced at the 60th ($P = 0.035$) and the 180th ($P = 0.022$) minutes of recovery in the SLE_{ACTIVE} group as compared to baseline levels. Between-group analyses revealed no significant differences between the $SLE_{INACTIVE}$ and HC groups. The SLE_{ACTIVE} group had higher levels of IL-6 at the 60th minute of recovery when compared with the HC group ($P = 0.036$).

TNF-α

TNF-α levels in response to the acute moderate exercise bout did not change in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups ($P > 0.05$). However, the between-group analysis showed higher levels of TNF-α in the $SLE_{INACTIVE}$ when compared with the HC and SLE_{ACTIVE} group throughout all the recovery period (except at the end of the exercise and at the 90th minute for recovery in comparison to the SLE_{ACTIVE} group). Despite the differences at baseline, the SLE_{ACTIVE} and HC group had similar TNF-α levels at the end of exercise and throughout the recovery period ($P > 0.05$).

IL-10

Serum IL-10 levels remained unchanged in response to a single bout of moderate aerobic exercise in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups ($P > 0.05$). Although the $SLE_{INACTIVE}$ group had higher levels of IL-10 than the HC group at baseline, there were no between-group differences between the $SLE_{INACTIVE}$ and HC groups in response to the acute moderate exercise bout ($P > 0.05$). Between-group analyses also revealed no significant changes between the SLE_{ACTIVE} and HC groups, nor were there any differences between the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups ($P > 0.05$).

sTNFR1

sTNFR1 levels did not change in response to the acute moderate exercise bout in the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups ($P >$

Table 1. Demographic, clinical and therapy data of SLE and health subjects.

	SLE_{ACTIVE} (n = 11)	$SLE_{INACTIVE}$ (n = 12)	HC (n = 10)	*P* SLE_{ACTIVE} *vs.* HC	*P* $SLE_{INACTIVE}$ *vs.* HC	*P* SLE_{ACTIVE} *vs.* $SLE_{INACTIVE}$
Age (years)	30.4 ± 4.5	35.3 ± 5.7	30.6 ± 5.2	1.000	0.117	0.080
Weight (kg)	66.8 ± 10.2	65.9 ± 8.8	63.9 ± 8.9	1.000	1.000	1.000
Height (cm)	160.4 ± 7.2	160.7 ± 5.2	162.6 ± 5.7	1.000	1.000	1.000
BMI (kg/m2)	26.1 ± 4.8	25.6 ± 3.4	24.1 ± 2.3	0.66	1.000	1.000
SLEDAI	5.8 ± 2.0	1.4 ± 1.0	-	-	-	0.037
Disease duration (years)	6.1 ± 3.5	11.1 ± 6.0	-	-	-	0.037
Drugs [nº(%)]						
Glucocorticoid	11 (100%)	0 (0%)	-	-	-	0.001
Antimalarial	10 (91%)	10 (83%)	-	-	-	0.596
Azathioprine	5 (45%)	1 (8%)	-	-	-	0.048
Methotrexate	2 (18%)	2 (16%)	-	-	-	1.000
Mycophenolate mofetil	4 (36%)	2 (16%)	-	-	-	0.357

Data are presented as mean ± standard deviation or n (%). BMI = body mass index; SLEDAI = systemic lupus erythematosus disease activity index; SLE: systemic lupus erythematosus; $SLE_{INACTIVE}$: women with inactive SLE; SLE_{ACTIVE}: women with active SLE.

Table 2. Cardiopulmonary data from active and inactive SLE women and HC subjects.

	SLE_{ACTIVE} (n = 11)	$SLE_{INACTIVE}$ (n = 12)	HC (n = 10)	*P* SLE_{ACTIVE} *vs.* HC	*P* $SLE_{INACTIVE}$ *vs.* HC	*P* SLE_{ACTIVE} *vs.* $SLE_{INACTIVE}$
VO_2peak (L/min)	1.70 ± 0.27	1.56 ± 0.16	1.95 ± 0.22	0.049	0.001	0.355
VO_2peak (mL/kg/min)	25.7 ± 3.7	23.9 ± 3.6	31.0 ± 5.1	0.021	0.001	0.874
HRpeak (bpm)	173 ± 23	178 ± 8	191 ± 9	0.024	0.131	1.000
RERpeak	1.08 ± 0.07	1.07 ± 0.07	1.10 ± 0.09	1.000	0.800	1.000
Time at VAT (min)	5.8 ± 0.9	4.8 ± 1.3	7.1 ± 1.1	0.043	0.001	0.111
Time at RCP (min)	9.5 ± 1.5	9.2 ± 1.8	11.3 ± 1.5	0.010	0.036	1.000
Time to exhaustion (min)	11.8 ± 1.4	11.5 ± 1.5	13.8 ± 1.6	0.013	0.003	1.000

Data are presented as mean ± standard deviation. VAT = ventilatory anaerobic threshold; RCP = respiratory compensation point; VO_2 = oxygen uptake, HR = heart rate; RER = respiratory exchange ratio; SLE: systemic lupus erythematosus; $SLE_{INACTIVE}$: women with inactive SLE; SLE_{ACTIVE}: women with active SLE.

