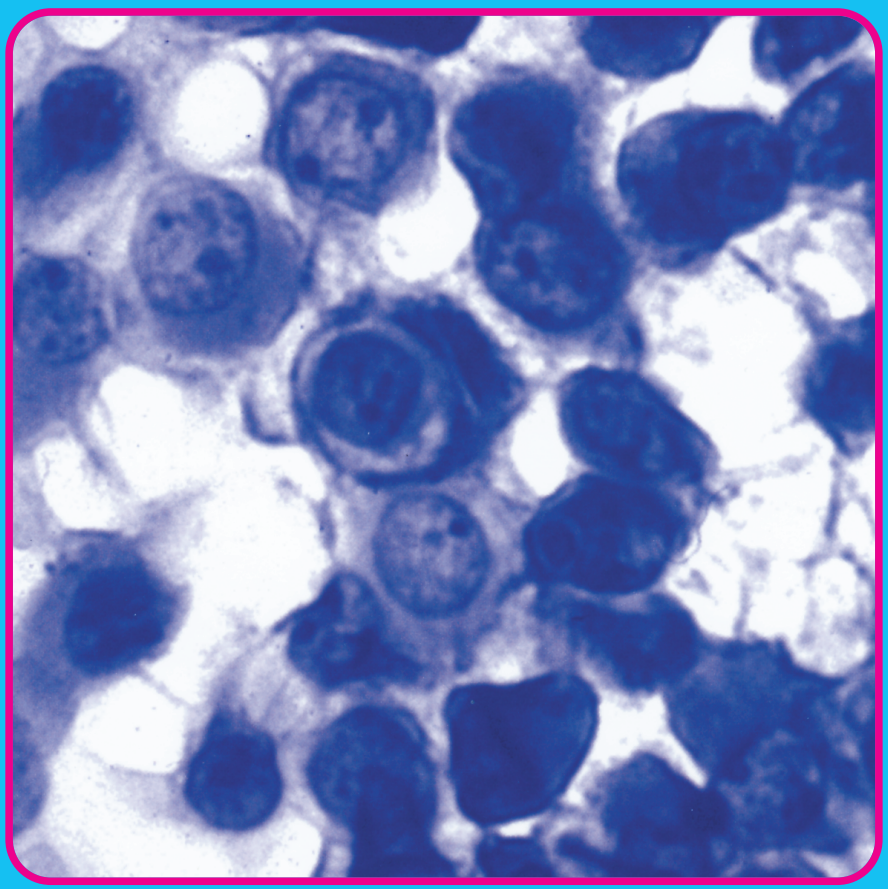
# EXERCISE IMMUNOLOGY REVIEW



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# EXERCISE IMMUNOLOGY REVIEW

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*Hinnak Northoff*
for the editorial team and all our readers

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*Exercise Immunology Review*

## 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*

This year´s issue of EIR contains eight scientific articles, six of which are concerned with Exercise Immunology itself while the last two have a decidedly clinical orientation. We would love to see this latter part increase a bit in future issues of EIR. It must however be acknowledged that all of the "nonclinical" articles carry substantial general implications for health beyond competitive exercise.

The first three articles by D. Freidenreich et al., by G. Paulson et al., and by G. Zhou et al. are solid, conventional reviews, focusing on resistance exercise, muscle damage and regeneration or effects of exercise intensity on $Th_1/Th_2$ balance.

The next three articles present novel results from three studies, two in humans, one in mice. A new role for IL-17 is discussed in the study by K. Sugama et al., linking it to post-exercise muscle damage. The next by A. Berg et al. elucidates effects of soy based supplements, linking them to metabolic but not inflammatory effects. The third by N. Pervaiz at al. defines differences between central and peripheral compartments concerning the cytokine response to exercise, in the mouse model.

The last two articles comprise the "clinical" compartment of this issue. The first article by J. Goh et al. is a review elucidating the role of tumour associated macrophages (TAMs) in the context of exercise. While TAMs of subtype $M_2$ are often referred to as bad guys, exercise, although also associated with M2 formation, is known to be beneficial in several tumour entities. This apparent paradox of the $M_2$ subtype may be a suitable topic for future research in our field. The last paper is an extension of a previously published study (EIR 16 - 2010) reporting the *ex vivo* reaction of immune cells from solid organ transplant recipients to bacterial stimuli – before and after strenuous exercise. Obviously, strenuous and high performance exercise is possible with an entirely curbed inflammatory response. While extending our general knowledge on exercise immunology, the findings may also serve to make patients in this condition more aware of possible risks of high performance exercise.

Finally, Lindy Castell honours the outstanding personality and great scientific achievements of Eric Newsholme who passed away in March 2011.

For the editors

*Hinnak Northoff*

# Immune Responses to Resistance Exercise

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## ABSTRACT

*Resistance exercise induces changes in leukocyte redistribution, phenotypical surface expression and leukocyte functionality. Several factors have been shown to alter the temporal pattern and/or magnitude of response including manipulation of acute program variables, the aging process, and nutritional supplementation. Rest period length and load can modify the temporal pattern and/or magnitude of leukocytosis post exercise. Aging diminishes both the duration and magnitude of the post exercise leukocytosis and reduces leukocyte functionality. The few studies that assessed the effects of nutritional supplements (e.g., carbohydrate, whey protein, caffeine) peri-resistance exercise showed minimal effects on leukocyte responses. Sex differences exist in the timing and magnitude of leukocyte infiltration into skeletal muscle. The immune response to resistance exercise is only a small part of the recovery paradigm. A better understanding of how acute program variables and other factors such as aging, sex and nutritional supplementation affect the immune response to resistance exercise is important in the context of improving recovery, performance and health.*

**Key Words:** resistance exercise; training; immune response; leukocyte; white blood cells; neutrophil; monocyte; natural killer cell; granulocyte; lymphocyte; sex differences; gender; supplementation; immunosenescence; aging; intensity; catecholamines; cortisol; lactate; metabolic stress

**Glossary of Terms:**
NK cells – Natural Killer Cells;
NKCA – Natural Killer Cell Cytotoxic Activity;
RE – Resistance Exercise;
IP – Immediate Post;
PE – Post Exercise;
TNF-α – Tumor Necrosis Factor α;
MIP-1α – Macrophage Inflammatory Protein 1α;
MIP-1β – Macrophage Inflammatory Protein 1β;
IFN-γ – Interferon γ;
GM-CSF – Granulocyte Macrophage Colony-Stimulating Factor;
G-CSF – Granulocyte Colony-Stimulating Factor;
M-CSF – Macrophage/Monocyte Colony-Stimulating Factor;
IL-1RA – Interleukin-1 Receptor Agonist;
IL-1β – Interleukin 1β;
IL-6 – Interleukin 6;
IL-10 – Interleukin 10;
VCAM-1 –Vascular Cell Adhesion Molecule 1;
ICAM-1 – Intercellular Adhesion Molecule 1;
VLA-4 – Very Late Antigen 4;
Ig – Immunoglobulin;
TLR – Toll-like Receptor;
$\beta_2$ ADR – $\beta_2$ adrenergic receptor;
GCR – Glucocorticoid Receptor;
$^{99m}$Tc – Technetium-99m;
MPO – Myeloperoxidase;
LPS – Lipopolysaccharide;
ACTH – Adrenocorticotropic Hormone

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## INTRODUCTION

Leukocytes mediate regeneration and repair of muscle tissue after resistance exercise induced damage. Following a bout of resistance exercise natural killer cells, monocytes and neutrophils increase in the circulation. Monocytes infiltrate the tissue and differentiate into macrophages (3). Macrophages are essential for muscle repair and perform several functions such as aiding satellite cells in recruiting monocytes, stimulating satellite cell proliferation and differentiation with monocytes, promoting myogenic precursor cell survival through cell to cell adhesion and mediating extracellular matrix repair (21, 95, 136, 143). Muscle cells release chemokines which attract neutrophils to damaged tissue (143). Neutrophils aid macrophages in muscle repair by inducing oxidative damage to muscle cell membranes and by removing cellular debris along with macrophages through phagocytosis (143). NK cells may function to maintain continued recruitment of monocytes and neutrophils into the circulation through cytokine cross-talk.

The majority of resistance exercise and immune literature has focused on post exercise changes in circulating leukocyte counts. The temporal pattern and magnitude of response in circulating leukocytes is altered by manipulation of the acute program variables, age and nutritional status (11, 20, 87). Shear stress and hormonal signals (e.g., catecholamines) induce the release of leukocytes from the marginated pool but the leukocyte response is not random. Specific cells must be redistributed for specific functional purposes. By analyzing the phenotypical and functional characteristics of the cells which increase in circulation, a better understanding of why certain cell populations are increased post exercise can be deduced.

The effects of endurance exercise on the immune system has been the focus of many review articles, yet in comparison the effects of resistance training on the immune system has received little attention (15, 47, 49, 50, 82, 100, 122, 133, 152, 153). The purpose of this review is to provide a comprehensive review that focuses on the immune response to resistance exercise in humans. The immune response to resistance training is operationally defined as changes in the magnitude and temporal pattern of circulating leukocytes, changes in circulating leukocyte phenotype (e.g., surface molecules such as Ig, TLRs, adhesion molecules, etc.) and functional parameters (NKCA and proliferation). We hope this discussion provides a foundation and inspires researchers to pursue additional work in this important area to better understand the relationships between skeletal muscle and leukocytes and how resistance training can benefit both young and elderly populations.

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## Leukocyte Patterns in Young Resistance Training Subjects

**Introduction**. A large number of studies inclusive of diverse training programs have examined the magnitude and temporal patterns of change in circulating leukocytes in response to resistance training (Table 1). Most of these studies measured leukocyte redistribution after a single bout of resistance exercise or used an acute testing exercise bout before, during and after a period of chronic training (6 months or more).

**Monocytes.** The most common pattern of response observed in monocytes is an increase during exercise, an early peak immediately post with a sustained monocytosis up to 120 min PE (87, 103, 116). Monocyte peak times occurred at the IP (87, 103, 134, 140) or 120 min PE time points (116). Most studies show a sustained monocytosis through the final measured time point (90-120 min), although a quicker return to baseline has been reported by 30 or 45 min PE (134, 140).

**CD4 T Helper Cells.** The response pattern for CD4 T helper cells to resistance exercise is variable. CD4 T cells have been shown to moderately but not significantly increase during exercise (116), result in no change (140), or result in a sustained lymphocytosis between the IP and 15 min PE time points before returning to baseline by 30 min PE (134). It is difficult to explain why CD4 T cells did not show a consistent pattern since details of the training programs used in each study were not always provided, but total volume of work may impact the CD4 T cell response (See Table 1).

**CD8 Cytotoxic T Cells.** The predominant CD8 T cell response is a lymphocytosis immediately post, followed by a return to baseline between 15-45 min PE

(134, 140). A different pattern of response was observed by Ramel et al 2003 where CD8 T cells decreased by approximately 14% during recovery before returning to pre-exercise levels by 120 min PE (116).

**B Cells.** The B cell response to resistance exercise depends on the subset being analyzed. Cells stained for CD19 represent both young immature and older mature B cells while CD20 staining represents mature B cells (78). The general acute response pattern for CD19 B cells is an increase in B cells immediately post followed by a return to baseline by 120 min PE (31, 93, 103). Since only one study measured beyond the IP time point, a narrower time frame for the return of CD19 B cells to baseline cannot be established. CD20 B cells show no significant changes in cell counts during the PE recovery period (140). It is possible that acute resistance exercise results in an increase in younger B cells in circulation.

**Natural Killer Cells.** NK cells respond to an acute bout of resistance exercise with a lymphocytosis during exercise (116) and immediately post (31, 93, 103, 134, 140) which is sustained until 15 min PE (134) and then either returns to baseline by 30 or 45 min PE (134, 140) or declines below baseline during the recovery period until the final time point at 120 min PE (116).

**Neutrophils.** The general acute response pattern of neutrophils is an increase above baseline immediately post with a sustained neutrophilia up to 120 min PE (87, 103, 116, 117, 134). Neutrophil peak times occurred immediately post (134) or at the last measured time point, either 90 (87) or 120 min PE (103, 116).

**Basophils.** Relatively few studies have measured and reported values for basophils after acute resistance exercise (67, 103, 134). The predominant response appears to be no change in basophils during the PE period (67, 134). Basophils have been reported to mimic the response of neutrophils, the predominant granulocyte, increasing above baseline immediately post and at the final 120 minute PE time point (103). The basophil response is associated with higher PE lactate (103).

**Eosinophils.** Three studies reported no changes in eosinophils within the first 30 minutes after exercise (67, 103, 134). However, one study measured eosinophils at 120 min PE and noted a decrease below baseline (103). Eosinophils show the weakest response to acute resistance exercise.

**Effects of Chronic Resistance Training.** Chronic resistance training is defined as performance of regular bouts of resistance exercise over a period of 6 or more months with a frequency of at least 2-3 times per week. It has been demonstrated that the type of routine (power or hypertrophy) and the workload (total body or upper body) do not affect the acute changes in the magnitude of circulating lymphocyte subsets, monocytes or neutrophils in response to a single exercise bout or augment resting levels of these leukocytes over a 6 month time period (91, 92). Since only pre to post measures were observed the possibility of differences exist-

ing between groups at later time points cannot be excluded. These studies only included women and so these results cannot be inferred to men.

**Absolute Versus Relative Changes in Leukocyte Patterns.** The method of reporting circulating cells can affect the results. For example Stock et al 1995 reported PE increases in total T cells, CD8 T cells, NK cells, total lymphocytes and total monocytes when expressed as absolute cell counts. However, when expressed as cell percentages, total lymphocytes, monocytes and CD8 T cells did not increase significantly PE, total T cells and CD4 T cells decreased PE and NK cells increased (140). The method of reporting changes in circulating leukocytes may especially affect lymphocytes since the dominant lymphocyte subset to change is NK cells. Immediately PE NK cells increase in magnitude by a much greater percentage than T cells or B cells, often increasing by over 200% (31, 93, 103, 140). The magnitude of the NK cell response can mask the relative response of other lymphocytes when expressed as cell percentages. Therefore absolute cell counts should be the preferred method of reporting exercise induced changes in leukocytes preferably in units of $10^9$ cells/L.

**Summary.** Resistance exercise impacts the magnitude of circulating leukocytes. (Figure 1). Monocytes and neutrophils show an early increase at the IP time point with a sustained leukocytosis through 120 minutes PE. NK cells appear to show an early increase followed by either a quick return to baseline or a decrease below baseline. The smaller granulocytes, basophils and eosinophils show little change , CD4 T cells do not have a general pattern and the B cell response depends on the subset. CD8 T cells show a lymphocytosis immediately PE and return to baseline as early as 15 minutes PE. Chronic resistance training appears to have no effect on circulating leukocyte counts at rest or after acute exercise regardless of training type (power vs hypertrophy) or workload (total body vs upper body) (91). Sex differences cannot be discerned chronically since these studies uti-

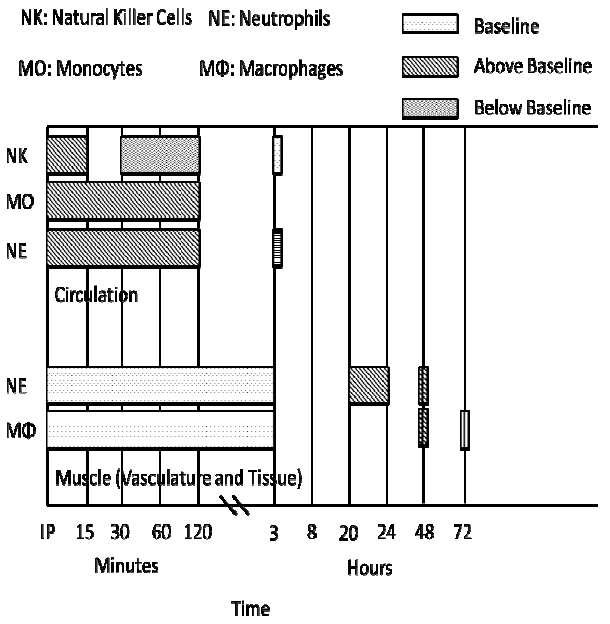


**Figure 1.** Temporal response patterns of selected leukocyte subsets in the circulation and muscle tissue.

lized only women. There appears to be no significant difference in the magnitude of leukocytosis after an acute bout of exercise between young trained and untrained individuals (111, 116). The method of reporting changes in circulating cell counts after RE can affect the results, especially when describing changes in lymphocytes.

## The Effects of Specific Program Variables on Leukocyte Redistribution

**Introduction.** Skeletal muscle adaptations and the acute hormonal responses to resistance exercise are impacted by the exercise program variables (i.e., loading, rest period length, set sequence, volume, total work, exercise choice and exercise order). Manipulating these variables can impact the intensity of the training program which can be measured by physiological changes such as heart rate and lactate and scales which rate perceived exertion such as the Borg RPE scale. Manipulating these variables can also modify the magnitude and perhaps temporal pattern of circulating leukocytes PE.

**Rest Period Length.** Rest period length is the interval of time taken to rest between subsequent sets in a resistance training routine. The effect of rest interval length (1 vs 3 min) while maintaining the load, total work, exercise choice and exercise order has been determined (87). Resistance exercise resulted in a similar temporal pattern of increase in total leukocytes, total lymphocytes, monocytes and neutrophils regardless of time between sets, but the short rest interval resulted in a significantly greater magnitude of response PE for all leukocyte subsets (87).

**Load.** Load is often defined as a percentage of 1RM and has demonstrated the potential to impact the leukocyte response to resistance training. Specifically, neutrophils displayed a different response pattern to different loading conditions, indicating that neutrophils may be sensitive to changes in loading conditions. Neutrophils increased more rapidly in response to a load of 55% 1RM than 65% 1 RM, however the cadence was also quicker in the higher load protocol but rest period length was the same (20).

**Metabolic Response.** The metabolic response to resistance exercise may impact the immune response as evidenced by a trend towards a greater increase in NK cells and significantly greater increases in CD19 B cells and CD8 and CD4 T cells in subjects who had a greater lactate response to resistance exercise (92). There were no differences in granulocytes or monocytes between high and low lactate responders (92). Lactate itself or the associated increase in acidity may impact leukocytes (59, 77, 92). Factors that determine lactate production such as inter-individual differences in muscle metabolism and manipulation of program variables may mediate the effect (35, 113, 121). Greater PE lactate levels are also associated with a greater catecholaminergic response which may also play a role in modifying cellular redistribution (113).

**Future Directions.** Manipulation of rest period length and load can alter leukocyte redistribution. The ideal immune response would be one that minimizes the risk of infection/onset of sickness/illness allowing for peak performance to be uninterrupted by immune compromise. This is especially crucial for athletes who compete and train during a season, a time at which maintenance of performance is crucial. The relationship between the degree of PE leukocytosis and prevention of illness/maintenance of health remains unclear. It would be valuable for future research studies to determine the impact of each program variable on leukocyte redistribution to be able to prescribe a resistance training program that will help maintain performance and health.

## Effects of Dietary Factors on Resistance Exercise-Induced Leukocytosis

Nutritional supplements can modulate physical performance and may impact the immune response to resistance exercise. The majority of studies that examined the impact of dietary factors on the immune response did so in the context of acute supplementation around a single bout of resistance exercise , but a few studies utilized acute testing sessions before and after a short term training period (8-21 weeks).

**Carbohydrates.** The temporal pattern of monocytes and eosinophils are altered by providing carbohydrate around a bout of resistance exercise while neutrophils and basophils are unaffected. The duration of PE monocytosis was truncated to 60 minutes, half the duration observed in most studies without carbohydrate supplementation (20, 61, 87, 101, 103, 116). A quick return to baseline by 30-45 min has been observed previously after unsupplemented exercise bouts (134, 140). Eosinophils displayed an immediate PE increase with carbohydrate supplementation, which contrasts the observances made without supplementation (61, 103, 134). Total lymphocytes largely mirrored the response pattern of natural killer cells observed without supplementation indicating that NK cells dictate the response of lymphocytes. An exception was seen by Carlson et al 2008 with an increase in lymphocytes at 90 min PE (20). Although there is inconsistency among studies, the magnitude of response for neutrophils (101), monocytes (101) and total lymphocytes (20) may be attenuated by carbohydrate supplementation. The addition of a 4h time point by Koch et al 2001 also revealed a sustained neutrophilia and a return of lymphocytes to baseline at 4h PE (61). Monocytes may be sensitive to nutritional intake since all the studies which reported a shortened period of PE increase for monocytes had some form of nutritional intake prior to exercise or failed to report it (20, 61, 101, 134, 140).

**Caffeine.** Caffeine is a central nervous stimulant that increases circulating catecholamines, promotes lipolysis, decreases pain perception, and sustains motor unit firing rates and neuro-excitability through inhibition of adenosine (29). Since maintenance of catecholamine levels has been shown to maintain force production during resistance exercise, it is possible that the increase in catecholamines stimulated by caffeine may aid in maintaining work capacity (44). The only study that has examined the effects of caffeine on the resistance exercise-induced

immune response showed no significant difference compared to placebo in the number of circulating neutrophils, basophils, eosinophils, monocytes total leukocytes and total lymphocytes measured immediately post (80). Differences in the temporal pattern of circulating leukocyte counts could not be determined since only a single PE time point was monitored. Since caffeine increases circulating catecholamines the primary cell type affected by catecholamines would be NK cells, but NK cells were not specifically measured. Based on the findings of this study, caffeine has no effect on the IP-resistance exercise immune response.

**Cystine and Theanine.** Glutamine/cystine and theanine can enhance glutathione concentrations in immune cells in vitro and in the liver in vivo respectively (73, 119). NKCA is correlated with glutathione levels (62, 63). Based on these predications it was determined that a combination cysteine and theanine supplement may have an effect on immune function after resistance training. Daily supplementation with cysteine and theanine maintained NKCA only when an unaccustomed increase in weekly training frequency occurred, but total leukocyte counts were unaffected (60).

**Whey Protein.** Whey supplementation has been shown to modify muscle damage, performance, perceived post-exercise fatigue and anabolic pathways in muscle fibers in conjunction with resistance training (6, 55, 57). These positive effects have mainly been attributed to the high content of essential amino acids, particularly leucine. Other whey components such as lactoferrin, glutamine, immunoglobulins, and other peptides (eg, lysozome, β-lactoglobulin, and β-lactalbumin) have demonstrated immunoregulatory functions in animals and humans (86). Hulmi et al 2010 observed no effect of whey supplementation on neutrophils, total lymphocytes and mixed cells (monocytes, eosinophils, basophils and immature precursor cells) in young trained, young untrained and elderly trained subjects (56). Another important finding was that young untrained subjects had higher relative neutrophils, mixed cells and absolute lymphocytes than older trained subjects (56). The interpretation of this study is complicated by the fact that whey was provided to both whey and control groups PE and a standardized breakfast was not consumed consistently in all group comparisons. Whey amino acid constituents can appear in the bloodstream within 5 minutes after administration and the breakfast could have impacted the number of circulating cells, particularly monocytes (19, 20, 61, 101).

**Summary.** Based on the limited studies conducted it appears that supplementation with carbohydrate, protein, and caffeine has a minimal effect on the immune response to resistance exercise. Carbohydrates may attenuate the magnitude of increase in neutrophils, monocytes and total lymphocytes, but the data are inconclusive. Cystine and theanine supplementation appears to maintain NKCA only during periods of rigorous resistance training to which the athlete is unaccustomed to. Whey and caffeine were shown to have no effect on circulating leukocytes. Conclusions about the effect of nutritional supplements on the immune response to resistance exercise are limited by supplement dosage between and/or within studies, monitoring and control of diet prior to acute exercise testing and the number of PE time points. The immune response is often only characterized

by changes in circulating cell counts, but other effects such as changes in surface expression of receptors and adhesion molecules or in nucleotide and protein synthesis may also be affected.

## Leukocyte Patterns in the Elderly: Effects of Immunosenescence

The aging process results in dysregulation of the immune system, known as immunosenescence, and altered hormonal responses. Immunosenescence is a two component model. The first component is a chronic increase in the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL- 1β, pro-inflammatory markers such as C-reactive protein and clotting factors known as "inflamm-aging"(105). The second component is a change in cell signaling. Lipid rafts are rigid platforms in cell membranes that facilitate cell signaling by bringing signaling complexes together (132). Although the surface expression of receptors often doesn't change (129), membrane fluidity changes with aging which could lead to decreased signal complex formation and modified internal cell signaling leading to decreased leukocyte functionality. The direction of change in membrane fluidity with age is dependent on cell type. Neurons and lymphocytes in the elderly show decreases in membrane fluidity, but neutrophils in both elderly humans and rats demonstrate an increase in membrane fluidity (2, 58, 107, 147). Changes in membrane fluidity are associated with changes in membrane cholesterol, phospholipids and fatty acids. Typically membrane cholesterol is inversely related to membrane fluidity (2, 58). There are also population shifts in some leukocyte subsets. Macrophages are skewed to the alternatively activated macrophage phenotype and there is an increase in the proportion of $CD56^{dim}$ NK cells (83). Endothelium dependent vasodilation is reduced with aging in both brachial and coronary arteries which may affect leukocyte redistribution from the marginated pool (34, 46). Aging results in changes to the circadian rhythms of several hormones such as catecholamines and cortisol (30, 146). Elderly individuals also demonstrate a modified hormonal response to resistance exercise as evidenced by diminished PE norepineprhine (11) and an increased sensitivity to ACTH, maintaining a higher level of cortisol (70). Altered leukocyte functioning, constant low grade inflammation decreased vascular plasticity, modified circadian patterns and different hormonal responses to resistance exercise all impact the differences in the immune response to resistance exercise between the young and elderly.

**T Cells and B Cells.** In elderly individuals, CD4 and CD8 T cells are unresponsive to resistance training. Neither resting nor the PE circulating cell numbers change after acute, short-term or chronic exercise (12, 39, 90, 115, 118). Even when controlling for total work, heavy and light loading acutely have no effect on circulating cell counts (98). Short-term training does not affect resting CD20 B cell counts, and neither short-term nor acute resistance training impacts the number of circulating CD19 B cells at rest or PE (12, 39, 115).

**Natural Killer Cells.** The predominant pattern of response acutely is an increase in NK cells immediately PE with a return to baseline by 2h PE (39, 90). This pattern was shown to be unaffected by short-term resistance training (39, 90). An

exception to this pattern was observed where untrained elderly subjects showed a decline in NK cell counts below baseline which returned to baseline by 6h PE (11). After short-term training the elderly subjects did not show a decrease in NK cell numbers at the IP time point and instead maintained NK cell numbers throughout the recovery period (11). Thus short-term training maintains NK cell counts after acute exercise. Several protocol differences could have contributed to the disparate results such as the prandial state of the subjects, the duration of short-term training, the acute resistance exercise tests and the sex of the subjects. Chronic resistance exercise does not affect resting NK cell levels (118). Further investigation into the morphology of circulating NK cells indicates that the predominant type of NK cells are the $CD56^{dim}$ subset and this was not altered by chronic training (118).

Overall the NK cell response to acute exercise is reduced compared to young subjects. Although NK cells have been shown to increase after acute resistance training in the elderly, the magnitude of the PE increase is diminished compared to young individuals (11, 31, 39, 90, 103, 116, 140). Not only is the magnitude of the response different in the elderly, but so is the temporal pattern of the PE response. Regardless of training status, elderly individuals display both a diminished magnitude of response and an altered temporal pattern, but training in the elderly may partially restore the NK cell response to resistance exercise.

**Neutrophils.** Short-term training affects the duration of neutrophilia after acute exercise but chronic training does not impact resting neutrophil cell counts (13). Untrained elderly individuals display a transient neutrophilia at the IP time point (56). After short-term training, the neutrophilia is maintained for 30 minutes into the PE period demonstrating that short-term training increases the duration of neutrophilia after acute exercise (56). In contrast to the elderly, the acute response to resistance training in the young is a sustained neutrophilia during the exercise recovery period up to 2h PE (87, 103, 116, 134). The elderly experience an attenuated time course for neutrophilia with age, but this can be partially recovered through resistance training.

**Monocytes.** Monocytes respond to an acute bout of resistance exercise, but short-term training has no effect on the pattern of response and chronic training does not impact the resting monocyte cell count (13). After acute resistance exercise, a transient monocytosis occurs at the IP time point, but short-term training has no impact on this response (Although monocytes were not measured directly, they were measured in a pool of mixed cells, of which they were the predominant cell type.) (40, 56). The acute response pattern of monocytes in the elderly is reduced compared to young individuals. Acute resistance exercise elicits a sustained monocytosis in the young for 120 minutes PE (87, 103, 116, 134). The duration of monocytosis observed in the elderly is far less than that observed in the young and training does not improve this response.

**Macrophages.** Macrophages demonstrate both impaired functioning and decreased numbers in muscle tissue of the elderly in comparison to younger counterparts. At rest in both young and old subjects, the anti-inflammatory CD163 or

alternatively activated macrophages are more prominent than the CD11b macrophages which are the classically activated pro-inflammatory macrophages (112). Young subjects possess greater numbers of total macrophages than older subjects including greater numbers of both CD163 and CD11b macrophages. Young and elderly subjects display no change in total macrophages (CD68) 72h after a single bout of resistance exercise but young subjects demonstrate increases in both CD163 and CD11b macrophage subsets while elderly individuals show no change in either macrophage subset. At 72h PE young subjects have 8 times more CD163 cells than CD11b cells (112) indicating that the predominant anti-inflammatory pool of macrophages before exercise is further augmented after exercise. Although both young and old at rest and post 72h after exercise show greater numbers of CD163 than CD11b macrophages, the older subjects have significantly fewer CD11b macrophages than younger subjects. This finding confirms that older individuals have a shift to the alternatively activated macrophage subset. Furthermore macrophage dysfunction is evident due to higher baseline levels of IL-1β and IL-1RA and no increase in anti-inflammatory cytokines PE as younger subjects demonstrated (112). The findings indicate a perturbed macrophage response to resistance exercise in older individuals.

**Summary.** The response pattern of several leukocyte subsets including monocytes, neutrophils, CD19 B cells, CD4 and CD8 T cells as well as NK cells are all diminished in the elderly compared to the young showing the impact immunosenescence with age has on the immune response to resistance exercise. Although exercise may improve functionality of NK cells, the tissue remodeling process may be slower due to the observed impairment in macrophage numbers and function as well as the known deficits in neutrophil and monocyte functioning.

## The Effects of Aging on Leukocyte Function

Decreased NKCA has been associated with poor lifestyle choices, illness and disease whereas maintenance of NKCA is associated with health and longevity in aging (16, 43, 74, 79, 94, 124).

**Young.** Although natural killer cell cytotoxic activity does not show resting differences based on training status, acute exercise attenuates NKCA later in recovery (102, 103). Immediately after exercise, no change in NKCA was determined despite a 225% increase in NK cells (103). However at 120 minutes PE NKCA decreased by 61% below baseline values when NK cells had decreased below baseline levels (103). NKCA does not appear to mirror changes in circulating NK cell numbers PE. Since the number of circulating NK cells does not appear to affect NKCA, it may be the subset of circulating NK cells that effect NKCA.

**NK Cell Subsets.** NK cells can be divided into two subsets based on the density of CD56 expression. $CD56^{bright}$ NK cells compose ~10% of circulating NK cells while $CD56^{dim}$ NK cells compose the remaining 90% (76). This distinction is important because of functional differences in these two NK cell subset populations. $CD56^{bright}$ NK cells are more responsive to monokines (monocyte derived cytokines) and produce more cytokines while being less cytotoxic at rest (23, 24).

$CD56^{dim}$ NK cells are more cytotoxic but produce less cytokines (23). The concomitant increase in $CD56^{dim}$ NK cells and decrease in $CD56^{bright}$ with aging may affect both the NKCA and cytokine regulatory capabilities of NK cells in the elderly.

**Elderly.** The variability of the NKCA response to exercise is a common motif in the literature and resistance training is no exception (39, 120, 131, 154). Resting NKCA has been shown to be unaffected by chronic training (118). The effects of acute and short term training are disparate. NKCA has shown both no changes and significant improvements after acute exercise and short-term training (39, 90). However, the significant improvements in NKCA were reported at all time points including baseline after short-term training. Seasonal variations may have caused or contributed to all the measured time points changing (53, 54, 97). Due to the variability in the NKCA response to resistance training in the elderly and only a single acute resistance training study performed in the young, it is difficult to make comparisons. In the elderly, there is either no change or an acute increase in NKCA after acute exercise (39, 90). In the young, there is no change until a decrease at 2h PE (103).

**Lymphocyte Proliferation.** Resistance exercise in young individuals has inconsistent effects on lymphocyte proliferation inducing either no effect, an increase or decrease (31, 91, 92, 103, 111). Lymphocyte proliferation consistently shows no response in the elderly possibly indicating a perturbed response to mitogenic signals in the elderly after resistance exercise (39, 115).

## Catecholamines and the Immune Response to Resistance Exercise

**Resistance Training and Catecholamines.** Catecholamines have been shown to respond to a variety of acute resistance exercise programs and have demonstrated dependence on total work, rest period length between sets and reps, anticipation of exercise, training status and the level of metabolic stress (18, 36, 44, 51, 65, 68, 71). Training status does not affect the pattern of the catecholaminergic response but does affect the magnitude of the response (68). Catecholamines can affect blood flow regulation, cardiac contractility, the secretion of other hormones, substrate mobilization and the maintenance of force development (44, 66). Epinephrine and norepinephrine have been shown to increase above baseline values prior to exercise, during exercise and up to 5 minutes during the recovery period (36, 44, 68, 71). Some research indicates that norepinephrine may increase up to 15 minutes PE while epinephrine returns to baseline by this time point (18, 51). NK cells are also elevated during the same timeframe as catecholamines (31, 103, 116, 140). This has been demonstrated with adrenaline infusion, after the cessation of which, circulating NK cells decrease below baseline (126). The parallel increases in circulating catecholamines and NK cells after resistance exercise suggest that catecholamines are necessary to maintain NK cells in circulation and play a role in cellular adhesion.

**Catecholamines and Cellular Adhesion.** Catecholamines, specifically epinephrine, decrease cellular adhesion (10). Epinephrine infusion has been shown to mobilize cells but does not increase soluble adhesion molecules or alter the surface

expression of adhesion molecules such as VCAM-1, ICAM-1 or E-Selectin (4, 7, 8, 9, 126). Resistance exercise increases circulating catecholamines, but does not increase soluble adhesion molecules or alter surface expression (93, 108). However, resistance exercise does cause a redistribution of cells preferentially expressing specific adhesion molecules, namely, VLA-4 on lymphocytes and L-Selectin on neutrophils (93). The increase of lymphocytes expressing VLA-4 is due to an increase in circulating NK cells PE (93). NK cells are most likely preferentially recruited since they have the highest density of $\beta_2$ ADR (75). When catecholamines bind to $\beta_2$ ADR they cause an increase in cAMP and induce changes in cytoskeletal organization which may modify the adhesion of cells to endothelial walls (110, 128, 150). This may lead to the preferential redistribution of cells with specific adhesion molecule expression.

Although NK cells may be preferentially recruited to the circulation, NK cell subsets may not be equally redistributed. A recent review on NK cell subsets and acute exercise (endurance exercise) indicated that $CD56^{dim}$ cells are preferentially redistributed into the circulation in greater magnitude than $CD56^{bright}$ NK cells (144). This may be due to differences in adhesion molecule expression. It has been demonstrated that $CD56^{bright}$ NK cells highly express CD62L (L-selectin), while $CD56^{dim}$ cells express low levels of this adhesion molecule (45). Based on the research of Miles et al 1998 $CD56^{bright}$ NK cells may not be preferentially recruited to the circulation due to their expression of L-Selectin. Therefore the RE literature may agree with the findings that $CD56^{dim}$ cells are preferentially redistributed to the circulation, however further work on differences in VLA-4 expression on both NK cell subsets needs to be performed. Since neutrophils expressing L-Selectin were preferentially recruited, a combinatorial signal of hormone receptors and adhesion molecules unique to each cell subset may control their redistribution.

## Cortisol and the Immune Response to Resistance Exercise

**Introduction.** Cortisol increases post resistance exercise especially bouts that utilize multiple sets with short rest periods between 60-120 sec (14, 17, 36, 88, 96, 148, 151). Plasma cortisol levels peak IP to 30 PE, then either remain elevated above baseline or begin to decline toward baseline 30-60 minutes PE (14, 69, 70, 96, 101, 116, 151). By 90-120 min PE cortisol either returns to baseline or decreases below it, followed by values below baseline at 4h PE (20, 61, 116). This timeline is important since neutrophils and monocytes have been shown to increase over this same time interval, and several studies have shown a relationship between changes in cortisol and leukocyte subsets after RE (20, 61, 87, 103, 116). Therefore it is important to clarify the effects of cortisol on leukocyte redistribution.

**The Glucocorticoids and Leukocyte Subsets.** Intravenous doses of cortisol have been used to isolate its effects on circulating leukocytes. However, this model is not reflective of the impact of cortisol after a bout of resistance exercise since other hormones with known effects on leukocyte adhesion are elevated in conjunction with cortisol PE. Cortisol administration induces neutrophilia between 1-2h

and 4-6h post injection and concomitantly induces lymphocytopenia and monocytopenia evident in the first hour and peaking 4-6h post injection (27, 37). After a bout of resistance exercise cortisol is correlated with increased neutrophil and monocyte counts, and decreases in CD8 T and NK cell counts (61, 103, 116). Although the relationship of neutrophils, lymphocytes and cortisol agree with cortisol administration studies, the directional relationship of cortisol and monocytes do not. There is evidence that cortisol can influence the transcription and therefore expression of adhesion molecules by endothelial cells suggesting a potential mechanism for the effects of cortisol on leukocyte adhesion (25). Since catecholamines which also increase post resistance training can influence the redistribution of cells with particular surface adhesion molecules it is plausible that some interaction effect between the influence of catecholamines and cortisol regulate the redistribution of monocytes. Similarly NK cells in the presence of elevated catecholamines early post exercise remain elevated, but after catecholamine levels decrease, cortisol becomes the dominant compartmentalizing factor. The ratio of these two hormones may be a crucial factor in determining the maintenance of leukocytes in the circulation.

## Infiltration of Muscle Tissue by Leukocytes

**Techniques Used to Quantify Leukocytes.** Radiolabelling of leukocytes and muscle biopsies are the two techniques used to determine changes in muscle tissue accumulation and infiltration of leukocytes in humans. Radiolabelling of leukocytes is performed using Technetium-99m, a radionucleotide tracer with a short half-life of 6h that restricts its use to a 24h period (28, 64). This short half-life leads researchers to forego a pre-exercise sample leading to comparisons of an exercised leg and a control leg (81, 106). Radiolabeling does not indicate muscle infiltration, but accumulation in a region whether it be adhesion to the walls of the microvasculature or penetration into the muscle tissue. Muscle samples obtained from biopsies are stained for leukocyte subsets to determine the magnitude of leukocyte infiltration (106, 112). However, the biopsy itself causes muscle damage leading to an inflammatory response (85).

**Technetium 99m Measures.** The general pattern of response is little to no increase in the accumulation of radiolabeled leukocytes during the first 4 to 8h PE followed by a significant increase above baseline 20-24h PE (81, 114). In an exception to this pattern, increased accumulation of radiolabeled leukocytes was seen in the quadriceps throughout the 24h PE period in a group of subjects which ranged from sedentary to physically active (106). The sedentary subjects showed markedly greater accumulation of $^{99m}$Tc-labeled leukocytes in the quadriceps (106). Comparisons of the sedentary participants to the other more fit subjects demonstrates that training status can influence the timing and magnitude of leukocyte accumulation with sedentary subjects accumulating a greater number of leukocytes beginning sooner after the cessation of exercise.

**Muscle Biopsy Measures of Tissue Infiltration.** No significant increase in muscle infiltration is detected during the first 3h PE, but by 48h PE there is a significant increase in both neutrophil and monocyte/macrophage infiltration (84).

Muscle damage determined by Z-disk streaming followed the same response pattern as leukocyte tissue penetration showing no increase at 3h PE but a significant increase by 48h PE (84). This common response pattern suggests the timing of leukocyte infiltration coincides with the development of muscle tissue damage. In contrast, increased leukocyte infiltration of the muscle tissue has been observed at all PE time points (0.5 to 168h PE) with the greatest accumulation of leukocytes at 4 and 7 days PE (106). This response pattern may result from the inclusion of subjects with diverse training experience.

**The Controversy of Infiltrating Neutrophils.** Although neutrophils have been shown to extravasate into tissue during acute inflammation, it is controversial whether this occurs after a bout of resistance exercise (33, 135). Paulsen et al 2010 determined that the amount of neutrophil infiltration into the muscle tissue after exercise was minimal and instead proposed that the neutrophils accumulate in the microvasculature of the quadriceps by adhering to endothelial cell walls and that only a small percentage of these cells extravasated into the muscle tissue (106). In contrast to these findings Mahoney et al 2008 reported a 14-fold increase in the presence of neutrophils (84). The reason for these dichotomous results may be at least partially explained by the difference in methods used to detect neutrophils. Mahoney et al 2008 stained for MPO to distinguish neutrophils, but Paulsen et al 2010 did not explicitly label neutrophils (84, 106). Instead the number of CD68 cells (monocytes/macrophages) was subtracted from the number of CD16 cells (which by majority are neutrophils and monocytes/macrophages) to estimate the level of neutrophil infiltration (106). However, the indefinite findings concerning the amount of tissue infiltration by neutrophils is a common paradox in the literature of exercise induced muscle injury (127).

**Location of Tissue Macrophages.** Macrophages located in the skeletal muscle are known as histiocytes, which are macrophages found in connective tissue (95). Several studies have confirmed the location of macrophages in the connective tissue of muscle (106, 139). This appears to be the normal pattern seen where macrophages can surround a muscle fiber through habitation in the connective tissue, mainly the endomysium and perimysium (106, 139). However, macrophages have been shown to penetrate the muscle fiber itself if the muscle tissue becomes necrotic (106, 139). After exercise wider regions of endomysium and perimysium around muscle fibers have been observed, even areas where the ECM detached from the muscle fiber surface on one or more sides (139). Muscle fiber infiltration was not observed where muscle fibers were detached from the ECM (139) demonstrating the use of connective tissue by macrophages as a path to damaged muscle fibers.