0.05), whereas sTNFR1 decreased in the HC group from the 90th to the 180th minute of recovery when compared with baseline ($P = 0.038$, $P = 0.028$, $P = 0.005$, $P = 0.037$, respectively). The between-group analyses revealed that sTNFR1 levels were not different between the $SLE_{INACTIVE}$ and HC group at baseline, at the end of exercise, and at the 60th and 150th minutes of recovery ($P > 0.05$). In contrast, the $SLE_{INACTIVE}$ group had higher levels of sTNFR1 than the HC group at the 30th, 90th, 120th, 180th minute of recovery, and 24 hours after the end of exercise ($P < 0.05$). The sTNFR1 levels were comparable between the SLE_{ACTIVE} and HC groups at all time points ($P > 0.05$). However, the sTNFR1 levels were higher in the $SLE_{INACTIVE}$ group when compared with the SLE_{ACTIVE} group only at the 30th and 60th minutes of recovery ($P = 0.027$, $P = 0.036$, respectively).

sTNFR2

Serum sTNFR2 levels did not change in response to acute moderate aerobic exercise bout in any of the three groups ($P > 0.05$). The $SLE_{INACTIVE}$ group showed higher levels of sTNFR2 when compared with both the SLE_{ACTIVE} and HC groups at all of the time points ($P < 0.05$), whereas sTNFR2 levels remained comparable between the SLE_{ACTIVE} and HC groups ($P > 0.05$) in response to acute exercise throughout the analysis period.

Effects of acute intense aerobic exercise on cytokine and soluble TNF receptor kinetics

Cytokine and soluble TNF receptor responses to a single bout of intense aerobic exercise in $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups are presented in Figure 2.

IFN-γ

Serum IFN-γ in the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups did not change in response to the acute intense aerobic exercise bout ($P > 0.05$), whilst the HC group showed decreased IFN-γ levels at the end of exercise ($P = 0.05$) returning to baseline levels at the 30th minute of recovery and remaining at comparable levels to those observed at baseline throughout the recovery period ($P > 0.05$). The between-group analyses did not show any significant differences in any of the comparisons ($P > 0.05$).