**Summary.** Radionucleotide labeling of leukocytes and muscle biopsies confirm that muscle tissue accumulation and infiltration by leukocytes does not occur for the first 3h of recovery PE and perhaps up to 8h. Muscle tissue accumulation and infiltration have been demonstrated between 20-48h PE, but the resolution of tissue infiltration and inflammation is still controversial and most likely depends on a multitude of factors including the exercise program variables and the training

status of the individuals (Figure 1). Although evidence indicates tissue infiltration by monocytes/macrophages, the degree of infiltration by neutrophils is still debatable. Macrophages use the connective tissue system as roads to reach damaged muscle fibers, but if necrotic cells are present they will infiltrate the muscle cell itself.

## Sex Differences

**Introduction.** Specific hormones that affect the immune system such as catecholamines and cortisol have been shown to respond differently to resistance exercise in men and women (72, 138). Sex differences in the extent of muscle damage after resistance exercise have been shown in studies using animal models but the effects of sex are mixed in humans (22). The purpose of this section is to highlight the sex differences in muscle damage and the endocrine system in response to resistance training and how these factors may affect the number of infiltrating leukocytes.

**Endocrine Responses.** There are many endocrine differences between sexes in the hormones that affect muscular adaptation to exercise and this information can be found elsewhere (72, 138). The hormones with the greatest effects on leukocyte redistribution are the primary focus here. The PE cortisol response after acute resistance exercise is mixed displaying either no sex difference or a greater increase during the PE recovery period in men (42, 72). No differences in the response pattern of norepinephrine have been demonstrated between sexes (41). Although the pattern of response for epinephrine is similar between men and women, men maintain higher epinephrine levels during the PE period than women (41).

**Receptor Expression.** Circulating leukocytes express several hormone receptors including $\beta_2$ ADR and GCR (41, 42). No significant sex differences in $\beta_2$ ADR have been observed for any leukocyte subsets. GCR has only been measured in B cells and demonstrated a greater density in men at baseline but not in respect to the PE response (42). Therefore, hormonal differences during the PE recovery period do not affect the temporal response pattern in GCR or $\beta_2$ ADR receptor density since it is similar between sexes. Since sex differences in hormone expression do not lead to altered receptor expression differential recruitment to muscle tissue may be independent of the influence of hormones and instead may be dependent on other signals.

**Different Microenvironments: Muscle Damage.** Some sex differences become apparent when determining the extent of muscle damage after exercise. No sex differences have been determined in the amount of muscle damage based on Z-disk streaming (141, 142). In contrast, the CK response shows either a greater response in men or that no differences exist. One study observed no differences in the CK response, although a trend existed for a lower response in women (141). In a second study men maintained a higher CK response for a more extended recovery period than women (142). However, the validity of using CK as a marker of muscle damage has been questioned (104). Sex differences in the CK

response may be due to the antioxidant effects of estradiol, but this effect is uncertain (5). Differences between sexes in the disruption of intracellular calcium concentrations within muscle fibers after exercise have been demonstrated in rats. After eccentric exercise, male rats demonstrated an increase in intracellular calcium concentration, that was significantly greater than female rats and ovariectomized female rats demonstrating an estrogen independent effect (137). Increased intracellular calcium in muscle cells is associated with muscle damage through several signaling pathways including activation of calpains and phospholipase A2 and increased production of reactive oxygen species (32, 48). The differences in calcium permeability in male and female muscle may explain differences in muscle damage.

**Muscle Infiltration.** Infiltration of muscle tissue by leukocytes and the level of tissue infiltration increases post resistance exercise (84, 106), but differences between sexes are less apparent. The day after a single bout of resistance exercise, men and women showed no differences in infiltration of neutrophils and macrophages by sex, but 24h after a 2$^{nd}$ repeated bout of resistance exercise women demonstrated significantly greater increases in neutrophils and macrophages than men (142). Increased muscle infiltration of neutrophils and macrophages has been observed 48h after a single bout of exercise, but only a trend was seen towards a greater increase in men than women (141). These studies indicate a sex difference in the timing of leukocyte infiltration leading to varying magnitudes of tissue infiltration at successive PE time points.

**Summary.** Although after resistance exercise sex differences exist in the levels of circulating hormones such as cortisol and epinephrine, there are no accompanying sex differences in the expression of $\beta_2$ ADR or GCR in circulating leukocyte subsets. No differences in the amount of Z-disk streaming are observed after a single bout of resistance exercise but women tend to display a lower CK response. Women may have a greater amount of leukocyte infiltration into muscle tissue than men, but this may be dependent on the timing of muscle biopsies and the level of ultra-structural damage and apoptotic cells. Differences in intracellular calcium accumulation between sexes may impact muscle damage or signaling between muscle fibers and leukocytes leading to differences in muscle tissue infiltration.

## Effects of Resistance Training on Leukocyte Phenotypical Expression

**Antigen Expression.** Leukocytes express several receptors to aid in the response to environmental stimuli. CD64 and CD11b/CD18 are two such receptors that aid the cells in recognizing antibodies and components of the complement system respectively. CD11b/CD18 together compose the type 3 complement receptor. The CD11b/CD18 receptor is a glycoprotein found on the plasma membranes of neutrophils, monocytes and NK cells. It binds to the 3rd component of the complement system, iC3b, and results in phagocytosis of iC3b opsonized cells and causes adherence to vascular endothelium (26, 155). CD64 is the receptor for the Fc portion of IgG and is found on neutrophils and monocytes. Receptor binding causes antibody dependent cytotoxicity, phagocytosis, superoxide production and

degranulation (1, 130, 149). The changes in surface expression of these antigens after exercise is dichotomous hence no general response pattern can be discerned. The expression of CD11b and CD64 surface antigens have been shown to increase PE on neutrophils and monocytes after a single bout of exercise and to a greater extent than after a second repeated bout (109). In contrast, a single bout of exercise has demonstrated no significant changes in any of these surface antigens (125). Major differences between the studies that could cause the discrepancies were differences in subject training status and differences in the eccentric exercise protocol (109, 125).

**TLR 4 and CD14 Expression.** Toll-like receptor 4 is a pattern recognition receptor. CD14 is a co-receptor that aids TLR4 in the detection of lipopolysaccharide, a bacterial cell membrane component. They are highly expressed on monocytes and macrophages. TLR4 plays a role in the inflammatory response and chronic exercise has been demonstrated to provide anti-inflammatory effects. Changes in the expression pattern of TLR4 and CD14 in response to resistance exercise have been demonstrated in elderly women. TLR4 and CD14 surface expression were significantly greater in untrained subjects than trained subjects (40, 89). In corroboration of these findings TLR4 and CD14 mRNA expression were greater in untrained than in trained subjects (40). Dividing participants into HI and LO TLR4 expression groups has demonstrated that HI TLR4 expression coincides with greater expression of the LPS stimulated cytokines TNF-α, IL-6 and IL-1β (89). Together these studies indicate that untrained subjects have higher TLR4 protein and mRNA expression than trained counterparts and that subjects who express higher levels of TLR4 also elicit a greater cytokine response to LPS stimulation. These findings show that chronic exercise may mediate some of its anti-inflammatory effects by affecting receptor expression for pathogen associated molecular patterns and by decreasing the cytokine response to stimulation. However, further research is required to determine if the decrease in TLR4 expression and subsequently lower cytokine secretion is beneficial in response to an actual infection.

## Hypotheses and Future Directions

**Initiation and Maintenance of the Cellular Innate Immune Response.** It is plausible that the initiation of leukocyte infiltration into damaged muscular tissue may be due to recognition of intracellular components from the muscle such as desmin in combination with cytokines released from the muscle tissue itself (99, 143). These initial signals may activate/recruit resident macrophages to areas of damaged muscle tissue and induce the release of cytokines in cooperation with muscle cell myokines and satellite cells. The combination of cytokines can direct monocytes and neutrophils to damaged muscle tissue to aid in regeneration and repair. Maintenance of monocytes and neutrophils in the circulation to supply these cells to muscle tissue may be a function of $CD56^{bright}$ NK cells. $CD56^{bright}$ NK cells have been demonstrated to release several cytokines in response to co-stimulation of monokines such as IL-10, MIP-1α, MIP-1β, TNF-α, IFN-γ and GM-CSF (38). $CD56^{bright}$ NK cells have also demonstrated the ability to produce G-CSF and M-CSF (123). Many of these cytokines promote a strong cellular

response acting as chemokines or activators for both macrophages and neutrophils as well as potential signals for hematopoietic release into the circulation of immature neutrophils and monocytes. $CD56^{bright}$ NK cells may mediate tissue repair from a distance by supporting the actions of macrophages, neutrophils and monocytes but without direct interaction since these cells do not infiltrate damaged muscle tissue after acute resistance exercise (106).

**Prevention of Self-Recognition Through Spatial Redistribution.** Although intracellular proteins and cytokines may aid innate cells of the immune system to be directed to the damaged tissue to facilitate repair and regeneration, there is a potential hazard for self-recognition by the adaptive immune system. This may be why acute exercise doesn't redistribute T and B cells into the circulation to the same extent as the cells of the innate arm. Furthermore, lymphocytes are demonstrated to be sequestered into the lymphatic system in the intestinal area according to animal studies due to the effects of cortisol (145). The timing of distributing lymphocytes into the lymphatic system may be to sequester these cells out of the circulation and away from damaged muscle tissue where they may come into contact with intracellular debris. Furthermore, their distribution back into the marginated pool (or their location in secondary lymphoid organs) may also be timed for when antigen presenting cells or debris that have the potential to induce self-recognition may enter the lymphatic system, thereby maintaining a safety net against self-recognition through spatial separation by timing where the lymphocytes are distributed in the body after exercise.

**Future Directions.** The current literature has formed a foundation for the effects of resistance exercise on leukocyte redistribution. By studying the effects of each of the program variables on resistance exercise, a better understanding of the relationship between exercise and leukocyte redistribution can be obtained. To better understand the causes behind why each program variable induces leukocyte redistribution, additional research needs to be performed on surface adhesion molecule expression of all leukocyte subsets, how surface expression is affected by resistance exercise and what role hormones such as cortisol and catecholamines may play in redistribution and adhesion molecule expression. Only a single paper distinguishes NK cell subsets (118). Considering the functional differences of these cell subsets, their impact on health and how their proportions change with age, it is imperative to delineate the effects of exercise on these cell subsets. Finally, to better understand functional changes after resistance exercise, the application of –omics must be applied to resistance exercise research. The study of genomics in NK cell subsets determined several functional distinctions based on gene expression patterns (52). Expanding the application of genomics and proteomics to study functional differences in leukocyte subsets in response to exercise is a promising future step. Overall this paper has tried to summarize the current literature on resistance training and the immune response to condense the literature into a foundation. Based on this foundation and the suggestions for future research, a more coordinated multidirectional front of research can be pursued to better understand the impacts of resistance exercise on immune function.

## Conclusion

After a bout of resistance exercise cells of the innate immune system, NK cells, monocytes and neutrophils are all preferentially elevated in the circulation PE, while cells of the adaptive immune system, the T cells and B cells, show a much lower magnitude of response. Changes in leukocyte redistribution generally follow a specific temporal pattern in young subjects. Monocytes and neutrophils aid in the repair and regeneration of muscle tissue as evidenced by their extravasation into muscle tissue or accumulation in the muscle vasculature. Increased circulating NK cells may function as supporting cells through cytokine mediation.

Elderly individuals display both an altered temporal pattern and magnitude of response for all leukocyte subsets. This disturbance is likely due to many factors including but not limited to immunosenescence, altered circadian rhythms, diminished catecholamine responses to acute resistance exercise, altered sensitivity to ACTH, and decreases in vascular plasticity. Although not all of the humoral and immune perturbations associated with aging can be restored through exercise, resistance training has the potential to improve the natural killer cell response to acute exercise, improve vasodilation and potentially shear stress stimulated leukocyte redistribution into circulation. Exercise alone may not restore leukocyte functionality, but the use of resistance exercise to study alterations to leukocyte function with age may shed light on new means to combat immunosenescence with aging.

Resistance training program variables such as rest interval length and load can impact the magnitude of leukocytosis after exercise. But further research needs to be done to determine the effects of each program variable independently. By understanding the impact each exercise program variable has on leukocyte redistribution, phenotype and function, a more optimal exercise prescription could be made. A more targeted exercise prescription that considers the effects on the immune system could be used to improve or maintain athletic performance during competition, and could be beneficial to health maintenance with age and the perturbation of disease.

Determining the potential therapeutic impact of resistance exercise on chronic illness is another important area of research, but it has received little attention (115). Although the study of resistance training and immunology is an important area of research on its own, studying the intersection of immunology and muscle physiology could also extend insight into the areas of sterile inflammation, muscular diseases and even autoimmune diseases. Resistance exercise provides a useful model for understanding how the immune system interacts with damaged and healthy tissue and how leukocyte redistribution is controlled.

**Table 1.** Summary of study designs and acute resistance exercise testing details of the research on leukocyte response patterns and resistance exercise.

| | Young Acute | | | | | Acute Testing Details | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **First Author** | **Second Author** | **Year** | **Gender** | **Age** | **Groups** | **SetsxReps** | **Load** | **Rest** | **Type** | **Exercises** |
| Ramel A | Wagner KH | 2003 | Male | 29.5 ± 7.1 | TR vs NRT | 2 sets | 75% 1RM | 1 min | Circuit | Bench Press, Leg Press, Lat Pulldown, Leg Extension, Shoulder Press, Triceps, Crunch, Vertical Row, Biceps Curls and Pullups |
| Ramel A | Wagner KH | 2004 | Male | 29.5 ± 7.1 | TR vs NRT | 1 set | 75% 1RM | 1 min | Circuit | Bench Press, Leg Press, Lat Pulldown, Leg Extension, Shoulder Press, Triceps, Crunch Vertical Row, Biceps Curls and Pullups |
| Nieman DC | Henson DA | 1995 | Male | 24.9 ± 1.2 | TR | 8x10 | 65% 1RM | 3 min | Lower Body | Squat |
| Mayhew DL | Thyfault JP | 2005 | Male | 22.2 ± 0.3 | 1 min vs 3 min rest | 10x10 | 65% 1RM | 1/3 min | Lower Body | Leg Press |
| Miles MP | Leach SK | 1998 | Female | 18-35 | NRT | 6x10 | 75% 1RM | 2 min | ARET | Squat |
| Petridou A | Chatzinikolaou A | 2007 | Male | L: 23.8 ± 1.1<br>O: 24.2 ± 1.0 | Lean vs Obese | 3x10-12 | 70-75% 1RM | 2 min | Total Body | Chest Press, Seated Row, Leg Press, Shoulder Press, Leg Extension, Leg Curl, Biceps Curs, Triceps Extension, Ab Curls, Back Extension |
| Dohi K | Mastro AM | 2001 | Female | HI: 23.6 ± 2.6<br>LO: 21.4 ± 3.5 | Hi vs Lo Strength | 6x10 | 10RM | 2 min | ARET | Squat |
| Nieman DC | Henson DA | 1994 | Male | TR: 23.6 ± 0.7<br>NRT:22.4 ± 0.5 | TR vs NRT | 1 RM squat testing | 1RM | NR | Lower Body | Squat |
| Potteiger JA | Chan MA | 2001 | Female | TR: 22.8 ± 1.6<br>NRT:22.9 ± 0.9 | TR vs NRT | 3x10 | 10RM | 3 min | Total Body | Leg Press, Bench Press, Leg Extension, Overhead shoulder Press, Seated Row, Leg Curls, Biceps Curls |
| Stock C | Schaller K | 1995 | Male | 22-29 | NRT | 3 sets | 70% by Reps | NR | Circuit | 5 different exercises |
| Kraemer WJ | Clemson A | 1996 | Male | 26.9 ± 4.8 | Hi vs Lo Cortisol | 8x10 | 10RM | 1/3 min | Lower Body | Squat |
| Miles MP | Naukam RJ | 1999 | Female | 21-36 | Hi CK vs Lo CK<br>TR and NRT | Arm Curls: 2x25<br>Leg Curls: 3x20 | Maximal<br>60°/ sec | 5 min | Eccentric | Leg Curls and Arm Curls |
| Simonson SR | Jackson CGR | 2004 | Male | 30 ± 7 | RT vs Con | 3x8-10 | 75% 1RM | 1:2, W:R | Total Body | Crunches, Chest Press, Pulldown, Leg Curls, Leg Extension, Leg Press, Seated Rows and Shoulder Press |
| Pizza FX | Davis BH | 1996 | Male | 20-37 | NRT | Arm Curls: 25 | NR | None | Eccentric | Arm Curls |
| Saxton JM | Claxton D | 2003 | NR | 20-37 | TR | Quadriceps: 2x25<br>Triceps: 2x25<br>Bench Stepping | 1.05 rad/sec<br>0.35 rad/sec<br>20 steps/min | 5 min<br>5 min<br>None | Eccentric<br>Bench Step | Leg Extensions and Triceps Extensions<br><br>Bench Stepping for 40 minutes |
| Stupka N | Tarnopolsky MA | 2001 | Male &<br>Female | 22 ± 2<br>23 ± 2 | NRT | Leg Press: 3x12<br>Leg Ext: 10x10 | 120%<br>Concentric<br>1RM | 3 min | Eccentric | Leg Press and Leg Extension |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Stupka N | Lowther S | 2000 | Male & | 22.75 ± 2.4 | NRT | Leg Press: 3x12 | 120% | 1 min/set | Eccentric | Leg Press and Leg Extension |
| | | | Female | 22.1 ± 2 | | Leg Ext: 9x12 | Concentric | every 3rd set | | |
| | | | | | | | 1RM | 3 min | | |
| Fragala MS | Kraemer WJ | 2010 | Male & | 24.63 ± 5.07 | Male vs Female | 6x5 | 90% 1RM | 3 min | Lower Body | Squat |
| | | | Female | 22.13 ± 3.09 | Exercise vs Control | | | | | |
| Fragala MS | Kraemer WJ | 2011 | Male & | 24.63 ± 5.07 | Male vs Female | 6x5 | 90% 1RM | 3 min | Lower Body | Squat |
| | | | Female | 22.13 ± 3.09 | Exercise vs Control | | | | | |
| MacIntyre DL | Reid DW | 1996 | Female | 20-33 | Exercise Leg vs | 30x10 | Maximal | 20 sec | Eccentric | Leg Extensions |
| | | | | | Control Leg | | 30°/sec | | | |
| Paulsen G | Cameri R | 2010 | Male | 28 ± 4 | Exercise Leg vs | 30x10 | Maximal | 30 sec | Eccentric | Leg Extensions |
| | | | | | Control Leg | | 30°/sec | | | |
| Raastad T | Risoy BA | 2003 | Male | 27.2 ± 2.7 | Exercise Leg vs | Leg Press: 5x3 | 100% 3RM | 3 min | Lower Body | Leg Press and Leg Extension |
| | | | | | Control Leg | Leg Ext: 5x6 | 100% 6RM | | | |
| Mahoney DJ | Safdar A | 2008 | Male | 23.4 ± 3.7 | NRT | 30x10 | Maximal | 1 min | Eccentric | Leg Extensions |
| | | | | | | | 120°/sec | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Young Chronic** | | | | | | | | | |
| Miles MP | Kraemer WJ | 2002 | Female | 18-30 | Strength & Power TB | No Acute Testing | | | | |
| | | | | | Strength & Power UB | | | | | |
| | | | | | Strength & Hyper TB | | | | | |
| | | | | | Stregth & Hyper UB | | | | | |
| | | | | | Non-exercise Con | | | | | |
| Miles MP | Kraemer WJ | 2003 | Female | 18-30 | TB vs UB | 6x10 | 75% 1RM | 2 min | ARET | Squat |
| | | | | | | | | | | |
| | **Supplementation** | | | | | | | | | |
| Carlson LA | Headley S | 2008 | Male | 21.1 ± 1.4 | CHO vs Placebo | 4x10 | 55/65% 1RM | 1 min | Total Body | Leg Press, Lat puldown, Bench Press and Leg |
| | | | | 21.0 ± 2.2 | | | | | | Curls |
| Nieman DC | Davis JM | 2004 | Male | 19- 27 | CHO vs Placebo | 4x10 | 1:40% 1RM | 2 min | Total Body | Flat Bench Press, Incline Bench Press, |
| | | | | | | | 3:60% 1RM | for UB | | Military Press, Upright Row, Bentover Bar |
| | | | | | | | | 3 min | | Row, French Curl, Biceps Curl, Back Squat, |
| | | | | | | | | for LB | | Front Squat and Deadlift |
| Koch AJ | Potteiger JA | 2001 | Male | 25 ± 2.8 | CHO vs Placebo | 5x10 | 65% 1RM | | Lower Body | Squat |
| | | | | | | 3x10 | 85% 1RM | | | Half Squat |
| Hulmi JJ | Myllymaki T | 2010 | Male | 23.9 ± 2.0 | Whey vs Placebo | 5x1 | 1RM | 3 min | Lower Body | Squat |
| | | | | | Study I: Young, TR | 3x10 | 10RM | 3 min | | Squat |
| | | | | | | 4x10 | 1RM | 2 min | | Leg Press |
| Hulmi JJ | Myllymaki T | 2010 | Male | Y: 22-26 | Whey vs Placebo | 5x10 | 10RM | 2 min | | Leg Press |
| | | | | O: 57-65 | Study II: NRT | | | | | |
| | | | | | Young and Old | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Young Chronic** | | | | | | | | | |
| Machado M | Koch AJ | 2010 | Male | 18.4 ± 0.8 | Caffeine vs Placebo | 3x10 | 10RM | 2 min | Total Body | Bench Press, Pullover, Biceps Curls, Triceps Extensions, Leg Extensions and Prone Leg Curls |
| Kawada S | Kobayashi K | 2010 | Male | 22.8 ± 4.0 | Cystine/Theanine vs Placebo | 4-6 sets, 6-10 reps | >80% 1RM | NR | NR | 3-5 free weight exercises<br>1st week: 3 sessions<br>2nd week: 6 sessions |
| | **Elderly Acute** | | | | | | | | | |
| Przybyla B | Gurely C | 2006 | Male | Y: 31.9±7.1<br>O: 71.4±4.6 | Young vs Old<br>NRT Males | 3x8<br>3rd set to failure | 80% 1RM | 2 min/set<br>5 min/exe | Lower Body | Leg Press, Leg Curl and Leg Extension |
| Neves SC | Lima RM | 2009 | Female | 67.5 ± 3.9 | S50%<br>S80%<br>SC: Control | 2x13<br>2x8<br>no exercise | 50% 1RM<br>80% 1RM | 80 sec | Total Body | Bench Press, Lat Pulldown, Seated Row, Leg Extension, Leg Curl and Leg Press (equated work volume) |
| | **Elderly Chronic** | | | | | | | | | |
| Rall LC | Roubenoff R | 1996 | Male & Female | Y: 25.8 ± 2.5<br>O: 70.3 ± 5.0<br>Con: 68.8±2.9 | Young<br>Old RT<br>Old Control | 3x8 | 80% 1RM | 2 min | Total Body | Abdominal, Back Extension, Chest Press, Leg Press and Leg Extension |
| Bermon S | Philip P | 1999 | Male & Female | 67-80 | Old RT<br>Old Control<br>Young Control | 3x8 | 80% 1RM | 2 min | Total Body | Leg Press, Knee Extension and Seated Chest Press |
| Bermon S | Philip P | 2001 | Male & Female | Y: 29.4 ± 0.4<br>O: 70.1 ± 1.0 | Old vs Young | 2x12<br>4x5 | 12RM<br>5RM | 2 min | Total Body | Leg Press, Chest Press and Leg Extension |
| McFarlin BK | Flynn MG | 2005 | Female | RT:71.8 ± 6.5<br>C:73.1 ± 6.0 | RT vs Con | 2x8 @ 80% 1RM<br>3rd set to failure | 80% 1RM | NR | Total Body | Seated Leg Press, Knee Extension, Knee Flexion, Seated Chest Press, Chest Flye, Lat Pulldown, Shoulder Press, Seated Rows, hip abduction and hip adduction |
| Raso V | Benard G | 2007 | Female | 60-77 | Con vs RT | No Acute Testing | | | | |
| Flynn MG | Fahlman M | 1999 | Female | RT:72.6 ± 3.5<br>C: 72.9 ± 4.9 | RT vs Con | 2x8<br>3rd set to failure<br>2x8 | 80% 1RM<br>8RM | NR | Lower Body | Leg extension, Leg Curl, Plantar Flexion and Dorsiflexion<br>Leg Abduction, Leg Adduction, Hip Extension and Hip Flexion |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bobeuf F | Labonte M | 2009 | Male & Female | 61-73 | Pre vs Post | 3x8 | 80% 1RM | NR | Total Body | 8 exercises for upper and lower body |
| Flynn MG | McFarlin BK | 2003 | Female | 65-85 | HRT | 2x8 | 80% 1RM | 1.5 min | Total Body | Seated Leg Press, Knee Extension, Knee Flexion, Upright Chest Press, Chest "flys", Lat Pulldown, Shoulder Press, Upright Row, Hip Abduction and Hip Adduction |
| | | | | | NHR | 3rd set to failure | | | | |
| | | | | | MIB | | | | | |
| | | | | | Control | | | | | |
| McFarlin BK | Flynn MG | 2004 | Female | TR: 67 ± 5 | TR vs NRT | NR | NR | NR | Total Body | Seated Chest Press, Chest Fly, lat Pulldown, Hip Abduction, Hip Adduction, Seated Rw, Knee Extension, Knee Flexion and Seated Leg Press |
| | | | | NRT: 69 ± 5 | Hi TLR4 vs Lo TLR4 | | | | | |