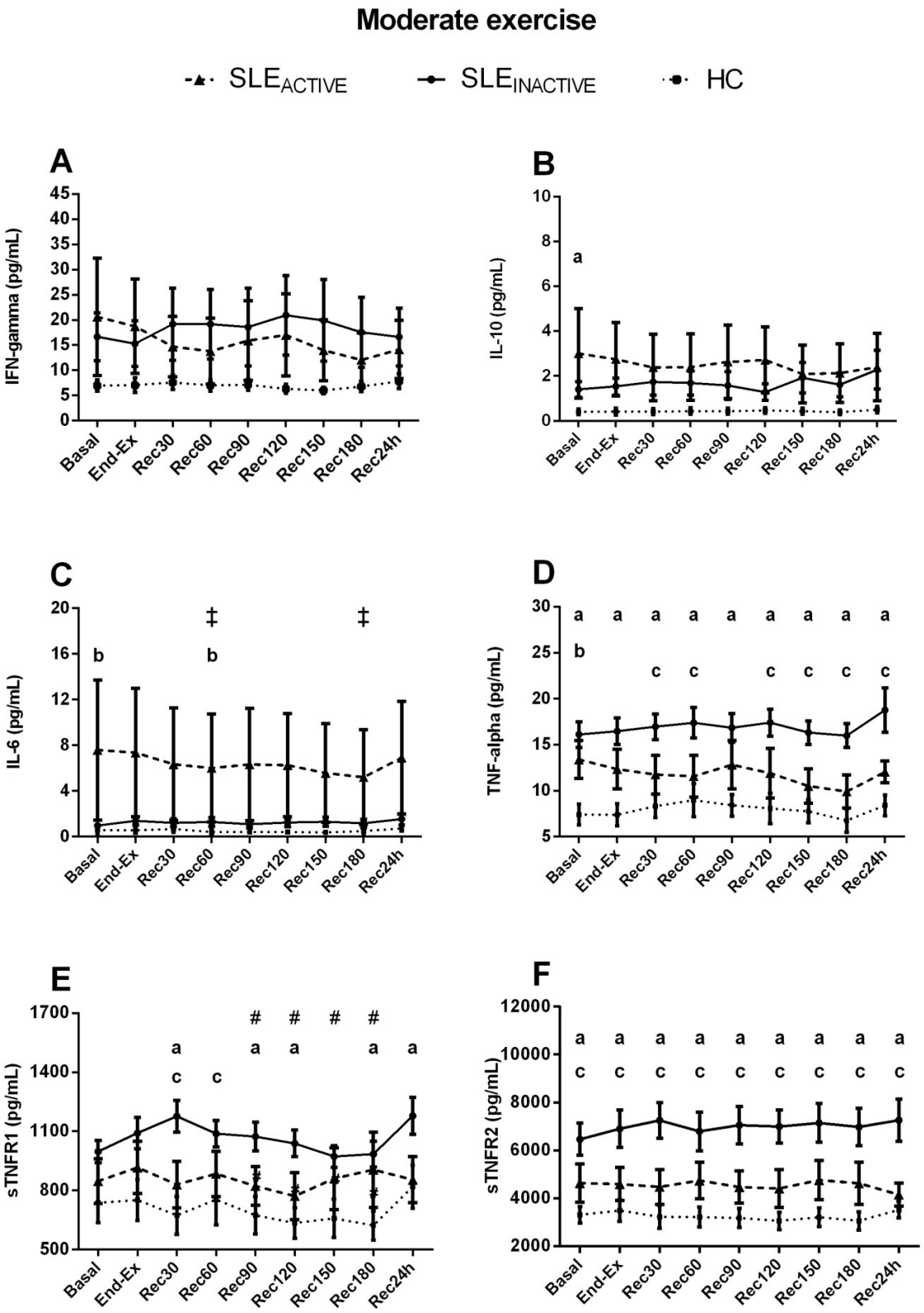

Figure 1. Cytokines and soluble TNF receptors responses to acute moderate aerobic exercise (30 minutes) in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups. * within-group differences in $SLE_{INACTIVE}$ when compared with baseline. ‡ within-group differences in SLE_{ACTIVE} when compared with baseline. ‡ within-group differences in HC when compared with baseline. a - between-group differences when comparing $SLE_{INACTIVE}$ vs. HC at the same time-point. b - between-group differences when comparing SLE_{ACTIVE} vs. HC at the same time-point. c - between-group differences when comparing $SLE_{INACTIVE}$ vs. SLE_{ACTIVE} at the same time-point. Panel A – Interferon-gamma; Panel B – Interleukin-10; Panel C – Interleukin-6; Panel D – Tumor necrosis factor-alpha; Panel E – soluble TNF receptor 1; Panel F – soluble TNF receptor 2.

IL-6

Serum IL-6 remained unchanged in response to the acute intense exercise bout ($P > 0.05$) in the $SLE_{INACTIVE}$ group. When compared with baseline, the SLE_{ACTIVE} group showed increased IL-6 levels at the end of exercise ($P = 0.028$), and decreased levels at the 60th, 120th, 180th minutes of recovery ($P = 0.047$, $P = 0.022$, $P = 0.028$, respectively). In the HC group, IL-6 levels increased at the end of exercise ($P = 0.008$)