Abbreviations: TR = Trained, NRT = Not Resistance Trained, RT = Resistance Training, Con = Control, TB = Total Body, UB = Upper Body, LB = Lower Body, CK = Creatine Kinase, W:R = Work:Rest Ratio, TLR4 = Toll-Like Receptor 4, HRT = Hormone Replacement Therapy, NHR: No Hormone Replacement Therapy, MIB = No hormones but on medications that could influence bone, CHO = Carbohydrate, Hi = High, Lo = Low, ARET = Acute Resistance Exercise Test, NR = Not Recorded (to indicate that the descriptor was not mentioned in the text)

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# *Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise?*

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## ABSTRACT

*Exercise-induced muscle damage is an important topic in exercise physiology. However, several aspects of our understanding of how muscles respond to highly stressful exercise remain unclear. In the first section of this review we address the evidence that exercise can cause muscle damage and inflammation in otherwise healthy human skeletal muscles. We approach this concept by comparing changes in muscle function (i.e., the force-generating capacity) with the degree of leucocyte accumulation in muscle following exercise. In the second section, we explore the cytokine response to 'muscle-damaging exercise', primarily eccentric exercise. We review the evidence for the notion that the degree of muscle damage is related to the magnitude of the cytokine response. In the third and final section, we look at the satellite cell response to a single bout of eccentric exercise, as well as the role of the cyclooxygenase enzymes (COX1 and 2). In summary, we propose that muscle damage as evaluated by changes in muscle function is related to leucocyte accumulation in the exercised muscles. 'Extreme' exercise protocols, encompassing unaccustomed maximal eccentric exercise across a large range of motion, generally inflict severe muscle damage, inflammation and prolonged recovery (> 1 week). By contrast, exercise resembling regular athletic training (resistance exercise and downhill running) typically causes mild muscle damage (myofibrillar disruptions) and full recovery normally occurs within a few days. Large variation in individual responses to a given exercise should, however, be expected.*

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*The link between cytokine and satellite cell responses and exercise-induced muscle damage is not so clear. The systemic cytokine response may be linked more closely to the metabolic demands of exercise rather than muscle damage. With the exception of IL-6, the sources of systemic cytokines following exercise remain unclear. The satellite cell response to severe muscle damage is related to regeneration, whereas the biological significance of satellite cell proliferation after mild damage or non-damaging exercise remains uncertain. The COX enzymes regulate satellite cell activity, as demonstrated in animal models; however, the roles of the COX enzymes in human skeletal muscle need further investigation. We suggest using the term 'muscle damage' with care. Comparisons between studies and individuals must consider changes in and recovery of muscle force-generating capacity.*

**Keywords:** Skeletal muscle, lengthening contractions, ultrastructural disruptions, necrosis, myokines, cyclooxygenase (COX1, COX2), non-steroidal anti-inflammatory drugs (NSAIDs)

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## INTRODUCTION

Exercise-induced muscle damage has been a popular topic in exercise science for many years. In particular, the term 'delayed onset muscle soreness' (DOMS) has been a recurring theme since the early work of Hough (127) (e.g., (3,12,15,66,68,123,210)). Human studies investigating the physiological responses to eccentric exercise (i.e., lengthening muscle actions) were conducted as early as the end of the 19th century and the beginning of the 20th century (references in (16)). More controlled experiments on eccentric exercise (e.g., 'backwards' cycling) were conducted by Abbott *et al.* (1,2) and Asmussen (16) in the 1950's. Extending the pioneering work of Hill (21,122), Katz (144) carefully explored the basic physiology of muscle lengthening. Concerning the potential for eccentric muscle actions to cause muscle damage, Katz wrote: 'Rapid stretching of an active muscle, beyond its optimum length, is apt to break or weaken permanently parts of the contractile substance' (p. 64, (144)). Important research into muscle physiology and sarcomere and myofibrillar mechanics continued through the work of Gordon, Huxley and Julian (108,109,132,133), and later Julian and Morgan (140,141). Morgan (205) put forward the 'popping sarcomere hypothesis' to explain the observations of disruptions to the myofibrillar machinery of skeletal muscle fibres after eccentric actions (reviews: (5,206,207,245)). Many other investigators have contributed to the paradigm of exercise-induced muscle damage, but some worth mentioning for their significant contributions include Salminen and Vihko (258-260,306,307), Armstrong *et al.* (11,12,14,315), Faulkner *et al.* (37,88,89), McArdle and Jackson (134,186-188), and Fridén and Lieber (157-159). These investigators mainly worked with muscle preparations (*in vitro* studies) and animals models (e.g., rodents and rabbits). Work on humans at the myocellular level was initiated by Fridén *et al.* (96,97,101-103) and Newham et al (137,210,213) in the 1980's. These authors investigated the effects of downstairs running, eccentric (backwards) cycling, stepping exercise (one eccentric working leg), isolated eccentric work for the elbow flexors and backward walking on a treadmill (targeting the calf muscles). They reported disruptions at the ultrastructural level and more gross cellular damage, comprising leucocyte accumulation and regeneration. Thereby, they confirmed many of the findings from previous studies on muscle preparation and animals. During the last few decades researchers have, with ever more advanced techniques, attempted to understand the aetiology of exercise-induced muscle damage. This work has focused on mechanical tearing, metabolic stress, the local and systemic inflammatory response, as well as the recovery process involving satellite cell activation and muscle regeneration (64,86,170,232,247,275,292,296,298).

In the first part we review the evidence for muscle damage and local inflammation (i.e., accumulation of leucocytes in the muscle tissue) following various types of eccentric exercise. In general, the assessment of muscle damage requires reliable and valid markers. This is indeed a major challenge, because in human research there are currently no markers that are considered the 'gold standard'. Histological observations (light or electron microscopy) and changes in muscle function (force-generating capacity) seem to be the most valid markers of muscle damage, although both have some limitations. Histological examination of muscle biopsy samples (typically, sections cut from a 5–20 mg muscle sample) can

identify abnormalities such as myofibrillar disruptions and the presence of inflammatory cells. However, the question remains as to whether this small piece is representative of the whole muscle (12,26,316). Changes in force-generating capacity are an indirect measure of muscle damage. Nevertheless, with appropriate testing, changes in muscle function give a good indication of the status of the whole muscle (316). Muscle damage is therefore best assessed by measuring changes in force-generating capacity and performing histological observations. Other proxy markers of muscle damage, such as DOMS and circulating creatine kinase (CK), are generally not considered sufficiently valid (66,280,316), but do provide some additional or complementary information. In this section, we focus on the relationships between changes in muscle function and histological evidence for myofibrillar disruptions, inflammation and myofibre necrosis in human studies.

In the second part of this review, we look at the exercise-induced cytokine response. The systemic inflammatory response comprises a leucocytosis and an acute-phase response (86). Cytokines (e.g., IL-1 and IL-6) are part of the acute-phase response, and strenuous exercise generally seems to increase the circulating levels of a number of different cytokines. Cytokines are traditionally regarded as messenger molecules associated with leucocytes and inflammatory and immunological reactions. However, more recent research demonstrates that these cytokines are not only produced by leucocytes, but also by myofibres and peritendinous tissue (151,238). This had led to the term 'myokines', which refers to muscle-derived cytokines and chemokines. Uncertainty persists concerning the physiological actions and the precise source of production for cytokines found in the circulation during and after exercise. We have reviewed the literature for studies that have investigated the cytokine/myokine response in relation to eccentric exercise.

In the final part of this review, we describe the satellite cell response to single bouts of eccentric exercise. Satellite cells are undeniably required for regeneration of gross muscle damage where segments of myofibres are lost, for example, after a strain injury with torn myofibres (136). Surprisingly, satellite cells seem responsive to a variety of exercise protocols, both muscle damaging and non-damaging exercise. We review the satellite cell response to eccentric exercise. Non-steroidal inflammatory drugs that inhibit the activity of cyclooxygenase enzymes in skeletal muscle can also inhibit the satellite cell response. We discuss the evidence for this effect in humans.

## 1. DEFINING EXERCISE-INDUCED MUSCLE DAMAGE

Regular exercise generally makes our muscles stronger and/or more resistant to fatigue. However, during intense exercise our muscles fatigue and weaken temporarily. If the exercise is unaccustomed and/or very vigorous, we may even damage the working muscles, and it may take days for the muscles to recover. This type of muscle damage has been named 'exercise-induced muscle damage' (143,226). Various types of eccentric exercise (i.e., lengthening muscle actions) have been used to induce muscle damage experimentally (49,64,86,282). Exercise-induced muscle damage also occurs after long distance running (121,272,273,310) and to some degree after traditional resistance exercise (i.e.,

lifting weights; (94,251,281,308))—especially if the exercise is unaccustomed, very intense and/or too frequent (69,148,194). However, exercise-induced muscle damage does not have any established definition; it is merely characterised by a set of signs and symptoms (49,66,159,282). DOMS is the most common symptom of exercise-induced muscle damage, whereas histological evidence of disruptions of the myofibrillar structure and, especially, myofibre necrosis and inflammation are the ultimate signs of muscle damage (if we disregard methodological uncertainties; discussed below).

Necrotic cells go through several stages (304), but necrotic segments of myofibres may appear as swollen and rounded (on cross-sections). Immunohistochemical staining for cytoskeletal (e.g., desmin and dystrophin) and myofibrillar proteins (myosin) is diminished (or absent), which indicates degradation of these proteins (71,158,166). Disruption of the sarcolemmal membrane causes influx of extracellular proteins, such as fibronectin and albumin (72,283). Segmental myofibre necrosis[1] is manifested by inflammatory cells (particularly macrophages) that have invaded these myofibres and accumulation of myoblasts that originate primarily from satellite cells ((136,154,158,230,257,284); see also Figure 1). Because necrotic cells will attract immune-competent cells through receptors, such as toll-like receptors (50,78,184), accumulation of inflammatory cells within myofibres is a strong sign of segmental myofibre degradation and necrosis.

Some studies have reported signs of necrosis in voluntarily exercised muscles of seemingly healthy subjects (72,74,121,135,137,226,230,310). In rare cases, experimental subjects and patients have been diagnosed with rhabdomyolysis after exercise. Rhabdomyolysis is broadly defined by severe muscle pain/tenderness, swelling and muscle weakness, elevated blood activity of muscle proteins such as CK (> 10,000 IU/L), and dark urine, indicating myoglobinuria (65,146,264,271). Serious cases of rhabdomyolysis (including myoglobinuria) have been observed after various types of exercise, but rhabdomyolysis has been most frequently reported after extreme military training (135,262).

In experiments involving eccentric exercise, relatively little evidence of severe myofibre necrosis exists (Table 1; see addendum). A more common finding is accumulation of inflammatory cells, primarily monocytes/macrophages, in the endomysium and especially in the perimysium ((92,119,129,229,283); see also Table 1). However, in response to 'extreme' exercise massive leucocyte infiltration and cellular accumulation inside myofibres can be demonstrated (60,224,230,257). These observations suggest that the leucocytes are recruited to remove cellular debris and prepare for regeneration of necrotic segments of myofibres (56,85). Thus, necrotic myofibre segments seem to induce a strong chemotactic signal to recruit leucocytes; however, exercised muscle tissue may also summon leucocytes in the absence if necrosis (Figure 1—moderate damage versus severe damage).

Leucocytes may start to infiltrate the muscle tissue immediately after exercise, but are typically detected in the extracellular space 24–48 hours after exercise (25,92,119,229). They infiltrate the intracellular space ~4-7 days after exercise—if some myofibres become necrotic (60,229,230). Leucocyte accumulation therefore appears to be a gradual process regulated by the extent of damage. In response to severe damage, leucocyte accumulation peaks in time with the pres-

[1] 'Segmental myofibre necrosis' implies that there are segments or parts of the fibre that are necrotic, not necessarily the whole myofibre (99,136,158).

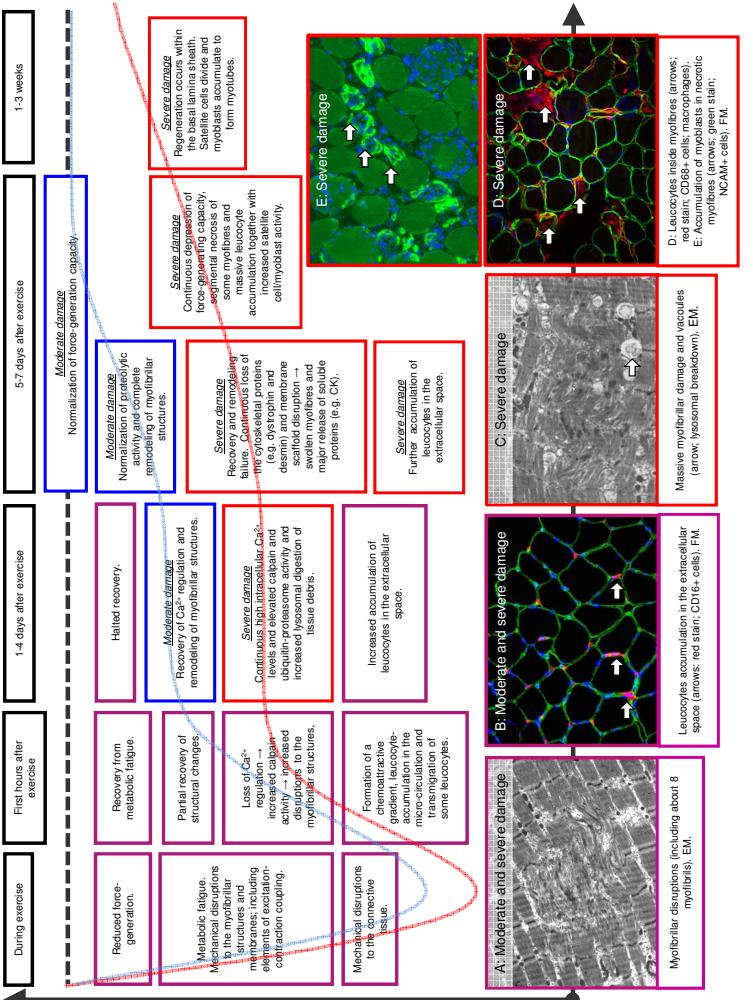


**Figure 1.** A model for central events in the recovery of the force-generating capacity after moderate (blue line) and severe (red line) exercise-induced muscle damage. Both moderate and severe exercise-induced muscle damage are accompanied by changes in the myofibrillar structure, together with an inflammatory response (purple boxes and images). The difference between moderate and severe damage becomes more prominent over time as the damage progresses in muscles with severe exercise-induced muscle damage. While the moderately damaged muscles enter the final phase of recovery (blue line and boxes), inflammation increases within the severely damaged muscles, probably in response to the presence of segments of necrotic myofibres (red line, boxes and images). Necrotic myofibres must be regenerated (by myoblasts), a process that may take several weeks and is manifested by a long-lasting reduction in the force-generating capacity. Note: large variation in individual responses has to be expected.
EM: Electron Microscopy (longitudinal sections).
FM: Fluorescence Microscope (cross-sections): Image B and D: Red stain: leucocytes; green stain: basal lamina (laminin); blue stain: nuclei (DAPI). Image E: Green stain: satellite cells/myoblasts (NCAM); blue stain: nuclei (DAPI).

ence of necrotic myofibres (60,137,229,230,257). In relation to muscle function, McCully and Faulkner (192) have reported a high correlation between gross muscle damage (apparent cellular infiltrate and myofibre necrosis) and reduction in the force-generating capacity.

Another histological characteristic of muscles following eccentric exercise is myofibrillar disruption. Fridén *et al.* (102,103) provided the first evidence of morphological changes in the 'contractile machinery' after eccentric exercise in humans (running down stairs and eccentric cycling). No gross damage or leucocytes were seen by light microscopy, but electron microscopy revealed subcellular disorganisation of the myofibrillar structure, especially that of the Z-bands (Z-band streaming and smearing (98)). Several subsequent studies have verified these findings (105,106,126,182,213,250), with (154,226,229) and without (71,223,324) simultaneous signs of inflammation and necrosis. Myofibrillar disruption is strongly associated with structural changes to the t-tubule system and the sarcolemma (245,289,315). Note that myofibrillar disruptions have been reported immediately after exercise, as well as one week after exercise, whereas the greatest disturbances are typically found after 1–4 days (103,121,213,250,325).

Whether myofibrillar disruptions indicate damage or remodelling and incorporation of new sarcomeres is debatable (71,97,126,154,213,324,325). Sarcomere disruption and myofibrillar disorganisation are linked to reduced force-generating capacity (106,154,162,176,250), and sarcomeres are disrupted following single eccentric muscle actions (39). These findings suggest that the myofibrillar disruption that occurs during or shortly after exercise represents damage. The changes in myofibrillar structures observed some days into recovery may, however, more appropriately be termed 'remodelling' (325). Minor myofibrillar disruptions can indeed occur without significant changes in the force-generating capacity (105,245). These findings raise the question of whether initial damage due to mechanical forces and activation of $Ca^{2+}$-dependent proteinases (e.g., calpain (161,250)) are required for remodelling. Nevertheless, disruption of intracellular $Ca^{2+}$ regulation probably links myofibrillar damage with necrosis. Hence, if $Ca^{2+}$ homeostasis is not re-established within a few days after exercise, the damage to the myofibrillar structure and the cytoskeleton may become irreparable, and segments of the myofibre become necrotic (Figure 1 and (5,98,107,245)).

Limited human research has examined changes to the extracellular matrix in response to eccentric exercise, but intracellular and extracellular events appear to occur simultaneously (40,73,173,250). Stauber *et al.* (283) reported that the extracellular matrix was separated from the myofibres after exercise. More recent studies have observed increased expression of tenascin C and PIIINP[2], which indicate remodelling of the extracellular matrix (73,250). Damage to the extracellular matrix may increase permeability of the sarcolemmal membrane, as indicated by increased efflux of CK and myoglobin, and influx of albumin and tetranectin (280,283,319).

Reports from various animal models show that exercise-induced muscle damage is linked to inflammation (88,200,274,275,296). Humans, however, can experience symptoms of exercise-induced muscle damage, such as DOMS and increased passive tension, without presenting classical signs of inflammation (i.e., leucocyte infiltration) in the muscle tissue (71,180,229,230,251). Based on

[2] N-terminal propeptide of Type III procollagen

well-controlled experiments, some researchers claim that voluntary eccentric exercise does not cause gross muscle damage (necrosis) or cellular infiltration in the exercised muscles (71,178,179,326). This concept challenges the validity of a series of human studies from the late 1980's and the 1990's (e.g., (60,92,119,137,226,257)). The main criticism of these classical 'muscle damage' studies is the risk of bias resulting from repeated biopsies from the same muscle (179). Malm *et al.* (180) reported no differences in leucocyte infiltration in muscle samples obtained from eccentrically-exercised muscle and resting muscle up to one week after exercise. Based on these observations they argued that the biopsy procedure itself, rather than exercise, increased inflammation in muscle. Electrical stimulation of human skeletal muscles causes significantly more damage than voluntary exercise (71,171). For these reasons, some researchers (71,179) also question the relevance of data from animal studies using rather 'non-physiological' muscle actions to inflict massive/gross muscle damage.

These problems arising from repeated biopsies, together with the results from electrical stimulation of human muscle, challenge the paradigm of exercise-induced muscle damage. Historically, this paradigm has been based on data from animal studies, and has muscle damage and inflammation as fundamental events (see introduction and (10,12,14,158,200)). Nevertheless, more recent studies that have collected samples from both exercised and resting muscle provide compelling evidence that eccentric exercise can lead to both accumulation of leucocytes and myofibre necrosis (154,229,230). Importantly, large variations in the individual responses to eccentric exercise were evident, and gross muscle damage did not occur in all individuals (229,230). The literature on exercise-induced muscle damage is therefore full of contradictory reports (e.g., see Table 1). Some of this variability is simply due to non-specific nomenclature used to identify and describe 'muscle damage' and inflammation.

Other confounding factors in this debate on exercise-induced muscle damage include variation in exercise protocols and inconsistent measurements of muscle function (i.e., the force-generating capacity) to assess muscle damage. Malm *et al.* (180,181) and Yu *et al.* (326) reported no signs of necrosis of inflammation after submaximal, eccentrically-biased exercise (i.e., backwards cycling, downhill running and running down stairs). Although they observed severe DOMS after these exercise protocols, changes in other markers of muscle damage were rather trivial compared with observations reported by others (e.g., (60,137,283)). Specifically, Malm *et al.* (181) reported that isometric strength (torque) decreased by only 15% in the first 24 hours after exercise. Isometric strength then returned to normal in the following 24 hours. Serum CK activity increased, but only about six times above pre-exercise values. By contrast, in other studies in which high-force eccentric exercise across large ranges of motion was used, isometric/concentric force-generating capacity decreased by about 50% after exercise. Recovery of muscle function in these other studies was also significantly slower, and plasma/serum CK activity reached more than 100 times pre-exercise values (see Table 1). These contrasting findings raise the question of whether it is appropriate to compare cellular responses from studies using quite different exercise protocols.

## 2. CHANGES IN MUSCLE FUNCTION REFLECT THE EXTENT OF MUSCLE DAMAGE

To assess muscle damage directly, histological analysis of muscle tissue is required (88). However, collecting tissue samples from humans can be unpleasant for the subjects and demanding on resources. Histological analysis of human muscle tissue can also be unreliable (26,115,156,316). For these reasons, proxy markers of muscle damage such as DOMS, range of motion, swelling, and serum CK activity are often used. But these markers do not always accurately reflect the extent of muscle damage, and do not always correlate with each other (100,128,210,218,219,255,280,316). By contrast, muscle function measured as force-generating capacity (e.g., maximal concentric or isometric strength) is relatively easy to measure, and is generally considered to be a reliable and valid marker for the degree of muscle damage (49,64,88,98,245).

Studies that have both obtained biopsies and measured changes in muscle function are summarised in Table 1 (see addendum). These studies point to an association between changes in force-generating capacity and histological observations such as myofibrillar disruptions, signs of necrosis and leucocyte accumulation. In those studies that report a minor reduction in the force-generating capacity (< 20% of pre-exercise values; Table 1A), few or no morphological/histological abnormalities are found. By contrast, those studies that report a large reduction in muscle function (> 50%; Table 1C) also report inflammation (leucocyte accumulation) and/or segmental myofibre degradation/necrosis. Eccentrically-biased exercise (e.g., downhill running) generally causes smaller changes in muscle function compared with isolated, eccentric muscle actions that involve a large range of motion (Table 1 and 2; see addendum).

Eccentrically-biased exercise and isolated eccentric exercise differ with regard to the mechanical characteristics of muscle-damaging exercise. The most critical factors for muscle damage are high force and large strain (i.e., muscle lengthening beyond the optimum length for force-generation (29,39,112,160,176,212,290,291)). Eccentric exercise that involves a large range of motion and high force-generation is very likely to cause substantial structural (myofibrillar) disruptions, and in turn, reduced muscle function. Other factors, such as joint angle velocity (51,52,192,227,312) and number of repetitions (41,162,219,221,291) may modify the degree of muscle damage. However, these factors seem secondary to the work (strain × force [J]) done to lengthen/stretch the muscles (38,88,167,321).

### *2.1 Mild exercised-induced muscle damage*

Among those studies that report a relatively small reduction in the force-generating capacity (i.e., < 20%) and rapid recovery (33,71,90,181), only one study reported accumulation of leucocytes in the tissue ((71); Table 1A; see addendum). None of these studies observed any signs of necrosis, and plasma/serum CK activity did not surpass ~1,000 IU/L.

In the study by Bourgeois *et al.* (33), subjects exercised with a load equal to 80–85% of 1RM (i.e., traditional resistance exercise), whereas in the study of Feasson *et al.* (90) and Malm *et al.* (181), subjects ran downhill (on a treadmill). In the study of Crameri *et al.* (71), subjects completed a bout of unilateral, single joint (knee), maximal eccentric actions. Compared with studies that have used

similar protocols (24,25,201), the reduction of muscle function was surprisingly low in the study by Crameri *et al.* (71). Nevertheless, Crameri *et al.* (71) did observe both myofibrillar disruption and accumulation of macrophages (CD68+ cells). Interestingly, the contralateral leg completed the same number of eccentric actions, but was stimulated electrically (not shown in Table 1A). Compared with the voluntarily exercised muscle, the electrically-stimulated muscle was clearly damaged. Necrosis was indicated by accumulation of intracellular leucocytes (CD68+ cells) and myofibres that did not express desmin or dystrophin. The authors suggested that these variations may be due to differences in the pattern of muscle activation between voluntary and electrically-stimulated muscle actions (71).

Although increased numbers of leucocytes are not found (by immunohistochemistry) within muscle fibres after these exercise protocols, it does not exclude the possibility that there are interactions between the exercised myofibres and the immune system. Circulating leucocytes may indeed accumulate in the micro-vessels of the exercised muscles (229). Raastad *et al.* (251) used radionuclide imaging (which involves radiolabelling of autologous leucocytes—primarily neutrophils—and scintigraphy). They documented an early accumulation of leucocytes in muscle 1–24 hours after resistance exercise. The force-generating capacity decreased by 16% shortly after exercise and returned to normal between 28 and 47 hours after exercise, indicating only mild muscle damage. Based on this evidence, we propose that the immune system immediately responds to muscle damage resulting from the stress of high-force exercise. However, when muscle damage is mild, blood borne leucocytes do not leave the circulation in significant numbers (229). Although there is no accumulation of leucocytes in the muscle tissue after exercise, resident stromal cells, such as macrophages, may become activated, and thereby play a role in the recovery and adaptation to exercise (246). Evidence to support this notion awaits further experiments on human subjects.

Figure 2 (upper curve) demonstrates typical changes in the force-generating capacity of trained subjects that have performed a bout of a heavy traditional resistance exercise (3–8 repetition maximum) or subjects that are 'low responders' to eccentric exercise. Typically the reduction in the force-generating capacity after exercise is less than 20% and recovery is completed within 48 hours. We suggest using the term 'mild exercise-induced muscle damage' if the reduction in the force-generating capacity is less than 20% and/or recovery is completed within 48 hours after exercise.

### *2.2 Moderate exercise-induced muscle damage*

Among those studies reporting a moderate reduction in the force-generating capacity (20–50%), only one study reported signs of necrosis (229), but five (24,25,129,229,287) of eight studies found accumulation of leucocytes in the exercised muscles (Table 1B; see addendum). Two of the three studies that did not report increased numbers of leucocytes also reported the smallest reduction in the force-generating capacity.

Paulsen *et al.* (229) reported very high serum CK activity in some subjects, and intracellular accumulation of leucocytes in four of eleven subjects. Although this histological observation was very infrequent (~1% of the counted fibres), it indicates that some degree of segmental necrosis did occur. Of note, tissue sam-

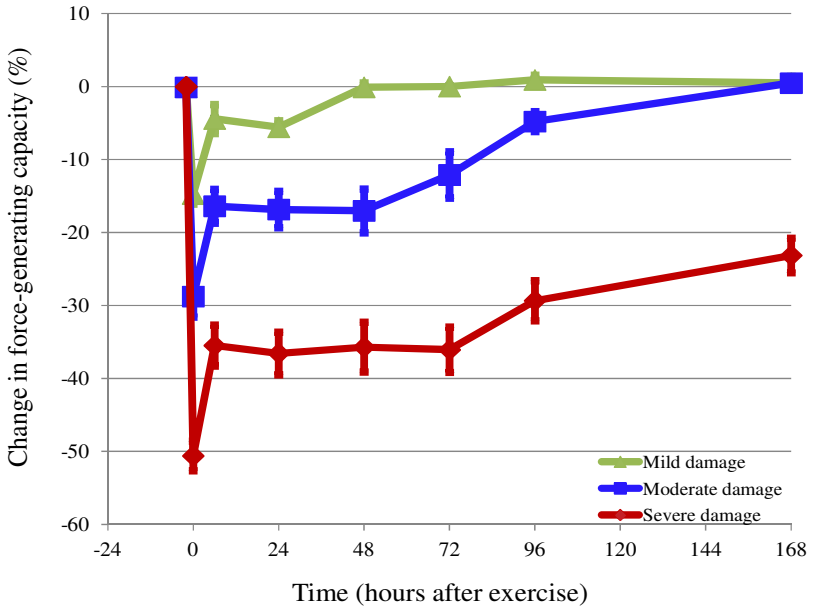


**Figure 2.** Recovery of the force-generating capacity of subjects that have performed heavy resistance exercise or maximal eccentric exercise (subjects from several studies are combined: (230,248-251), as well as unpublished data). The subjects are organized so that those who recover their force-generating capacity within 48 hours are represented as mild exercise-induced muscle damage (34 subjects). Those who recover between 2 and 7 days are presented as moderate exercise-induced muscle damage (17 subjects). Finally, subjects that do not recover within one week are presented as severe exercise-induced muscle damage (21 subjects). See further comments in the text. All data are gathered at the Norwegian School of Sport Sciences by Professor Truls Raastad. Data are presented as means ± standard error of the mean.

ples that exhibited intracellular leucocyte accumulation were typically collected from 'high responder' subjects who showed a substantial decline in muscle function (50–73% lower immediately after exercise, and 17–42% lower 1 week later), indicating they had suffered severe muscle damage.

Beaton *et al.* (25) did not observe necrotic myofibres, but did observe reduced immunohistochemical staining of both desmin and dystrophin, as well as increased numbers of macrophages between myofibres 4 and 24 hours after exercise. It is likely that necrosis was not evident at these early time points. The authors suggested that these findings were related to increased activity of the $Ca^{2+}$-dependent calpain system, as mentioned in Figure 1.

Figure 2 (middle curve) demonstrates the typical recovery of the force-generating capacity in subjects that have performed unaccustomed eccentric exercise and some subjects that have performed heavy traditional resistance exercise. We suggest using the term 'moderate exercise-induced muscle damage' if the largest reduction in the force-generating capacity is between 20–50%, and/or recovery is completed between 2 and 7 days after exercise.

### *2.2 Severe exercised-induced muscle damage*

All studies reporting large loss of force-generating capacity (≥ 50% reduction) and long-lasting recovery (> 1 week) also reported accumulation of leucocytes in the exercised muscle tissue, and all but one found signs of segmental myofibre necrosis (Table 1C; see addendum). All studies that assessed serum/plasma CK activity also observed high CK activity (i.e., subjects with values > 10,000 IU/L). Although changes in CK activity have been reported to correlate poorly with other damage markers in some studies (100,182,280), very high CK activity does appear to accompany severe reductions in force-generating capacity (218,229) and evidence of severe muscle damage (137). Changes in CK activity in these cases follow a similar time course to histological observations of severe muscle damage (about 4 days after exercise; (60,137,230)). Thus, CK activity measurements may be used to separate subjects with mild muscle damage (< 1,000 IU/L) and severe muscle damage (> 10,000 IU/L; (66)). Considering the combination of reduced muscle function and increased CK activity with severe soreness and massive muscle swelling after exercise, many of the subjects in these studies would probably be diagnosed with rhabdomyolysis if they had attended an emergency room.

Muscle samples from subjects with very severe exercise-induced muscle damage may display a long-lasting regeneration process between 1 and 3 weeks after exercise (137,154,230,257). Increased numbers of macrophages (or other CD68 positive stromal cells) are present even after 3 weeks, but these cells are primarily located in the extracellular matrix around regenerated myofibres (230). The regeneration process therefore exceeds 3 weeks, and corresponds with incomplete recovery of muscle function at this time point. Sayers and Clarkson (263) have reported recovery times of 33–47 days for subjects with an immediate reduction in muscle function of > 70% after eccentric exercise with the elbow flexors. Foley *et al.* (93) observed that muscle volume of the elbow flexors decreased by about 10% for a period from 2–8 weeks after eccentric exercise. These two studies did not obtain muscle biopsies; but it seems very likely that necrosis of myofibres delayed muscle recovery and caused significant atrophy of the exercised muscles. The cross-sectional area of regenerating fibres is typically much smaller compared with undamaged adjacent myofibres 2–3 weeks after exercise (137,230,281).

Myofibrillar disruption was evident in several of the studies in Tables 1 (A–C) that also reported minor changes in muscle function. It appears that myofibrillar disruption is not directly—or at least not linearly—related to the accumulation of leucocytes. Some studies indicate that myofibrillar disruption can occur in the absence of leucocyte accumulation (90,103,326). Other studies, however, do show a significant correlation between myofibrillar disruption and leucocyte accumulation (92,229). Therefore, we suggest that when intracellular damage exceeds a certain level, leucocytes accumulate in the damaged tissue. The factors that regulate this degree of damage could include degradation and damage to the sarcolemma. As myofibrillar disruption worsens during the first days after exercise (probably due to failure to re-establish intracellular $Ca^{2+}$-homeostasis), the cytoskeletal framework eventually collapses, thereby leading to sarcolemmal damage (245). Significant leakage of intracellular proteins to the extracellular milieu stimulates inflammation (22,79,95,295). If the remodelling process fails to

restore the subcellular structure within a few days, further damage occurs and eventually segments of myofibers will become necrotic (Figure 1—severe damage).

Figure 2 (lower curve) displays the typical time course of changes in the force-generating capacity for subjects who do not recover within one week after exercise. Such changes are exclusively observed after unaccustomed eccentric exercise and are typically observed together with high circulating CK activity, histological signs of myofibrillar damage and leucocyte accumulation. We suggest using the term 'severe exercise-induced muscle damage' if the greatest reduction in the force-generating capacity is larger than 50%, and/or recovery is not completed within 7 days after exercise.

### *2.3 Muscle damage is dependent on the choice of exercise protocol*

Unfortunately, relatively few studies investigating inflammation in muscle following exercise have examined changes in muscle function. However, it is possible to assess the degree of damage by considering the exercise protocols that were used in these studies (Table 2; see addendum). For the studies summarised in Tables 1A–C and Tables 2A and B, resistance exercise (with equal concentric and eccentric loads) and eccentrically-biased exercise, such as downhill running (~8°) and running down stairs (33,90,102,181,223,326), generally do not cause severe muscle damage or significant leucocyte accumulation in the exercised muscles. By contrast, tissue accumulation of leucocytes occurs consistently after single joint, maximal eccentric exercise across a large range of motion (24,25,60,137,177,283,320). Stepping exercise (i.e., isolated eccentric work for one leg (213,224)), very steep downhill running (e.g., 16° in Fielding *et al.* (92)) and very long distance running (74,121) appear to induce moderate or severe muscle damage and leucocyte accumulation (at least if the exercise is unaccustomed). Studies involving eccentric cycling have produced mixed findings (103,119,150,180,226).

Although single joint, maximal eccentric exercise across a large range of motion usually inflicts considerable muscle damage, responses to this form of exercise do vary. MacIntyre *et al.* (168,169) used an exercise protocol that consisted of 300 eccentric knee-extensions. Several other more recent studies have adopted this protocol (20,25,71,209,229). MacIntyre *et al.* (168) and Murphy *et al.* (209) observed that force-generating capacity decreased only moderately by 20–25% immediately after exercise. In the latter study, recovery was actually complete between 3 and 24 hours after exercise. By contrast, Paulsen *et al.* (229) and Beaton *et al.* (25) found that force-generating capacity decreased by about 50% shortly after exercise, and was about 30% below baseline at 48 hours after exercise. These measurements of muscle function point to differences between studies at the cellular level, despite the similarities in exercise protocol. The reasons for the rather large differences between studies are difficult to determine, but we provide some plausible explanations below.

### *2.4 Low and high responders make interpretations difficult*

The differences between studies (Table 1 and 2; see addendum) are a challenge for understanding the physiology and/or pathology behind exercise-induced muscle damage. The inter-individual variation in each study is also problematic, especially in stud-

ies with low subject numbers. Large inter-individual variation in response to eccentric exercise is commonly reported (54,58,67,113,131,211,218,229,263,265,268). Individuals are sometimes characterised as 'low', 'medium/moderate' or 'high' responders, based on changes in muscle function (229,265), CK activity (58) and signs of necrosis and regeneration (230). The factors contributing to this interesting phenomenon are uncertain. One possibility is that so-called 'low-responders' show less impairment of muscle function because they have recently (i.e., within some months (220)) performed high-force eccentric work using the same muscle(s) (commonly referred to as the 'repeated-bout effect' (194)). Other contributing factors to the large variation in individual responses may include: age (53,138,155,183), gender (63,263,287), certain genetic factors (62,81,82,130,323) and training status (10,87,105,229,308), as well as flexibility (57,195), eccentric peak and end-range torque (265) and angle of peak torque (cf. the joint angle-torque relationship (207)). In combination with wide variation in exercise protocols and challenges with the biopsy analyses (26), this unpredictable inter-individual variability may explain much of the diverse findings and debate on the aetiology of exercise-induced muscle damage. Future studies on exercise-induced muscle damage should consider these factors. The most important action would probably be to carefully evaluate the number of subjects needed. Probably no more than one third of individuals are likely to be 'high' responders who display a clear local inflammatory reaction—even when using maximal voluntary eccentric exercise across a wide range of motion (72,229,230).

We would like to emphasise that using traditional statistics such as means and standard deviations to describe and illustrate the response to eccentric exercise can mask important and interesting observations, because of large inter-individual variations. We recommend to report individual data and classifying subjects as 'low', 'moderate' and 'high' responders, because this will allow for better presentation and interpretation of the data (229,265). Therefore, studies on exercised-induced muscle damage should be designed (with power estimations) for detecting different responders.

### *2.5 Inconsistencies between animal studies and human studies*

The data summarised in Tables 1 and 2 (see addendum) suggest that a certain level of damage is required to initiate a detectable inflammatory reaction with leucocyte accumulation in exercised muscle tissue. This level of damage seems rather high, because it is mainly unaccustomed maximal eccentric exercise that induces extensive muscle damage and inflammation. By contrast, more applied modes of exercise, such as traditional resistance exercise, cause less or no gross muscle damage and inflammation.

Somewhat surprisingly, the relationship between the degree of muscle damage and inflammation (leucocyte accumulation) in animals seems even less clear. In experiments with rodents, leucocyte accumulation is evident after passive stretches and isometric actions that supposedly do not cause damage (147,164,243), and also after low mechanical impact exercises, such as swimming (208) and level running (252). Hence, there appears to be important differences between humans and (caged) mice with respect to exercise-induced muscle damage. Although the sequence of events is similar in humans and animals after muscle damaging exercise, the time course seems much faster in animals. For exam-

ple, in humans, plasma/serum CK activity and histological abnormalities seem to peak after 4–7 days (67,154,210,229,230), whereas in typical animal models, these events occur 1–3 days after exercise (13,37,100,153,192,193,204). These observations suggest that caution is advised when comparing data on inflammatory reactions to exercise-induced muscle damage between humans and animals.