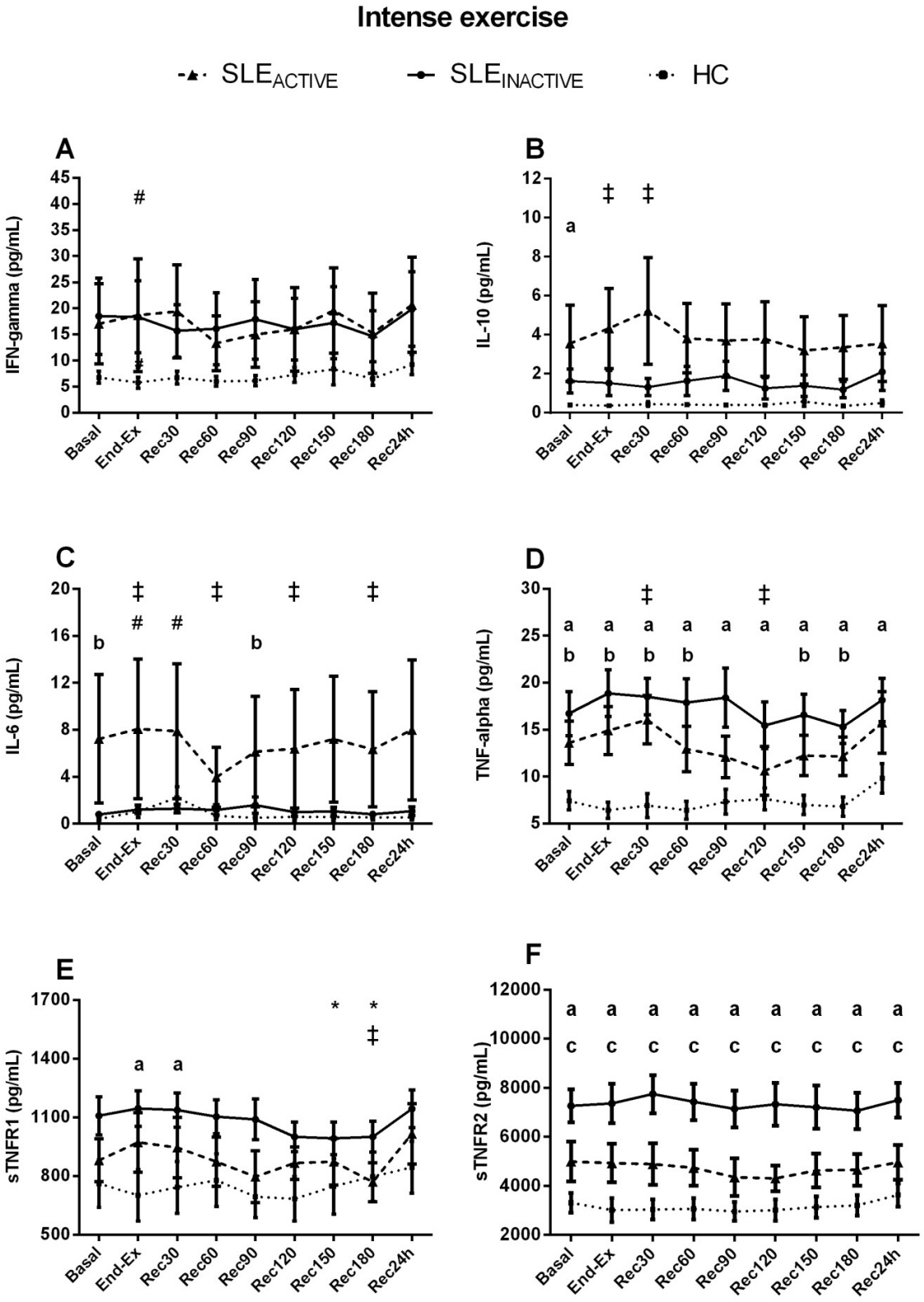

Figure 2. Cytokines and soluble TNF receptors responses to acute intense aerobic exercise (30 minutes) in the $SLE_{INACTIVE}$, SLE_{ACTIVE}, and HC groups. * within-group differences in $SLE_{INACTIVE}$ when compared with baseline. ‡ within-group differences in SLE_{ACTIVE} when compared with baseline. ‡ within-group differences in HC when compared with baseline. a – between-group differences when comparing $SLE_{INACTIVE}$ vs. HC at the same time-point. b - between-group differences when comparing SLE_{ACTIVE} vs. HC at the same time-point. c - between-group differences when comparing $SLE_{INACTIVE}$ vs. SLE_{ACTIVE} at the same time-point. Panel A – Interferon-gamma; Panel B – Interleukin-10; Panel C – Interleukin-6; Panel D – Tumor necrosis factor-alpha; Panel E – soluble TNF receptor 1; Panel F – soluble TNF receptor 2.

and at the 30th minute of recovery ($P = 0.005$), returning to baseline levels from the 60th minute to the 24th hour of recovery ($P > 0.05$). Between-group comparisons revealed no significant differences in IL-6 levels either between the $SLE_{INACTIVE}$ and HC groups, or between the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups at any of the time points ($P > 0.05$). IL-6 levels in the SLE_{ACTIVE} group were higher at baseline ($P = 0.043$), but similar during recovery when compared with the HC group ($P >$

0.05), except for higher IL-6 levels seen in the SLE_{ACTIVE} group at the 90th minute of recovery ($P = 0.024$).

TNF-α

Serum TNF-α levels did not change in response to the acute intense aerobic exercise bout in the $SLE_{INACTIVE}$ and HC groups ($P > 0.05$), whereas in the SLE_{ACTIVE} group, TNF-α levels increased at the 30th minute of recovery ($P = 0.038$), decreased at 120th minute of recovery ($P = 0.037$), and returned to baseline levels from the 150th minute to the 24th hour of recovery ($P > 0.05$). Between-group analyses showed that TNF-α levels were higher in the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups when compared with the HC group at all time points ($P < 0.05$), except for comparable TNF-α levels seen between the SLE_{ACTIVE} and HC groups at the 90th, 120th minute and 24th hour of recovery ($P > 0.05$). There were no significant differences between the $SLE_{INACTIVE}$ and SLE_{ACTIVE} groups at any time point ($P > 0.05$).

IL-10

IL-10 levels did not change in the $SLE_{INACTIVE}$ and HC groups in response to the acute intense aerobic exercise bout ($P > 0.05$), whereas the SLE_{ACTIVE} group showed increased levels at the end of exercise ($P = 0.034$) and at the 30th minute of recovery ($P = 0.039$), returning to baseline levels from the 60th minute of recovery to the end of the recovery period ($P > 0.05$). The between-group analyses revealed that despite differences in the IL-10 levels at baseline, there were no significant differences between the $SLE_{INACTIVE}$ and the HC groups from the end of exercise to the 24th hour of recovery ($P > 0.05$). Also, no significant differences were observed between the SLE_{ACTIVE} and HC groups and between the SLE_{ACTIVE} and $SLE_{INACTIVE}$ groups at any of the time points ($P > 0.05$).

sTNFR1

Serum sTNFR1 levels were reduced at the 150th and 180th minutes of recovery ($P = 0.041$, $P = 0.034$, respectively) in the $SLE_{INACTIVE}$ group, and at the 180th minute of recovery ($P = 0.05$) in the SLE_{ACTIVE} group when compared with baseline levels. The HC group did not show significant changes in the sTNFR1 levels after the acute intense exercise bout ($P > 0.05$). In the between-group analyses, the sTNFR1 levels were higher in the $SLE_{INACTIVE}$ when compared with the HC group only at the end of exercise ($P = 0.009$) and at the 30th minute of recovery ($P = 0.011$), while no significant differences were observed either between the SLE_{ACTIVE} and HC groups or the SLE_{ACTIVE} and the $SLE_{INACTIVE}$ groups throughout the protocol ($P > 0.05$).

sTNFR2

Serum sTNFR2 levels did not change significantly in response to the acute intense aerobic exercise bout in any of the three groups ($P > 0.05$). Between-group analyses showed higher levels of sTNFR2 in the $SLE_{INACTIVE}$ group when compared with both the SLE_{ACTIVE} and the HC groups ($P < 0.05$) at all of the time points. No significant differences were observed between the SLE_{ACTIVE} and HC groups ($P > 0.05$) throughout the protocol.

Effect of exercise intensity on cytokines and soluble TNF receptors kinetics

There were no effects of exercise intensity (moderate vs. intense) on cytokines and soluble TNF receptors kinetics in the $SLE_{INACTIVE}$, SLE_{ACTIVE} and HC groups at any time point ($P > 0.05$).