***Summary***

Exercised-induced muscle damage is characterised by a set of symptoms and signs ('damage markers'). These markers typically include DOMS, increased passive tension, decreased range of motion, increased levels of circulating proteins such as CK and myoglobin, and decreased force-generating capacity (muscular strength), as well as histological evidence of myofibrillar disruption, cellular infiltration, and necrosis. The presence and severity of the different symptoms/signs varies widely between studies. A troubling fact is the relatively weak association between these damage markers (219,229,255,280,316). For example, DOMS does not reflect histological observations of myofibrillar disruptions or accumulation of inflammatory cells. However, as others have suggested (88,316), changes in muscle function appear to be the best marker for the degree of exercise-induced muscle damage. Although the capacity to activate the exercised muscles may change in the recovery phase (27,42,244), reduced force-generating capacity seems to reflect myofibrillar disruption, inflammation and necrosis better than any other markers of muscle damage. Based on our review of the literature, we suggest the following scheme for assessing the extent of muscle damage:

- 'mild exercise-induced muscle damage' corresponds with a decline in force-generating capacity of no more than 20% (during the first 24 hours), and/or full recovery within 48 hours;
- 'moderate exercise-induced muscle damage' corresponds with a 20–50% decline in force-generating capacity, and/or full recovery between 48 hours and 7 days;
- 'severe exercise-induced muscle damage' corresponds with a decline in force-generating capacity of more than 50%, and/or that recovery of force-generating capacity exceeds 1 week.

We further suggest that muscle function should be assessed as concentric actions at a slow velocity, e.g., 30–60°/s, and across a large range of motion. Peak torque, total work and angle of peak torque should be reported. Isometric contractions may also be used, but exercise-induced changes in the angle of peak torque can easily over- or underestimate the changes in peak torque when only one joint angle is tested (49,245). Baseline levels of force-generating capacity should be carefully established (≥ 1 familiarisation session), and muscle function should be monitored repeatedly (daily) until full recovery. Note, we advise caution about merely evaluating the immediate reductions in force-generating capacity (217), because this measurement may reflect muscle fatigue rather than muscle damage (88).

## 3. CYTOKINE RESPONSES TO EXERCISE-INDUCED MUSCLE DAMAGE

Researchers in exercise immunology have used various exercise protocols to investigate cytokine responses to muscle damage. These protocols include downhill running, eccentric actions of the leg or arm muscles, and traditional resistance exercise. Most studies have reported that these modes of exercise increase plasma

IL-6 concentration for several hours after exercise (see Table 3). Some studies have also reported that the plasma concentrations of interleukin-1 receptor antagonist (IL-1ra), monocyte chemotactic protein (MCP)-1 and granulocyte-colony stimulating factor (G-CSF) increase in the hours after exercise. Changes in the plasma concentrations of IL-8, IL-10, IL-12 and soluble tumor necrosis factor α receptor 1 (sTNF-αR1) are more variable. The plasma concentrations of IL-1β, IL-2, IL-5, IL-13, IL-15, IL-17, TNF-α, leukemia inhibitory factor (LIF) and interferon (IFN)-γ do not change at all following exercise. In skeletal muscle, following resistance exercise, mRNA expression of IL-1β, IL-6, IL-8 and TNF-α increases for up to 24 hours after exercise (see Table 4). IL-6, IL-8 and MCP-1 mRNA expression also increases for several hours after downhill running and eccentric actions of the quadriceps. Changes in IL-10 mRNA are more variable, while IL-2, IL-5 and IL-12 mRNA expression does not change after exercise. No research to date has investigated alterations in the anti-inflammatory cytokines IL-4 and IL-13 in skeletal muscle following acute exercise.

Table 3. Summary of systemic cytokine responses to eccentric and resistance exercise.

| Reference | Exercise mode | Immediately post-exercise | 1–4 h after exercise | 4–24 h after exercise | ≥ 24 h after exercise |
|---|---|---|---|---|---|
| (145,235,236, 239,277,294) | Downhill running | ↑ IL-1ra, IL-6, IL-8, G-CSF, MCP-1 | ↑ IL-1ra, IL-6, IL-12p40, MCP-1 | ↑ IL-6, IL-7, IL-8, IL-10, MCP-1, MIP-1β | ↑ IL-1ra |
| | | | ↓ IL-8 | | ↓ IL-8 |
| | | ↔ IL-10, IL-12p40 | ↔ IL-10 | ↔ IL-2, IL-4, IL-5, IL-12p70, IL-13, IL-17, IFN-γ, IL-1β, TNF-α, G-CSF | ↔ IL-6, G-CSF |
| (43,297) | Eccentric cycling | ↑ IL-1ra, IL-6, sTNF-αR1 | ↑ IL-1ra, IL-6, sTNF-αR1 | | ↑ IL-1ra, IL-6, sTNF-αR1 |
| | | ↔ TNF-α | ↔ TNF-α | | ↔ TNF-α |
| (77,228,256,322) | Eccentric exercise of the quadriceps | ↑ IL-6, MCP-1, G-CSF, M-CSF | ↑ IL-6, MCP-1, G-CSF | ↑ G-CSF | |
| | | | ↔ IL-1ra, IL-8, IL-10, TNF-α, G-SCF, M-CSF, sTNF-αR1 | ↔ IL-6, MCP-1, M-CSF | |
| (61,124,234,242) | Eccentric exercise of the elbow flexors | ↑ IL-6 | ↑ IL-6, IL-10, G-CSF, sTNF-αR1 | ↑ G-CSF, IL-10 | ↑ IL-6, IL-10, G-CSF |
| | | ↓ IL-8, IL-10 | ↓ IL-8 | ↓ IL-8 | ↓ IL-8, TNF-α |
| | | ↔ TNF-α, IL-1ra, IL-8, IL-10, G-CSF | ↔ TNF-α, IL-1ra, IL-8, IL-10 | ↔ IL-1ra, IL-6, IL-8, IL-10, TNF-α, sTNF-αR1 | ↔ IL-1ra, IL-6, IL-8, IL-10, TNF-α, sTNF-αR1 |
| (35,36,214,215,276,305) | Resistance exercise | ↑ IL-6, IL-8, IL-10 | ↑ IL-6, IL-8, IL-10 | | ↑ IL-6, IL-10, M-CSF |
| | | | | ↓ IL-1β | ↓ IL-1β |
| | | ↔ IL-6, IL-10, IL-15, TNF-α, LIF | ↔ IL-6, IL-10, TNF-α | ↔ IL-15 | ↔ IL-6, IL-10, IL-15, TNF-α |

Table 4. Summary of intramuscular cytokine mRNA responses to eccentric and resistance exercise.

| Reference | Exercise mode | Cytokine | 1–4 h after exercise | 4–24 h after exercise | ≥ 24 h after exercise |
|---|---|---|---|---|---|
| (36,45,80,139,165,215,246,253,303) | Resistance exercise | IL-1β | ↑ | ↑ | ↑ |
| | | IL-2 | ↔ | | |
| | | IL-5 | ↔ | | |
| | | IL-6 | ↑ | ↑ | ↔ |
| | | IL-8 | ↑ | ↑ | |
| | | IL-10 | ↔ | | ↑ |
| | | IL-15 | | ↑ | ↔ |
| | | TNF-α | ↑, ↔ | ↑ | ↔ |
| | | LIF | | ↑ | |
| (45,116) | Downhill running | IL-1β | ↔ | ↔ | ↔ |
| | | IL-6 | ↑ | ↑ | ↑ |
| | | IL-8 | ↑ | ↔ | |
| | | TNF-α | ↔ | ↔ | ↑ |
| | | TGF-β | | | ↑ |
| (59,129,177,203,256) | Eccentric exercise of the quadriceps | IL-1β | ↑ | | ↑ |
| | | IL-6 | ↑ | | ↔ |
| | | IL-8 | ↑ | | |
| | | MCP-1 | ↑ | | ↑ |
| | | TNF-α | ↔ | ↑ | ↔ |
| (172) | Electrical stimulation of *gastrocnemius* | IL-1β | | | ↔ |
| | | TNF-α | | | ↔ |
| | | MCP-1 | | | ↑ |

### *3.1 Cytokines as mediators of exercise-induced muscle damage*

As described previously, exercise induces systemic and local cytokine responses in skeletal muscle. Over the past decade or so, considerable attention has focused on the biological role of cytokines derived from muscle (so-called 'myokines') in regulating metabolism in skeletal muscle and adipose tissue (237). Less is known concerning the role of cytokines in regulating inflammatory responses and adaptation to exercise-induced muscle damage. To examine whether cytokines are a cause or a by-product of exercise-induced muscle damage, a small number of studies have investigated the relationship between cytokine responses and markers of muscle damage. Three studies report that plasma cytokine concentrations correlate with plasma CK activity and myoglobin concentration after exercise (43,124,216). Other research has investigated the relationship between cytokines and muscle damage more directly by comparing cytokine responses to concentric versus eccentric actions, submaximal versus maximal eccentric actions and single versus repeated bouts of eccentric exercise.

#### *3.1.1 Eccentric versus concentric muscle actions*

Several studies have examined cytokine responses to eccentric exercise, which causes greater muscle damage than concentric exercise. Bruunsgaard *et al.* (43) demonstrated that serum IL-6 concentration and CK activity are higher after eccentric cycling compared with concentric cycling (Table 5; see addendum). Clarkson *et al.* (59,129) have also reported greater strength loss and gene expression of both IL-1R and MCP-1 after eccentric actions compared with concentric actions of the quadriceps. However, others have reported no differences in the plasma cytokine responses to level running versus downhill running, despite higher plasma CK activity and myoblobin concentration after downhill running

(235,236). Variation in exercise protocols, training status of study participants, and sampling times may account for some of these inconsistent findings.

#### *3.1.2 Submaximal versus maximal eccentric exercise*

As a variation to research comparing eccentric and concentric exercise, other studies have compared muscle damage and cytokine responses to submaximal and maximal eccentric exercise, which cause differing degrees of muscle damage. Malm *et al.* (181) observed that loss of strength was greater 1 d after downhill running at a gradient of 8° versus 4°, but they detected no changes in serum or muscle cytokines after either exercise trials. In another study, muscle damage (as demonstrated by loss of muscle strength) was greater after maximal versus submaximal eccentric actions of the elbow flexors, but cytokine responses were similar between the two trials (234).

#### *3.1.3 Repeated bouts of eccentric exercise*

Several studies have investigated whether adaptations to repeated bouts of eccentric exercise are associated with alterations in cytokine responses. However, this research has also produced equivocal findings (Table 5; see addendum). The results of two studies indicate less muscle damage, smaller changes in circulating IL-6, IL-8 and MCP-1, and greater changes in circulating IL-10 and macrophage inflammatory factor (MIF)-1β in the days following two bouts of eccentric exercise (124,277). In contrast with these observations, other groups have found no difference in plasma IL-6 concentration following repeated bouts of eccentric actions of the knee extensors/flexors, despite evidence of less muscle damage (77,322). Once again, these discrepant findings may be due to differences in exercise protocols, training status of study participants, and sampling times. Two studies have reported that MCP-1 gene expression in *m. vastus lateralis* is higher following a repeated bout of eccentric actions (129), but lower after electrically-stimulated muscle actions (172) performed four weeks after an initial bout of the same exercise. As discussed previously, this discrepancy may reflect differences in the pattern of muscle fibre recruitment between voluntary and electrically-stimulated muscle actions.

### *3.2 Experimental considerations for examining the role of cytokines in muscle damage*

Research to date has examined muscle cells and leucocytes as potential sources of cytokines during exercise; however, the dominant cellular source of circulating cytokines remains uncertain, for two main reasons. First, *in vitro* cell culture methods do not take into account the complex array of interactions between humoral factors produced by multiple organs during exercise. Although certain types of cells may generate large amounts of cytokines *in vitro*, cytokine synthesis i*n vivo* may depend on the presence of other inhibitory (or stimulatory) factors in the local or systemic environment. Second, molecular analysis of isolated RNA or protein extracts is often performed using homogenised muscle, which makes it difficult to identify specific cell sources of cytokines.

To clarify the role of cytokines in muscle damage and adaptation in greater detail, more complex experimental procedures are required. Analysis of the circulating concentrations of cytokines is arguably insufficient to examine the role of

cytokines in muscle damage for two reasons. First, most cytokines are produced locally within skeletal muscle during exercise. Second, with the exception of IL-6, these cytokines are not released into the circulation (285). Regular muscle sampling in the first few hours and days after exercise provides the most direct evidence as to whether cytokines regulate muscle damage and regeneration. However, due to the invasive nature of muscle biopsies, most studies to date have collected no more than four biopsy samples at various time points after exercise. Variation in the time points for muscle sampling, coupled with different exercise protocols, makes it difficult to obtain a clear understanding of the time course of inflammatory responses to exercise-induced muscle damage. As discussed previously, some researchers have also questioned whether the biopsy procedure itself causes more inflammation than exercise (180,181).

Another alternative approach is to modulate or block cytokine activity prior to muscle damage, and then examine subsequent muscle regeneration. These procedures are obviously difficult to implement in human studies. Several studies have examined muscle regeneration following freeze injury in mice lacking cytokine activity. Compared with wild-type mice, recovery of muscular isometric strength is lower between 7–28 days post-injury in $CCR2^{-/-}$ mice (314), at 12 days post-injury in mice depleted of TNF-α or its receptors (313), and at 14 days post-injury in $MCP^{-/-}$ mice (317). These findings indicate that rather than causing muscle damage, cytokines such as TNF-α and MCP-1 and their receptors are required for successful muscle regeneration to occur. Conversely, whether over-expression of these cytokines and their receptors increases muscle injury is currently unknown. MCP-1 deficiency causes more rapid recruitment and activation of neutrophils (through increased expression of neutrophil chemoattractants) and delays recruitment of macrophages in injured muscle tissue. These effects delay the formation of new muscle fibres and increase lipid accumulation and necrosis in regenerating muscle tissue (270,317). The mechanisms through which TNF-α deficiency impairs muscle regeneration are less clear, but may also involve a decline in infiltrating neutrophils and macrophages (240) and/or the expression of myogenic regulatory factors (313). In contrast with TNF-α and MCP-1, IL-6 deficiency does not appear to alter muscle regeneration, even though IL-6 expression in muscle increases following injury (313) and IL-6 regulates the proliferation and differentiation of myoblasts (17,267). Taken together, these findings do not necessarily exclude IL-6 as a regulatory factor in muscle regeneration, but suggest that other factors such TNF-α and MCP-1 and their receptors play more important roles. Few studies have reported any change in TNF-α mRNA expression in muscle after exercise, so its role in human skeletal muscle remains uncertain. IL-6 may be more active in regenerating tendon tissue (8). The research described above implicates TNF-α and MCP-1 and their receptors in muscle regeneration following acute muscle injury. They may play a different—and potentially negative—role in chronic diseases that involve muscle wasting.

***Summary***

In comparison with cellular inflammatory responses to exercise-induced muscle damage, much less is known about changes in local cytokine responses and their functional significance. Most studies have only collected muscle biopsies at one or two time points after exercise, which precludes any detailed assessment of the

role of cytokines during different phases of muscle inflammation and regeneration. Definitive evidence exists that muscle cells produce a variety of cytokines and chemokines *in vitro*, whereas it is more likely that various cell types synthesise cytokines in muscle following exercise. The results of studies that have used freeze or crush injury in animal muscle to investigate the role of cytokines are not necessarily applicable to humans, because this type of injury is generally more severe and localised than exercise-induced muscle damage. Although cytokines and chemokines regulate a variety of metabolic, endocrine and immunological functions, it remains unclear whether they are a cause or by-product of exercise-induced muscle damage. Until we gather more precise information about their functional role in exercise-induced muscle damage, there seems little rationale for athletes to attempt to attenuate cytokine responses to exercise through nutritional or pharmacological means.

## 4. SATELLITE CELL RESPONSE TO ECCENTRIC EXERCISE

Muscle cells are multi-nucleated, and can be five orders of magnitude larger than mononucleated cells (44,114). Because adult muscle nuclei (myonuclei) do not divide, new myonuclei must come from other sources when required. Satellite cells serve this role as so-called stem cells of skeletal muscle or myogenic precursor cells during both skeletal muscle adaptation and regeneration (55,118). During muscle hypertrophy, the growing myofibre may require additional myonuclei, because the area of the cytoplasm that each myonucleus can control is traditionally regarded as fairly constant (6,278). However, hypertrophy can proceed without satellite cell activity, probably because existing myonuclei are able to control larger areas of cytoplasm when stimulated (28,190,191).

Satellite cells are situated beneath the basal lamina, but in contrast to regular myonuclei, they are located outside the plasma membrane (sarcolemma). In response to an appropriate stimulus, satellite cells are activated, and then proliferate. Some activated satellite cells help to replenish the satellite cell pool. Other satellite cells migrate to areas where they differentiate and fuse with existing myofibres or produce new fibres. Exercise can stimulate satellite cells to re-enter the cell cycle and proliferate, as shown in several human training studies lasting 2–3 months (see summary in (174)). Interestingly, satellite cells may become activated and proliferate after a single bout of exercise that induces neither hypertrophy nor damage to myofibres (71,72,174,202). In this situation, although the satellite cells are activated, they do not necessarily fuse with myofibres to become myonuclei (no increase in number of myonuclei) or accumulate to generate new myofibres. This 'low threshold' activation of satellite cells may primarily serve to replenish the satellite cell pool, because a reduced satellite cell pool would diminish the regeneration potential of the muscle (261).

### *4.1 Satellite cell response to a single bout of eccentric exercise*

Human studies investigating the skeletal muscle satellite cell responses to a single bout of eccentric exercise are summarised in Table 6. Figure 3 demonstrates the quantitative satellite cell responses in these studies. The proportion of satellite cells increases quickly within the first 24 hours after exercise and may remain elevated for 8 days or more.

Table 6: Human studies investigating the satellite cell (SC) response to a single bout of eccentric exercise in young, healthy subjects.

| Reference | Exercise mode (thigh muscle if not indicated) | Subjects' training status | Sampling time points | Measure of SC given: | Context and comments |
|---|---|---|---|---|---|
| Crameri *et al.* (72) | Eccentric exercise:<br>50 one-leg 'drop down' jumps<br>8x10 reps at 30°/s<br>8x10 reps at 180°/s | Sedentary | 2 d<br>4 d<br>8 d | NCAM<br>% MN | First study to show increased SC number with single bout of exercise |
| Dreyer *et al.* (84) | Max eccentric:<br>6x16 reps at 60°/s | No resistance training | 1 d | NCAM<br>% MN /fibre | Larger SC response in younger than in older subjects |
| Crameri *et al.* (71) | Max eccentric:<br>10x10 reps at 30°/s<br>11x10 reps at 180°/s | No regular training | 4 d<br>8 d | NCAM, Pax7<br>% MN | Larger response with electrical stimulation.<br>No baseline data given |
| O'Reilly *et al.* (225) | Max eccentric:<br>30x10 reps 180°/s | No resistance training | 4 h<br>1 d<br>3 d<br>5 d | NCAM<br>% MN, /fibre | Association with HGF response |
| McKay *et al.* (196) | Max eccentric:<br>3.14 rad/s<br>30 x 10 reps | No resistance training | 4 h<br>1 d<br>3 d<br>5 d | Pax7<br>% MN | Association with IL-6 signalling |
| Mikkelsen *et al.* (202) | Max eccentric:<br>10x10 reps at 30°/s<br>10x10 reps at 120°/s | Well trained | 5 h<br>28 h<br>8 d | NCAM, Pax7<br>% MN, /fibre | SC response reduced by NSAID infusion |
| McKay *et al.* (197) | Max eccentric:<br>3.14 rad/s<br>30 x 10 reps | No resistance training | 1 d | NCAM, Pax7<br>% MN, /fibre | Compared with FACS, similar results |
| Paulsen *et al.* (230) | Max eccentric (elbow flexors):<br>14x5 reps at 30°/s | Physically active | 1 h – 7 d (combined) | NCAM<br>% MN, /fibre | Biopsies from m. biceps brachii |

NB: To quantify satellite cells, the number of positive cells is expressed relative to fibre number (per fibre) or as a proportion of the total number of myonuclei (MN), the latter calculated as (NCAM+ cells / [myonuclei + NCAM+ cells] × 100) or (Pax7+ cells / [myonuclei + Pax7+ cells] x 100).

In Figure 3, the relative satellite cell responses (normalised to pre-exercise values) observed in the studies from Table 6 are shown together. This figure shows that the observed responses are highly variable, even at similar time points and despite similarities between the exercise protocols. The satellite cell response does not appear to correlate with the stress and damage to the exercised muscle. For example, the study that reported the most damage after exercise (230) also reported the smallest increase in the number of satellite cells. When gross muscle damage does occur—as demonstrated in Paulsen *et al.* (230) and Crameri *et al.* (71)—the satellite cells leave their location and migrate as myoblasts to areas of need for regeneration (136,266). Note, however that in humans, the signs of severe damage and necrosis are first observed after about 4 days (71,137,230), while a strong satellite cell response is evident after only 24 hours (Figure 3). Further research is warranted to clarify the function and time course of changes in satellite cell activity in response to exercise-induced muscle damage.