DISCUSSION

To our knowledge, this is the first study to assess cytokine and soluble TNF receptor kinetics in response to both acute moderate and intense aerobic exercise bouts in $SLE_{INACTIVE}$ and SLE_{ACTIVE} women. Our main results indicated that 30 minutes of an acute aerobic exercise bout, irrespective of its intensity (*i.e.*, roughly 50% or 70% of VO_2peak), caused only minor disturbances in cytokines and soluble TNF receptors, which were normalized after a 24 hour of recovery, suggesting that the acute exercise modes tested in the current study did not exacerbate the disease. In addition, there was no exercise-intensity effect on the responses of cytokines and soluble TNF receptors in both groups.

The effects of a single bout of acute moderate aerobic exercise on cytokines and soluble TNF receptors have been previously assessed in other chronic diseases, with contradictory results. For example, Gomes et al. (23) showed higher levels of sTNFR1 and lower levels of sTNFR2, but did not observe any changes in IL-6 and TNF-α levels in response to a single bout of acute moderate exercise (*i.e.*, 20 minutes of walking at 2 mph) in patients with knee osteoarthritis. Conversely, Rabinovich (50) showed increased levels of TNF-α and unchanged levels of soluble TNF receptors and IL-6 levels after a single bout of acute moderate exercise (*i.e.*, 40% of peak power on a cycle ergometer) in patients with chronic obstructive pulmonary disease. These findings reveal a disease-specific response in relation to exercise-induced changes in cytokines and soluble TNF receptors levels. The current results add to the literature by showing no alteration in these inflammatory parameters in response to the single bouts of acute moderate and intense aerobic exercise in $SLE_{INACTIVE}$ and SLE_{ACTIVE} women. Considering that SLE patients with active and inactive diseases often show very discrepant features as regard to clinical symptoms and drug therapy (61), the results of this study will be discussed separately according to the disease activity.

Effects of acute moderate and intense aerobic exercise on cytokines and soluble TNF receptors kinetics in $SLE_{INACTIVE}$

A single bout of acute moderate aerobic exercise elicited similar cytokine responses in inactive SLE patients and HC subjects, except for the reduction in sTNFR1, which was only observed in the HC subjects. In contrast to the present findings, Drenth et al. (18) and Ostrowski et al. (36) found increased levels of sTNFR1 and sTNFR2 in physically active subjects after more exhaustive/prolonged exercise protocols (i.e., a 5-km time trial or a marathon). A longer exercise duration in these previous studies (18, 36) has been related to increased TNF-α levels, and consequently, increase sTNFRs levels (7). In fact, the short duration of the exercise protocol in the current study may also explain the lack of changes in IL-6 levels in HC. Supporting this hypothesis, Scott et al. (53)

found an increase in IL-6 only after longer periods of moderate-intensity exercise (*i.e.*, > 40 minutes). The INF-γ, TNF-α and IL-10 responses to a single bout of acute moderate aerobic exercise in the HC subjects observed herein were in line with previous reports (9, 21, 32, 37, 38, 53).

In response to a single bout of acute intense exercise, IL-6 increased in HC and, subsequently, returned to baseline levels at 60th minute of recovery, in agreement with other findings (20, 41, 42). Although the chronic increase of IL-6 has been classically associated with exacerbated inflammation in chronic diseases, it has been postulated that transitory rises in IL-6 levels after acute exercise bouts may, in fact, exert anti-inflammatory effects (22, 41, 42, 62, 63). Supporting this notion, *in vitro* and *in vivo* observations (1, 5, 65) suggest that the transitory IL-6 elevation is followed by an increase in anti-inflammatory cytokines, such as IL-10 and soluble TNF receptors, ultimately blocking TNF-α actions. In the $SLE_{INACTIVE}$ women, however, a single bout of acute intense exercise did not promote any significant alterations in IL-6 levels. In addition to the already discussed effect of the exercise duration, which was shorter in the current study as compared to others involving healthy subjects (21, 38, 54), the absolute intensity of the single bout of acute intense aerobic exercise in $SLE_{INACTIVE}$ women was considerably lower than that of the healthy subjects. As IL-6 has been thought to act as an energy sensor, the magnitude of its change in response to exercise is known to respond to substrate availability, particularly to muscle glycogen levels (34). Thus, it may be that the lower absolute intensity achieved by the $SLE_{INACTIVE}$ women led to a lower glycogen depletion during acute exercise when compared with HC, which may have attenuated the IL-6 response. Alternatively, one may speculate that this "blunted" response may be somehow related to the inflammatory profile in $SLE_{INACTIVE}$ women and/or its pharmacological treatment, although the clinical relevance of these findings remains to be elucidated. From a clinical standpoint, the fact that no changes were observed (except for a slight reduction in sTNFR1) in any of the inflammatory markers suggest that even more intense exercise may pose no risk to $SLE_{INACTIVE}$ women, at least acutely.

Effects of acute moderate and intense aerobic exercise on cytokines and soluble TNF receptors kinetics in SLE_{ACTIVE}

A single bout of acute moderate aerobic exercise did not lead to cytokines and soluble TNF receptors changes, except for minor reductions in IL-6. Similarly as observed in $SLE_{INACTIVE}$, all cytokines and soluble TNF receptors remained stable in response to a single bout of acute moderate exercise in SLE_{ACTIVE}. Thus, one may suggest that acute moderate exercise did not exacerbate the disease in either active or inactive SLE patients. The lack of a transitory increase in IL-6 levels usually seen after a single bout of acute aerobic exercise in healthy subjects (32, 53) may be partially attributed to the characteristics of the acute moderate exercise protocol (*i.e.*, low intensity and/or duration), which may have been insufficient to induce such an effect.

Importantly, the single bout of acute intense exercise did not induce changes in IFN-γ levels in SLE_{ACTIVE} women. This finding is of particular relevance as this cytokine seems to play an essential role in human systemic autoimmunity, particularly in SLE with active disease (47). In support to this notion, there is evidence showing that IFN-γ is uniformly required in both spontaneous and induced animal models of SLE (2). Even though the single bout of acute exercise did not decrease IFN-γ levels as previously showed in multiple sclerosis patients (12), the absence of changes in this cytokine suggests that an acute exercise bout does not exacerbate inflammation in SLE_{ACTIVE} women.

In addition, the IL-10 increase in SLE_{ACTIVE} women after the single bout of acute intense exercise is in accordance with previous observations in Parkinson's patients (10). Considering that IL-10 has an inhibitory action upon nuclear factor kappa B (NF-κB) (29), the transient increase in this cytokine observed after the single bout of acute intense aerobic exercise has been interpreted as an anti-inflammatory response to exercise (22, 39, 41, 42, 62, 63). In contrast to healthy subjects, who seem to require a more prolonged and intense exercise to elevate IL-10 production (38), a relatively shorter-duration and lower-intensity exercise protocol (*i.e.*, 30 min at 70% of VO_2max) was shown to be sufficient in eliciting an IL-10 increase in SLE_{ACTIVE} women. Whether this response translates into a chronic anti-inflammatory effect remains to be elucidated.

Another interesting result of the present study refers to the IL-6 response. SLE_{ACTIVE} women showed a transient increase - although smaller than that of the healthy individuals (23% *vs.* 368%, respectively). This followed by a substantial reduction in IL-6 levels 60 minutes after the single bout of acute intense exercise, with a progressive return to baseline. This partially "blunted" response regarding the exercise-induced increase in IL-6 is intriguing, and may be explained by some hypotheses. First, in accordance with previous reports (33, 49), SLE_{ACTIVE} women showed lower physical capacity than HC, implying that their absolute workload was lower than that of the healthy subjects. In theory, this may have led to an insufficient stimulus to stimulate IL-6 production, as previously showed in healthy subjects (32, 53). Alternatively, one may speculate that the pharmacological treatment may have inhibited this response. Corroborating this assumption, it has been demonstrated that 20 mg of prednisolone abrogated the exercise-induced IL-6 increase in healthy subjects (3). Further studies should investigate the mechanisms by which the IL-6 response to exercise is dissonant in SLE_{ACTIVE} and healthy subjects, as well as the clinical repercussions of this phenomenon.

Notably, an increase in TNF-α levels—which was not paralleled by a concomitant increase in soluble TNF receptors—was seen in SLE_{ACTIVE} women at the 30th minute of recovery. A similar increase in TNF-α after the single bout of intense exercise was also observed in patients with chronic obstructive pulmonary disease (50). In healthy subjects, an exercise-induced increase in TNF-α is not usually expected (39, 41, 42, 62, 63), unless large amounts of exercise are performed (*e.g.*, marathon running) (36). TNF-α acts as a growth factor for B cells by stimulating the production of IL-1. Moreover, TNF-α promotes increased IFN-γ production via NF-κB activation. Its role in SLE pathogenesis has been debatable. For example, increased serum levels of TNF-α have been observed in SLE patients and associated with disease activity and some clinical manifestations (51). Conversely, the deletion of a fragment of the TNF-α gene, which reduces TNF-α serum levels, led to a delayed disease onset in a murine "lupus" model (*i.e.*,

NZB/W) (27); in addition, a replacement therapy with recombinant TNF-α delayed the development of nephritis (26, 27). Notwithstanding the controversial involving the role of TNF-α in SLE, it is important to note that in the current study, TNF-α levels peaked at the 30th minute of recovery. TNF-α consistently decreased thereafter (below baseline levels), returning to baseline 24 hours after the single bout of acute intense exercise. This response suggests that a single bout of acute intense exercise does not disrupt TNF-α response permanently, reinforcing the notion that acute exercise bout does not exacerbate the disease.

Study limitations and concluding remarks

It is important to highlight that this study is not without limitations. First, our sample was relatively small and heterogeneous, particularly with respect to the disease-related morbidities and the drug therapy. Whether these are factors affected the inflammatory response to exercise must be further examined. Second, despite the fact that SLE is much more prevalent in females (*i.e*., female to male ratio ranging from 4.3 to 13.6) (45), our sample was composed only of women, which limited our ability to extrapolate our findings males. Third, our conclusions must be confined to the exercise type (i.e., aerobic exercise) and its respective intensities (*i.e.*, ≤ approximately 70% of VO_2peak) tested in the current study. The effects of other acute exercise types (*e.g.,* resistance training, high-intensity interval training, circuit training) on inflammation needs to be carefully evaluated in future studies. Finally, we have assessed neither the full spectrum of cytokines implicated in the pathogenesis of SLE, nor the impact of a long-term exercise program on the inflammatory profile in SLE patients, which should also be assessed in future studies.