### *4.1.1 Satellite cell identification*

Satellite cells were first identified in 1961 using electron microscopy (185). Today, specific antibodies are generally used to identify and quantify satellite cells. Most human studies have used an antibody against NCAM (also known as CD56 and Leu19 (266)) to identify satellite cells (see Table 6). Because NCAM is a cell surface glycoprotein expressed on the membrane of satellite cells, this anti-

body marks the outer border of satellite cells. The transcription factor Pax7 is traditionally used to identify satellite cells in cell culture. Pax7 is a transcription factor that is expressed in the nuclei of satellite cells; thus, Pax7 antibodies only label the nucleus of the satellite cell. Lindström and Thornell (163) reported that 94% of all human satellite cells are both NCAM and Pax7 positive.

### *4.2 The role of the COX-pathway and NSAIDs in satellite cell activation signalling*

Many factors are proposed to control satellite cell activity, yet the precise regulatory mechanisms in human skeletal muscle are not fully understood (9,30,327). Animal studies have identified several factors that influence satellite cells at different stages of their activity (for detailed reviews see (30,327)). Among these factors, the cyclooxygenase (COX) pathway is one of the most important (31,32,199). Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit this pathway. In relation to muscle soreness and/or muscle damage, athletes often use NSAIDs, which highlights the importance of understanding their effect on muscle regeneration (7,91,328).

COX2 inhibitors and the non-selective NSAID ibuprofen reduce hypertrophy of mice and rat skeletal muscle (222,279). In humans, NSAIDs attenuate the satellite cell response to exercise (174,202), which is discussed in more detail below. How NSAIDs or prostaglandins exert their effect on satellite cells is not known.

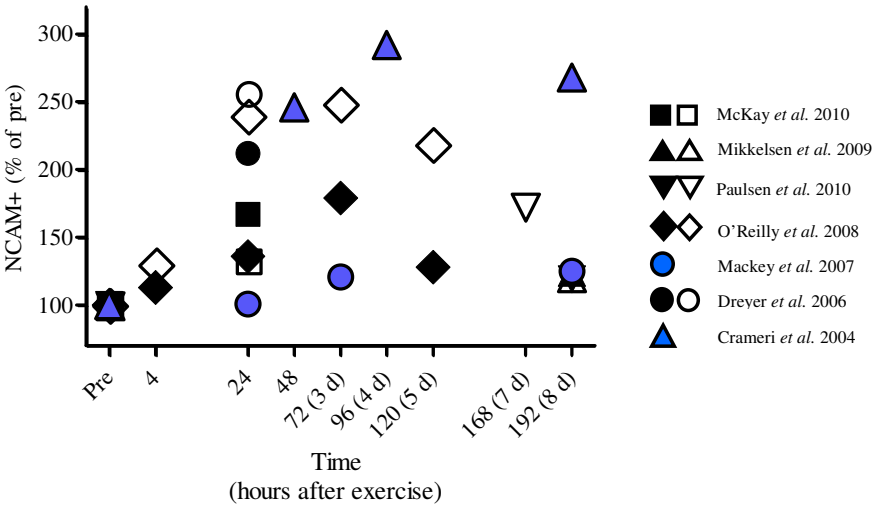


**Figure 3:** Satellite cell response to a single bout of maximal exercise. The satellite cells were identified by antibodies against NCAM on muscle cross sections from biopsies obtained at different timepoints after exercise. Eccentric exercise was used in all studies except from Mackey *et al.* 2007 (36 km run). Biopsies were obtained from *m. vastus lateralis* except from Paulsen *et al.* 2010 (*m. biceps brachii*). The number of NCAM+ cells is expressed as proportion of total myonuclei (filled symbols) or per muscle fibre (open symbols) and shown as percentage of pre-exercise values.

### *4.2.1 COX and prostaglandins*

The primary function of the COX enzymes is to generate prostaglandins. Prostaglandins (e.g., PGE and PGF) are ubiquitous lipid compounds derived from membrane phospholipids. They regulate smooth muscle tissue, acting as vasodilators to enhance blood flow in a wide range of tissues including the kidneys. They also sensitise nociceptors (pain response), regulate inflammation and fever, and protect the mucus layer in the gastrointestinal tract (104). Among the various COX enzymes, COX1 and COX2 are the most common (83,104). COX1 is constitutively expressed in several cell types, and synthesises prostaglandins that are important for homeostasis. COX2 is induced during cell injury, inflammation and mechanical stretch, and it synthesised prostaglandins that mediate inflammation and pain. Traditional NSAIDs inhibit both COX1 and COX2 isoforms (120). COX2 selective inhibitors have been developed to reduce the adverse effects of traditional NSAIDs on the gastrointestinal tract and the kidneys, while maintaining their analgesic and anti-inflammatory effects (34,309).

### *4.2.2 COX expression and prostaglandin response to exercise in healthy human muscle*

Several studies have investigated the expression of the different COX isoforms in human skeletal muscle, although mainly at the mRNA level. COX1 mRNA is highly expressed in human skeletal muscle (COX1v1 and COX1v2), but it does not respond to exercise or NSAIDs (47,203,318). By contrast, COX2 mRNA is expressed at very low levels at rest, and is either unchanged (47,203) or induced with exercise (45,46,318) and some NSAIDs (47,203). COX3, which constitutes three different splice variants of COX1, is also expressed at very low levels (318). COX1 protein expression remains unchanged with exercise and NSAID treatment (47). COX2 protein is less abundant in skeletal muscle. Immunohistochemical studies have identified both COX1 and COX2 in human skeletal muscle (288,293). However, it is uncertain whether the antibodies used in these studies are specific for the COX enzymes, particularly because COX2 protein expression in human skeletal muscle is very low (47,293). The level of prostaglandins in skeletal muscle increases with resistance exercise, as indicated by the presence of $PGF_{2\alpha}$ in muscle homogenate (300), and $PGE_2$ efflux from muscle using microdialysis (142). By contrast, $PGE_2$ does not seem to change following the first hours after eccentric exercise (230).

### *4.2.3 Effect of NSAIDs on healthy human muscle*

#### *4.2.3.1 Muscle function and DOMS following exercise*

The effects of NSAIDs on DOMS and recovery of muscle function following exercise have been widely investigated during the last decades (see reviews (4,18,19)). Research on the use of NSAIDs to relieve DOMS has yielded conflicting results. Likewise, the evidence for the effects of NSAIDs on circulating CK activity is inconclusive. Considering the widespread use of NSAIDs among athletes (110,198,311), the effects of NSAIDs on adaptation to exercise training are important to consider. Whether short-term NSAID therapy affects muscle adaptation to training in humans is largely unknown. The discussion below focuses on healthy young humans, but this issue is just as relevant to the elderly and clinical patients who may consume NSAIDs regularly for medicinal purposes.

*4.2.3.2 Cellular effects of NSAIDs on human muscle*

The cellular effects of exercise combined with NSAIDs on skeletal muscle are not well known. Bourgeois *et al.* (33) demonstrated that consuming the NSAID naproxen for 48 hours following knee extensor exercise did not affect the number of total leucocytes in *m. vastus lateralis* (leucocyte common antigen, CD45+ cells). However, muscular strength returned to baseline more rapidly in response to naproxen compared with the placebo treatment. Treatment with the COX2 specific inhibitor diclofenac for a total of 27 days before and after 20 min step exercise reduced histological abnormalities in muscle (foci of inflammation or necrotic fibres), DOMS and plasma CK activity (224). Animal studies point to a negative effect on skeletal muscle regeneration and adaptation (31,152,204,222,279), whereas evidence from human studies is sparse.

Trappe *et al.* (301) reported that ingestion of ibuprofen for 24 hours following one session of intense eccentric exercise suppressed the increase in mixed muscle protein synthesis rates that normally occurs following exercise. The protein synthesis rate measured 24 hours after exercise, increased by 76% in the placebo group, whereas it remained unchanged in the ibuprofen group. Ibuprofen also suppressed the $PGF_{2\alpha}$ response to exercise (300). Mackey *et al.* (174) showed that ingestion of NSAIDs in humans attenuates the satellite cell response to endurance exercise. In this study, satellite cell number remained unchanged 8 days after a 36-km run in athletes who consumed NSAIDs after exercise. By contrast, satellite cell number was elevated by 27% 8 days after exercise in athletes who consumed placebo. This finding points towards a negative effect of NSAIDs on satellite cell proliferation *in vivo* in humans. This is consistent with reports from animal models that COX enzyme activity is necessary for satellite cell activity and muscle regeneration.

We have also shown that infusion of NSAIDs before, during and for 4.5 hours after eccentric exercise (a total of 7.5 hours) reduces the satellite cell proliferation observed 8 days after exercise (202). A moderate dose of ibuprofen (400 mg/d) during 6 weeks training does not, however, alter muscle hypertrophy, strength or soreness (149). The few human studies using COX2 specific inhibitors have not observed any effect on satellite cells (230) or mixed muscle protein synthesis (48). Burd *et al.* (48) administered celecoxib (600 mg) for 24 hours following a single session of heavy eccentric exercise. Treatment with celecoxib did not alter mixed muscle protein synthesis following exercise (0.06 to 0.11 %/h) compared with the placebo treatment (0.07 to 0.09 %/h). Following eccentric exercise of the elbow flexors, Paulsen *et al*. (230) observed that treatment with celecoxib (400 mg/d) for 7 days did not suppress the increase in numbers of satellite cells or inhibit myofibre regeneration after exercise, when compared to a placebo treated group.

Animal and cell culture studies mainly show that NSAIDs negatively affect satellite cells, hypertrophy and regeneration of skeletal muscle. Results from studies on young, healthy humans indicate either a negative effect (301) or no effect (203) of traditional NSAIDs on muscle protein synthesis (300). Two studies report negative effects of NSAIDs on satellite cells (174,202). Hypertrophy is not reduced following moderate (400 mg/day) doses of ibuprofen (149). Furthermore, COX2 specific inhibitors do not alter muscle protein synthesis (48) or satellite cells (230).

### *4.3 Inflammation in skeletal muscle: friend or foe?*

Current evidence suggests that in healthy young individuals, reducing inflammation by use of NSAIDs may interfere with muscle regeneration or hypertrophy. In contrast, NSAIDs may be beneficial under conditions of excessive or prolonged inflammation. For example, in the elderly, low-grade systemic inflammation may contribute to the loss of muscle mass (termed 'sarcopenia'). In this context, NSAIDs may benefit maintenance of muscle mass (254). Low-grade systemic inflammation that accompanies ageing has attracted a lot of attention during recent years. Levels of inflammatory markers, such as IL-6 and CRP increase slightly with ageing, and these higher levels are correlated with disability and mortality in humans (23,117,231). During 12 weeks of resistance training in elderly people, ibuprofen (3 × 400 mg/day) promoted gains in quadriceps muscle volume and strength (299).

In many chronic disease states, systemic inflammation may contribute to loss of muscle mass (termed 'cachexia'). In animal models of diseases like cancer (125,189) and arthritis (111), NSAIDs help to maintain muscle mass. In these studies, indomethacin (inhibiting COX1 and COX2) and COX2 specific NSAIDs reduce the negative effects of arthritis on body mass, muscle mass and muscle gene expression. Similarly, ibuprofen and indomethacin help to preserve muscle mass in tumor-bearing mice with reduced muscle mass (189).

***Summary***

Satellite cells are necessary to repair muscle damage. Animal studies show that the COX pathway is essential for this, and that NSAIDs inhibit repair and regeneration. Likewise, in healthy young humans, NSAIDs seem to reduce the capacity to repair muscle damage, since the number of satellite cells is reduced. The biological significance of these findings is that consumption of NSAIDs to alleviate soreness and expedite muscle repair after damage may be contraindicated. Inflammation (or at least a functional COX-pathway) may be necessary for muscle adaptation and regeneration in young, healthy people. Consequently, inhibiting inflammation using NSAIDs may have negative effects on skeletal muscle. Evidence from human studies indicates that consumption of large doses of traditional NSAIDs may negatively affect skeletal muscle in healthy young humans, whereas moderate doses of NSAIDs or COX2 selective inhibitors have apparently no effect. Contrary to this, inhibition of inflammation using NSAIDs may be beneficial in elderly or in individuals with chronic diseases that cause muscle atrophy. The recommendations on use of NSAIDs are therefore likely to differ between subjects, and it seems important to consider health, age and training status of individuals when considering the use of NSAIDs.

## CONCLUSIONS

Currently there is no common definition or accepted way to measure the degree of exercise-induced muscle damage. However, both animal and human studies have repeatedly demonstrated that severe exercise-induced muscle damage encompasses myofibrillar disruptions, local inflammation with leucocyte accumulation, segmental myofibre necrosis, and subsequent regeneration involving satellite cell

activation. In humans, the extent of damage and inflammation varies considerably, in contrast to more consistent findings in animal models. There is reasonably solid evidence that unaccustomed, isolated, maximal eccentric actions over large range of motion are necessary to inflict severe exercise-induced damage, including necrosis. Still, some subjects do not display severe damage even after such 'extreme' protocols. Exercise protocols that are closer to exercise used in regular athletic training (e.g., traditional resistance exercise) generally cause minor damage, although some myofibrillar disturbances seem to occur to some degree. Irrespective of the exercise protocol, changes in muscle function (force-generating capacity) seem to be the best marker for the overall level of damage. Thus, if the reduction in muscle force-generating capacity is less than 20% after exercise and recovery is complete in the following 48 hours, the degree of damage is likely to be minor and signs of classical inflammation (i.e., leucocyte accumulation in the muscle tissue) are hardly detectable or absent. Nevertheless, local cytokine production may still occur. By contrast, reductions of muscle force-generating capacity that surpass 50% and prolonged recovery requiring more than one week, indicate severe exercise-induced muscle damage. Consequently, we recommend that investigations of exercise-induced muscle damage should always monitor muscle function until full recovery.

The cytokine response to exercise seems robust, but complex. The circulating concentrations of various cytokines increase during and after exercise, yet the cellular sources of these cytokines are difficult to ascertain. Myofibres have the potential to produce cytokines, but with the exception of IL-6, there is no evidence that other cytokines are released from skeletal muscle into the circulation during exercise. Leucocytes also seem an unlikely major source of circulating cytokines following exercise-induced muscle damage. In the exercised muscles, the increased mRNA expression of cytokines is poorly supported with evidence of increased protein expression. One exception is MCP-1, but this chemokine appears to be produced by stromal cells (macrophages) and satellite cells, rather than by myofibres. Most evidence indicates that the cytokine response to exercise is not necessarily an acute inflammatory response to muscle damage. Instead, cytokines may play a greater role in mediating glucose metabolism and muscle regeneration.

Satellite cells are activated by various types of exercise, both damaging and apparently non-damaging exercise. Thus, the threshold for satellite cell activation is rather low, and the satellite cell response does not seem to be directly related to muscle damage markers. However, only if the initial muscle damage induces a necrotic process in segments of myofibres will the satellite cells leave their position and migrate to the area of damage as (differentiated) myoblasts. The COX pathway by which prostaglandins are synthesised is associated with the satellite cell response, because blocking this pathway in animals reduces the regeneration and growth of skeletal muscle. Although COX2 is essential in animal muscles, selective COX2 inhibitors do not always inhibit muscle regeneration in humans. This could be linked to the fact that both the mRNA and protein levels of COX2 are very low in human skeletal muscle. Non-selective NSAIDs (blocking both COX1 and COX2) can inhibit satellite cells and may affect muscle regeneration and adaptation in young healthy individuals; yet when combined with resistance training, NSAID supplementation facilitate muscle hypertrophy in elderly people.

# ADDENDUM

Table 1A: Human studies that have investigated the presence of inflammatory cells (leucocytes) in biopsy samples obtained after types of exercise that apparently inflicted *'mild' exercise-induced muscle damage*. In studies with treatment groups, data from the control/placebo group are presented.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Acute ↓ in muscle function | Recovery of muscle function | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Group response* | *Individual response* | | | | | |
| Bourgeois *et al.* (33) | Resistance exercise (concentric/eccentric): Leg press and knee-extension (single leg), 6 x 10 repetitions; 80-85% of 1 RM. | VL: 1 d | 1 d (contra-lateral leg) | No | ? | ~9% at 1 d | ~3% below baseline 2 d after exercise | NA | NA | ~750 (1 d) |
| Féasson *et al.* (90) | 30 min downhill running at 12° (~11 $km \cdot h^{-1}$; ~54% of $VO2_{max}$) | VL: 0 h and 1 and 14 d | Pre | No | ? | ~15% (max power during cycling) | Within 7 d (no tests between day 1 and 7) | Yes | No | ~1000 (1 d) |
| Malm *et al.* (181) | 45 min downhill running at either 4° (50% of $VO_{2max}$) or 8° (max tolerated speed) | VL: 2 d | Control group | No | Higher levels in the epimysium of subjects with DOMS | ≤15% (isometric; 1 d; at 8°) | Within 2 d | NA | No | ~1000 (1 d; at 8°) |
| Crameri *et al.* (71) | 210 max eccentric actions using the knee-extensors in one randomly chosen leg. The exercise had two phases: 100 actions at $30° \cdot s^{-1}$ and 110 actions at $180° \cdot s^{-1}$. ROM: 10-90°. | VL: 5 h, 1, 4 and 8 d | Pre | Yes (CD68) | - | ~16% (isometric) | Within 2 d | Yes | No | NA |

Abbreviations: 0° = extended joint; 0 h, immediately after exercise; BB, m biceps brachii; DOMS, delayed onset muscle soreness; EM, electron microscopy; LCA, leucocyte common antigen; MC, mononuclear cells; MPO, myeloperoxidase; NA, not analysed/assessed; VL, m. vastus lateralis; Sol, m. soleus; Gast, m. gastrocnemius; PL, m. peroneus longus; TA, m. tibialis anterior.

Table 1B: Human studies that have investigated the presence of inflammatory cells (leucocytes) in biopsy samples obtained after types exercise that apparently inflicted *'moderate' exercise-induced muscle damage*. In studies with treatment groups, data from the control/placebo group are presented. See Table 1A for abbreviations.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Acute ↓ in muscle function | Recovery of muscle function | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Group response* | *Individual response* | | | | | |
| Fridén *et al.* (103) | Eccentric cycling exercise for 30 min at an intensity corresponding to 80-100% of $VO_{2max}$ during concentric cycling | VL: 0 h and 3 and 6 d | Pre | No | ? | ~24% (isometric) | Within 6 d | Yes | No | NA |
| Crenshaw *et al.* (75,76) | Max eccentric actions ($60° \cdot s^{-1}$) with the knee-extensors in one leg until exhaustion; the other (randomly chosen) leg performed concentric work. ROM: 30-120°. | VL: 2 d | 2 d ('concentric leg') | No | ? | NA | ~20% below baseline 2 d after exercise (isometric/ concentric) | Yes | No | NA |
| Stupka *et al.* (287) | 36 eccentric actions of leg press and 100 isolated eccentric actions at 120% of max concentric strength, using the knee-extensors in one leg. ROM: 15-90° (both exercises). The weakest leg evaluated from a pre-test) was exercised. | VL: 1 d | 1 d (contra-lateral leg) | Yes (CD68) | - | ~31% (concentric) | Within 7 d | Yes | NA | ♀: 200-1400, ♂: 300-2100 (range; 4 d) |
| Beaton *et al.* (24) | 240 max eccentric actions ($30° \cdot s^{-1}$) using the knee-extensors in one leg. ROM: 50-110°. | VL: 1 d | 1 d (contra-lateral leg) | Yes (CD68; no MPO) | - | ~50% (concentric/ isometric) | Within 4 d | Yes | No | 120-1300 (range; 3 d) |

Table 1B: Human studies that have investigated the presence of inflammatory cells (leucocytes) in biopsy samples obtained after types exercise that apparently inflicted *'moderate' exercise-induced muscle damage*. In studies with treatment groups, data from the control/placebo group are presented. See Table 1A for abbreviations.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Acute ↓ in muscle function | Recovery of muscle function | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Group* | *Individual* | | | | | |
| Beaton *et al.* (25) | 300 max eccentric actions ($30° \cdot s^{-1}$) using the knee-extensors in one randomly chosen leg. ROM: 60-120°. | VL: 4 h and 1 d | Pre | Yes (CD68; no MPO and elastase) | - | ~54% (isometric) | Within 7 d | Yes | NA (reduced desmin and dystropin staining) | ~300 (2 d) |
| Hubal *et al.* (129) | 300 eccentric and concentric actions. The subjects rose from a chair with one randomly chosen leg and lowered the body weight back to a seating position with the other leg (20 cm hip displacement). | VL: 6 h | 6 h ('concentric leg') | Yes (MAC387) | - | ~32% (isometric) | ~23% below baseline 5 d after exercise | NA | NA | NA |
| Mikkelsen *et al.* (202) | 200 max eccentric actions using one leg (knee-extensors); both legs were exercised. The exercise had two phases: 100 actions at $30° \cdot s^{-1}$ and 100 actions at $120° \cdot s^{-1}$. ROM: 10-90°. | VL: 8 d | Pre | No | Yes, 2 of 8 subjects (CD16 and CD68) | ~28% (isometric) | Within 8 d | NA | No | ~1000 (1 d) |
| Paulsen *et al.* (229) | 300 max eccentric actions using the knee-extensors in one randomly chosen leg. ROM: 30-110°. | VL: 30 min, 4 and 8 h and 1, 4 and 7 d | VL: 30 min, 4 and 8 h and 1, 4 and 7 d | Yes (CD16, CD68) | - | ~47% (concentric) | Within 7 d | Yes | Yes (~1%) | ~200-25000 (range; 4 d) |