Importantly, both single bouts of acute moderate and intense aerobic exercise led to comparable (minor) changes in cytokines and soluble TNF receptors in $SLE_{INACTIVE}$ and SLE_{ACTIVE} women. This observation is in line with the findings of Scott et al. (53), who found similar IL-6, TNF-α, and IL-1ra kinetics in response to 60 minutes of running either at 55 or 65% VO_2max. Nonetheless, the same authors found that running at 75% VO_2max led to greater IL-6 and IL-1ra levels following acute exercise in comparison with the lower-intensity exercise protocols. Likewise, Peake et al. (38) observed higher levels of cytokines (*i.e.*, IL-6, IL-10, IL-12, and IL-1ra) in response to a single bout of acute intense exercise (*i.e*., 60 minutes at 85% VO_2max) when compared with a lower-intensity one (*i.e*., 60 minutes at 60% VO_2max) in healthy subjects. Altogether, these results suggest that only a single bout of higher-intensity (≥ 75% VO_2max) longer-lasting (>40 minutes) acute exercise, which induces a greater amount of glycogen depletion (34, 40), may lead to further increases in IL-6 and, consequently, anti-inflammatory cytokine levels (*e.g.*, IL-10). Further chronic studies should be performed to investigate the safety and efficacy of exercise program with different intensities in SLE patients.

Noticeably, cytokine kinetics in response to a single bout of acute exercise, regardless of its intensity, were very similar in both SLE_{ACTIVE} and $SLE_{INACTIVE}$ women. Perhaps an exception was the lower level of soluble TNF receptors observed at some time points (especially in response to moderate exercise) in the SLE_{ACTIVE} women, possibly reflected by the lower levels of TNF-α in this group. The mechanisms by which acute exercise bout may induce differential responses in TNF-α and its soluble receptors in SLE patients with active and inactive disease remain elusive. However, one may speculate that glucocorticoid treatment might have attenuated TNF-α levels in response to the single bout of acute exercise in SLE_{ACTIVE} women, which is corroborated by *in vitro* experiments showing that dexamethasone can inhibit lipopolysaccharide-induced TNF-α production in a dose-dependent manner (55). Further investigations regarding the possible interaction between drugs and exercise upon inflammation are required.

As evidence against the concern that a single bout of acute exercise could exacerbate the disease, there was some evidence suggesting that exercise could, in fact, alleviate inflammation (39, 44). In this regard, it is noteworthy that the single bout of acute exercise was able to restore, at least temporarily, IL-6 and TNF-α levels in SLE_{ACTIVE} women, which reached comparable levels to those of the HC group. This observation warrants further investigation for the potential anti-inflammatory effects of chronic exercise in SLE.

In conclusion, single bouts of acute moderate and intense exercise led to minor and transient changes in the cytokines and soluble TNF receptors levels, which were fully restored after 24 hours of recovery in $SLE_{INACTIVE}$ and SLE_{ACTIVE} women and their healthy counterparts. Collectively, the current findings demonstrated that single bouts of acute moderate and intense exercise did not exacerbate the inflammatory state of both $SLE_{INACTIVE}$ and SLE_{ACTIVE} women.

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The evidence of exercise-induced bronchoconstriction in endurance runners; genetic basis and gender differences

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Exercise is one of the most common triggers of bronchospasm in persons with and without chronic asthma. Exercise-induced bronchoconstriction (EIB) is defined as transient, reversible bronchoconstriction that develops after strenuous exercise (23). It is a heterogeneous syndrome occurring in a variety of settings, ranging from the asymptomatic military recruit (whose condition is detected by diagnostic exercise challenge) to the leisure-time athlete with known asthma to the elite athlete for whom EIB may represent an overuse or injury syndrome. If exercise is the only identified trigger for bronchoconstriction, it is called EIB. However, when it is associated with known asthma, then it is defined as EIB with asthma. It is unclear if EIB in those with and without chronic asthma results from the same mechanism. One of the new approaches for evaluating of the pathogenesis of EIB or exercise-induced asthma is analysis of the cellular responses and cytokine production in the airways. When the natural mucosal warming and humidification processes are disturbed / overrun by exercise-associated hyperventilation, this results in changes in osmolarity which will then trigger the release of inflammatory mediators causing bronchospasm.

Furthermore, this cascade of events may be exacerbated by pre-existing airway inflammation and airway remodeling. Evidence suggests that histamine, leukotrienes and prostanoids are likely central mediators involved in this response. Recent studies continue to demonstrate heterogeneity in the airway inflammatory response to EIB, reporting correlations of bronchospasm with eosinophils and eosinophil cationic protein (ECP), lipoxin A4, phospholipase A2, and endothelin-1 (24).

With this letter, we like to draw the attention to some findings from our recent work which may have relevance for this question (EIB/EIA) but have not been discussed in an integrative, comprehensive fashion. One hour of high intensity aerobic exercise, corresponding to 93% VIAT (21), or a half-marathon (1) significantly induced the up-regulation of genes such as Prostaglandin D2 receptor (PTGDR), interleukin-18 receptor-1 (IL-18R1), interleukin-18 receptor accessory protein (IL-18RAP), β2-adernergic receptor (ADRB2), arachidonate 5-lipoxygenase (ALOX-5), Endothelin-1 (EDN1, in LPS-stimulated cultures), and Cysteinyl leukotriene receptor-1 in healthy athletes, with females in luteal phase having either more dramatic or more prolonged regulation than male athletes. These observations are in good agreement with studies which have shown that female mice (19, 3) and rats (5,6) are more susceptible to induction of allergy and asthma, due to female hormone-induced cytokine release. In addition, clinical studies also have shown an important role of female sexual hormones regulating airway inflammation and allergic reactions in asthmatic women (18, 27). In summary, these studies point out that female hormones can induce a switch of Th1 to Th2 response, increasing allergic airway inflammation, in addition to an increase in the production of pulmonary nitric oxide, a classical marker of airway inflammation and hyperresponsivenes in asthmatic individuals (9,17).While previous studies have clearly demonstrated the involvement of Cysteinyl Leukotrienes (CYS-LTs) and their receptors in the development of airflow obstruction and in the pathophysiology of EIB (10,11,15), the functions of PTGDR, IL-18RAP, IL-18R1, and EDN1 in exercise-induced bronchoconstriction and/or asthma have not been described elsewhere so far. Recent studies have clearly pointed to the role of these genes in the pathophysiology of asthma, especially their roles in airway inflammation and bronchial hyperresponsiveness (2,22). For example, PTGDR (D prostanoid receptor) which is a classic type of transmembrane receptor specific for PGD2 has been shown to play an important role in allergic inflammation of the airways and asthma (22). In addition, IL-18RAP and IL-18R1 genes, which are specific receptors for IL-18 have been identified as candidate genes associated with increased susceptibility to airway hyperresponsiveness, bronchopulmonary dysplasia, and asthma (4,7,25,29,31). Moreover, increased serum levels of soluble IL-18 receptor complex in patients with allergic asthma have also been shown (14). It has been suggested that the co-expression of IL-18R1 and IL-18RAP is required for the activation of NF-κB and MAPK8 (JNK) in response to IL-18. The induction of both signaling pathways results in secretion of cytokines, a number of which (IL-8, MCP-1/2/3, G-CSF, and IL-6) have been associated with bronchoconstriction and bronchopulmonary dysplasia (7). Furthermore, IL-18-driven asthmatic responses via NF-κB have been associated with increased Th2 differentiation and activation, leading to release of IL-4, IL-5 and IL-13 (16,20).

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The strong exercise induced up-regulation of EDN1 which occurred when cultures were co-stimulated with pathogen (low dose endotoxin/LPS) also deserves our attention. Endothelin-1 is a potent vasoconstrictor that is produced by vascular endothelial cells and has been shown to play an important role in the pathogenesis of atopic asthma, airway obstruction and exercise-induced bronchoconstriction (26,12). It seems that EDN1 is involved in the pathophysiology of atopic asthma through the induction of serum IgE (12). The serum concentration of IgE is a well-stablished marker for the evaluation of asthma and bronchoconstriction (28,32).

Besides these observations, genetic association studies have also revealed a positive linkage of the genetic polymorphisms in PTGDR, IL-18RAP, IL-18R1, ADRB2, END-1 and ALOX-5 with asthma phenotypes (4,7,8,13,22,25,30), suggesting that the strong up-regulation of these genes may have some roles in the pathophysiology of EIB or EIA.

These results can be considered from several perspectives. First, our synopsis shows that exercise can significantly induce mRNA expression of a row of asthma-related genes, for instance, PTGDR, IL-18R1, IL-18RAP, ADRB2, ALOX-5, EDN1 (1,21). Given the dynamic nature of gene expression and the given the fact that microarrays can only reflect single time points, more can be expected to come in the future. Second, many of those genes (e.g. PTGDR, IL-18RAP) were only changed significantly in female athletes who were in the luteal phase of their menstrual cycle. Such findings suggest that the women who exercise in their luteal phase might be more susceptible to exercise-induced bronchoconstriction (EIB). Here it should be noted that all our female athletes were on a normal menstrual cycle. Of course hormonal regulation can be disturbed by excessive exercise/hard training up to the degree of amenorrhea. Although this was not the case of our set of female athletes, we cannot exclude that exercise-induced hormonal changes below the ”amenorrhoeic threshold” were involved in the mechanisms underlying our findings. Hormonal values which were assessed before exercise were however in normal ranges corresponding the second half of menstruation.

Third, our observation concerning EDN1, underlines that exercise and effects of concomitant pathogens can cooperate in the induction of important asthma-related genes.

These results accentuate a need for careful consideration of the pulmonary functions of athletes when programming the exercise training. While the strong association of the mentioned genes with asthma and bronchoconstriction has been demonstrated very well, we have to note that, unfortunately, there was no parallel evaluation of the pulmonary functions (i.e. FEV1, VO2, maximal expiratory flow) of athletes in our studies, which should be addressed in further studies.

Future studies are needed to measure the symptoms of exercise-induced bronchoconstriction and correlate these symptoms with the changes in asthma-related genes following exercise program. Clinical measurements of exercise-induced bronchoconstriction will enable a more precise discussion of the association of prolonged, exhaustive exercise and exercise-induced bronchoconstriction. This letter may help to draw the attention of the exercise immunology community to this open question.

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