Table 1C: Human studies that have investigated the presence of inflammatory cells (leucocytes) in biopsy samples obtained after types of exercise that apparently inflicted *'severe' exercise-induced muscle damage*. In studies with treatment groups, data from the control/placebo group are presented. See Table 1A for abbreviations.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Acute ↓ in muscle function | Recovery of muscle function | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Group response* | *Individual response* | | | | | |
| Hikida *et al.* (121,269) | Marathon (running) | Gast: 0 h and 1, 3, 5, and 7 d | Pre | Yes (EM) | - | ~47% (concentric) | > 7 d | Yes | Yes | NA |
| Jones *et al.* (137,257) | 1) Eccentric exercise using the elbow flexors until 50% loss of force<br>2) Backwards downhill walking (large strain on the calf muscles) | BB and Sol/Gast: 4, 5, 7, 8, 9, 10, 12, 14 and 20 d | No | Yes (MC) | - | >50% (eccentric) | NA | NA | Yes | ~750-80000 (range; 4-6 d) |
| Child *et al.* (60) | 70 max eccentric actions ($100°\cdot s^{-1}$) with the knee- extensors in one randomly chosen leg. ROM: almost full extension to almost full flexion (subjects in prone position). | VL: 4 and 7 d | Pre (randomly selected) | Yes (MC) | - | ~50% (isometric) | NA | NA | Yes | ~16000 (4 d) |
| Hellsten *et al.* (119) | Max eccentric cycling exercise; 5 x 5 min | VL: 45 min, 1, 2 and 4 d | Pre, 45 min and 2 d | Yes (CD11b) | - | ~54% (isometric) | ~45% under pre-value 4 d after exercise | NA | NA | ~13300 (4 d) |
| Paulsen *et al.* (154,230) | 70 max eccentric actions using the elbow flexors in one randomly chosen arm. ROM: 145-5°. | BB: 1 h and 2, 4 and 7 d | BB: 1 h and 2, 4 and 7 d | Yes (CD68, EM) | - | ~50% (isometric) | > 7 d, but within 3 weeks | Yes | Yes | ~300-25000 (range; 4 d) |

Table 2A: Studies in which *no inflammatory cells (leucocytes) were found* after voluntary exercise in humans. Muscle function was not assessed. In studies with treatment groups, data from the control/placebo group is presented. See Table 1A for abbreviations.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | | | | *Group response* | *Individual response* | | | |
| Fridén *et al.* (102) | Running down stairs: 10 x 10th floor to ground floor. | Sol: 2 and 7 d | Pre | No | ? | Yes | No | NA |
| Kuipers *et al.* (150) | 30, 45 or 60 min of eccentric cycling exercise at ~80% $VO_{2max}$ | VL: 0 h and 1 d | Pre | No | Yes, in two subjects exercising for 45 and 60 min | No | NA | < 200 (unchanged) |
| Warhol *et al.* (310) | Marathon (running) | Gast: Some hours, 1, 2, 3, 5, 7, and 10 d and 2, 3, 4, 6, 8, 10 and 12 weeks | Control group | No | ? | Yes | No | NA |
| Nurenberg *et al.* (223) | 30 min downhill running at 8°; 8 $km \cdot h^{-1}$ | Sol, Gast, TA, PL: 2 d | No | No | ? | Yes | No | 200-1200 (range; 12-36 h) |
| Malm *et al.* (180) | Eccentric cycling exercise for 30 min at an intensity corresponding to 80-100% of $VO_{2max}$ during concentric cycling (250-300 W). | VL: 0 and 6 h and 1, 2, 4 and 7 d | Pre and control group | No | ? | NA | No | ~120 (1 d) |
| Yu *et al.* (324-326) | 1) Running down stairs: 15 x 10th floor to ground floor; 2) Eccentric cycling exercise; 3) Downhill running (8°) | Sol: 1 h, 2-3 and 7-8 d | Control group | No | ? | Yes | No | < 1000 |
| Crameri *et al.* (72,73) | 50 one-leg drop down jumps (45 cm) followed by 80 eccentric actions at 30°·$s^{-1}$ and 80 eccentric actions at 180°·$s^{-1}$. | 0, 2, 4 and 8 d | Contra-lateral leg | No | Yes (CD68; 1 of 8 subjects) | NA | No (1 of 8 subjects) | NA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dennis *et al.* (81) | Resistance exercise (concentric/eccentric): Leg press and knee-extension (both legs): 4 x 8 reps (to failure in the 4th set) at 80% of 1 RM. | VL: 1 and 3 d | Pre | No | Yes (individual changes were found) | NA | NA | NA |
| Przybyla *et al.* (246) | Resistance exercise (concentric/eccentric): Leg press and knee-extension (both legs): 4 x 8 reps (to failure in the 4th set) at 80% of 1 RM. | VL: 3 d | Pre | No | ? | NA | NA | NA |

Table 2B: Studies in which *inflammatory cells (leucocytes) were found* after voluntary exercise in humans. Muscle function was not assessed. See Table 1A for abbreviations.

| Study | Exercise | Muscle biopsy | Control biopsy | Leucocytes | | Myofibrillar disruptions | Signs of necrosis | Peak CK ($IU \cdot L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | | | | *Group response* | *Individual response* | | | |
| O'Reilly *et al.* (226) | Eccentric cycling for 45 min (3 x 15 min) at 70-90% of $VO_{2max}$ (~180-220 W) | VL: 0 h and 10 d | Pre | Yes (EM) | - | Yes | Yes | NA |
| Costill *et al.* (70) | Eccentric actions using the knee-extensors in one leg; 10 x 10 repetitions with 120% of 1 RM. 30 min after the eccentric exercise the subjects performed a cycling exercise to deplete their glycogen stores (both legs). | VL: 1.5 h and 1 and 3 d | 1.5 h and 1 and 3 d (contra-lateral leg) | Yes (MC) | - | NA | NA | ~7000 (3 d) |
| Stauber *et al.* (283) | 70 max eccentric action (120°·-1) using the elbow flexors in the non-dominant arm. ROM: 120°. | BB: 2 d | 2 d (contra-lateral arm) | Yes (MC) | - | NA | Yes (~2%) | NA |
| Widrick *et al.* (320) | Eccentric actions using the knee-extensors in one leg; sets of 6 repetitions until failure with 120% of 1 RM. Subjects had the evening before performed a cycling exercise to deplete their glycogen stores (both legs). | VL: 0 and 6 h and 1 and 3 d | 0, 6 h and 1 and 3 d (contra-lateral leg) | Yes (MC) | - | NA | Yes | < 300 (unchanged) |
| Crenshaw *et al.* (74) | Ultramarathon footrace (160 km) | Gast: 1 d | No | Yes (EM) | - | Yes | Yes (~1%) | NA |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Fielding *et al.* (92) | 45 min downhill running at 16°; 75% max heart rate. | VL: 45 min and 5 d | Pre | Yes (neutrophils) | - | Yes | NA | ~300 (1 d) |
| O'Grady *et al.* (224) | 20 min eccentric stepping exercise; step height vas 110% of the lower leg length. | VL: 12 d (right leg) | Pre (left leg) | Yes (MC) | - | NA | Yes | ~1800 (3 d) |
| Stupka *et al.* (286) | Eccentric actions using the knee-extensors in one leg; leg press 3x12 and knee-extension 9x12 at 120% of 1 RM. | VL: 2 d | VL: 2 d | Yes (LCA; increase only in males) | - | Yes | No | ♀: ~600<br>♂: ~1000<br>(6 d) |
| Peterson *et al.* (241); Trappe *et al.* (302) | 10-14 sets of 10 actions with the knee-extensors in the non-dominant leg.<br>Load: 120% of max concentric strength.<br>ROM: 0-90°. | VL: 1 d | Pre (contra-lateral leg) | Yes (CD68, not CD15) | - | NA | NA | ~2250 (5 d) |
| Mahoney *et al.* (177) | 300 max eccentric actions ($120°\cdot s^{-1}$) using the knee-extensors in the non-dominant leg. ROM: 30-90°. | VL: 3 h and 2 d | Pre | Yes (CD68, MPO) | - | Yes | NA | ~300 (2 d) |
| MacNiel *et al.* (175) | 150 max eccentric actions ($120°\cdot s^{-1}$) using the knee-extensors in the right leg. ROM: 30-90°. | VL: 3 h and 2 d | Pre (left leg) | Yes (CD68, MPO) | - | NA | NA | ~500 (2 d) |

Table 5. Summary of changes in cytokines and markers of muscle damage after exercise.

| Reference | Exercise mode | Cytokine | Time of peak Δ | Function | Time of peak Δ | Protein | Time of peak Δ |
|---|---|---|---|---|---|---|---|
| Buford *et al.* (45) | Downhill running | IL-6 mRNA<br>IL-8 mRNA | 6% ↑, 3 h<br>8% ↑, 3 h | | | Serum CK | 6× ↑, 1 d |
| Kingsley *et al.* (145) | Downhill running | Serum IL-6 | 1.2× ↑, 0 h | | | Plasma CK<br>Plasma Mb | 2× ↑, 1 d<br>80% ↑, 0 h |
| Malm *et al.* (181) | Downhill running | | | | | | |
| | 4° gradient | IL-6, LIF protein in muscle | No change | Strength<br>Soreness [b] | No change<br>2, 0 h | Serum CK | 70% ↑, 1 d |
| | 8° gradient | IL-6, LIF protein in muscle | No change | Strength<br>Soreness [b] | 15%↓, 1 d<br>3, 0 h | Serum CK | 4.6× ↑, 4 d |
| Malm *et al.* (180) | Eccentric cycling | IL-1β protein in muscle | 60% ↑, 0 h | | | Plasma CK | 1.2× ↑, 1 d |
| Chen *et al.* (59) | Eccentric contractions of the quadriceps | MCP-1 mRNA | 25×↑, 4–8 h | Strength | 9% ↓, 3 d | | |
| Ross *et al.* (256) | Eccentric contractions of the quadriceps | MCP-1 mRNA<br>IL-6 mRNA<br>IL-8 mRNA | 1.3× ↑, 3 h<br>70% ↑, 3 h<br>1.2× ↑, 3 h | | | Serum Mb | 2.3× ↑, 3 h |
| Paulsen *et al.* (228) | Eccentric contractions of the quadriceps | Plasma M-CSF<br>Plasma IL-6<br>Plasma G-CSF<br>Plasma MCP-1 | 50% ↑, 0 h<br>4× ↑, 6 h<br>70% ↑, 6 h<br>1.6× ↑, 6 h | Strength | 34%↓ 6 h<br>24%↓ 4 d | Serum CK | 50× ↑, 4 d |
| Childs *et al.* (61) | Eccentric contractions of the elbow flexors | Plasma IL-6 | 4× ↑, 2 d | | | Serum CK<br>Serum Mb<br>Serum LDH | 20× ↑, 3 d<br>4× ↑, 2 d<br>5× ↑, 3 d |
| Phillips *et al.* (242) | Eccentric contractions of the elbow flexors | Serum IL-6 | 1.5× ↑, 1 h | Soreness [a] | 35 mm | Serum CK | 2.4× ↑, 3 d |
| Peake *et al.* (234) | Eccentric contractions of the elbow flexors | | | | | | |
| | Submaximal | Serum IL-6<br>Serum sTNFαR1 | 80% ↑, 3 h<br>40% ↑, 3 h | Strength<br>Soreness [a] | 10% ↓, 1 d<br>15 mm, 2 d | Plasma CK<br>Plasma Mb | 2.4× ↑, 4 d<br>100% ↑, 3 h |
| | Maximal | Serum IL-6<br>Serum sTNFαR1 | 40% ↑, 3 h<br>50% ↑, 3 h | Strength<br>Soreness [a] | 40% ↓, 1 d<br>21 mm, 2 d | Plasma CK<br>Plasma Mb | 1.3× ↑, 4 d<br>60% ↑, 3 h |
| Bruunsgaard *et al.* (43) | Concentric cycling<br>Eccentric cycling | Serum IL-6<br>Serum IL-6 | 50% ↑, 2 h<br>4.5× ↑, 2 h | | | Serum CK<br>Serum CK | No change<br>55× ↑, 4 d |
| Peake *et al.* | Level running | Plasma IL-6 | 4× ↑, 0 h | | | Plasma CK | 20% ↑, 0 h |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (233,235,236) | | Plasma IL-1ra | 1.3× ↑, 1 h | | | Plasma Mb | 1.1× ↑, 1 h |
| | | Plasma IL-10 | No change | | | | |
| | Downhill running | Plasma IL-6 | 4.6× ↑, 0 h | | | Plasma CK | 60% ↑, 0 h |
| | | Plasma IL-1ra | 2.4× ↑, 1 h | | | Plasma Mb | 18× ↑, 1 h |
| | | Plasma IL-10 | No change | | | | |
| Croisier *et al.* (77) | Eccentric contractions of the quadriceps | | | | | | |
| | Bout 1 | Plasma IL-6 | 7× ↑, 0.5 h | | | Serum Mb | 290× ↑, 0.5 h |
| | Bout 2 | Plasma IL-6 | 7× ↑, 0.5 h | | | Serum Mb | No change |
| Hirose *et al.* (124) | Eccentric contractions of the elbow flexors | | | | | | |
| | Bout 1 | Plasma G-CSF | 60% ↑, 3 d | Strength | 43% ↓, 2 d | Plasma CK | 60× ↑, 4 d |
| | | | | Soreness [a] | 37 mm, 2 d | Plasma Mb | 20× ↑, 4 d |
| | Bout 2 | Plasma G-CSF | 25% ↑, 3 d | Strength | 19% ↓, 2 d | Plasma CK | No change |
| | | | | Soreness [a] | 20 mm, 2 d | Plasma Mb | No change |
| Hubal *et al.* (129) | Contractions of the quadriceps | | | | | | |
| | Bout 1 | | | | | | |
| | Concentric | MCP-1 mRNA | 9.2× higher (vs. concentric) | | | | |
| | Eccentric | | | Strength | 35% ↓, 3 d | | |
| | | IL-1R mRNA | 5× higher (vs. concentric) | | | | |
| | Bout 2 | | | | | | |
| | Concentric | MCP-1 mRNA | 0.7× higher (vs. Bout 1) | Strength | 22% ↓, 3 d | | |
| | | IL-1R | 1.1× higher (vs. Bout 1) | | | | |
| | Eccentric | MCP-1 mRNA | 2.6× higher (vs. Bout 1) | | | | |
| | | IL-1R | 1.9× higher (vs. Bout 1) | | | | |
| Smith *et al.* (277) | Downhill running | | | | | | |
| | Bout 1 | Serum IL-6 | 6 pg/mL, 12 h | | | Serum CK | ~900 U/L, 1 d |
| | | Serum IL-10 | 0.8 pg/mL, 12 h | | | | |
| | | Serum MCP-1 | 103 pg/mL, 12 h | | | Serum CK | ~340 U/L, 1 d |
| | | Serum MIP-1β | 42 pg/mL, 12 h | | | | |
| | Bout 2 | Serum IL-6 | 12 pg/mL, 12 h | | | | |
| | | Serum IL-10 | 1.6 pg/mL, 12 h | | | | |
| | | Serum MCP-1 | 93 pg/mL, 12 h | | | | |
| | | Serum MIP-1β | 50 pg/mL, 12 h | | | | |

N.B. Only muscle function > 1 day post-exercise is reported because changes in strength < 1 day post-exercise most likely represent acute changes in muscle fatigue rather than damage *per se*. [a] Soreness was assessed using a visual analogue scale measured in millimeters (mm). [b] Soreness was assessed on a scale from 0–10.

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# *Effects of moderate and high intensity exercise on T1/T2 balance*

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## ABSTRACT

*Type 1 (T1) and Type 2 (T2) lymphocytes promote cell-mediated immunity and humoral immunity respectively. Evidence accumulated over the past two decades has demonstrated diverse responses of T1 and T2 cells to acute exercise or long-term training at moderate and high intensities. This brief review highlights the current findings from animal and human experimental models on the relationship between the T1 and T2 cell counts and the cytokines these cells produce, in response to moderate and high intensity exercise. The potential of using the T1/T2 balance as an indicator of immune function changes in response to exercise is discussed.*

**Key words:** T1 cell, T2 cell, interferon-γ, interleukin-4, IFN-γ/IL-4 ratio

## INTRODUCTION

Cytokines produced by T lymphocytes play a critical role in the development of host immunity against infection. It has been established that intracellular pathogens initiate a strong cellular immune response resulting in the differentiation of naive $CD4^+$ and $CD8^+$ T lymphocytes into type 1 T lymphocytes (T1), which consist of T helper type 1 (Th1) and T cytotoxic type 1 (Tc1) phenotypic cells (figure 1). These cells are characterised by production of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-2 (28, 34). In contrast, extracellular pathogens initiate a humoral immune response resulting in the differentiation of naive $CD4^+$ and $CD8^+$ T lymphocytes into type 2 T lymphocytes (T2),

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which consists of Th2 and Tc2 phenotypic cells characterised by the production of IL-4, IL-5, IL-6, and IL-10 (figure 1) (28, 34). T1 and T2 cell responses are mutually inhibitory. T1-derived cytokines (notably IFN-γ) inhibit T2 cell development, while T2-derived cytokines (IL-4, IL-10) suppress T1 responses (15, 29). T1/T2 balance (or Th1/Th2 balance, Tc1/Tc2 balance) has been used as an indicator of the changes in immune function and has become a research focus during the past decades (23, 25, 44, 48). There is currently no consensus on the definition of T1/T2 balance in the literature. In this review T1/T2 balance refers to a dynamic change between the numbers of T1 and T2 cells or between the concentrations of cytokines secreted by T1 and T2 cells. A significant up-regulation or down-regulation of any subset of the T1 or T2 cells or their cytokines indicates T1/T2 imbalance. The T1/T2 imbalance has been reported in acute and chronic infections or several diseases in humans such as cancer and asthma (6, 13, 33, 49, 51).

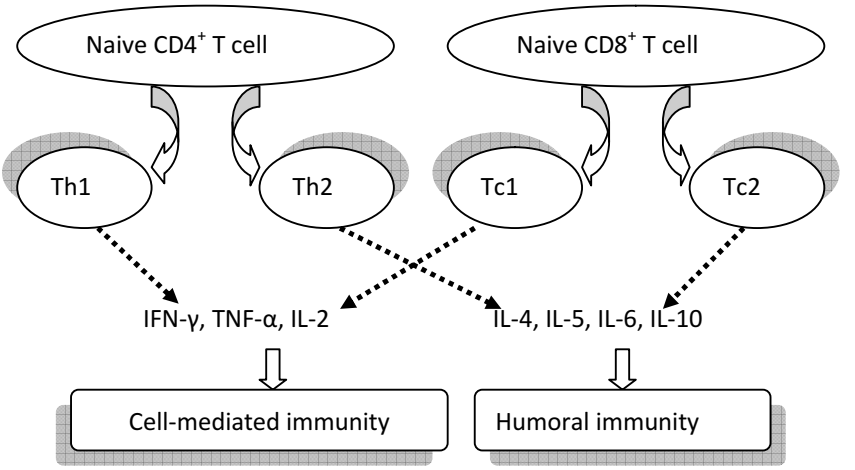


**Figure 1.** Development of T cells from T precursors.

In theory, T1 cells are associated with cell-mediated immunity and play a more important role in clearing infections (28), whereas T2 cells are associated with humoral immunity and play a key role in preventing infections (11). Thus, a severe depression of T1 cells caused by acute or chronic stress may lead to a failure in completely clearing viral infections, while an immune polarisation toward T1 phenotype may indicate an increased ability for clearing infections.

In the current literature the consensus is that the responses of T1 cells to infection are suppressed after high intensity exercises that may provide an explanation for the increased susceptibility to infection following prolonged high intensity exercise (18, 21, 46). In contrast, the T1 cell responses are strengthened following moderate intensity exercises which may enhance the immune responses to infectious agents (17, 19). However, these suggestions are based only on the results of

a small number of experiments. There have been no systematic investigations on the alterations of T1/T2 balance in response to exercise of various types, intensities and durations.

Given that the T1/T2 balance is considered to be closely associated with the immune function in response to different forms of exercise, the primary purpose of this brief review is to analyse the current literature regarding the specific effect of exercise intensity on T1 and T2 counts and function in humans or animals. Furthermore, acute and chronic effects of exercise on T1/T2 balance in different subject populations will be analysed. Whether the alterations in the T1/T2 balance following exercise can be used as an indicator for monitoring immune function and health of exercise participants will also be discussed.

## T1 AND T2 CELLS AND THEIR CYTOKINES

The innate immune system activates T cells and induces differentiation of naive T cells into polarised subsets of effector T cells, in response to infection, disease or physical exercise (9, 31, 47). The type 1/type 2 (Th1/Th2 and Tc1/Tc2) paradigm is a good indication of T cells polarisation. In simple terms, both Th1 and Th2 cells are differentiated $CD4^+$ T cells with Th1 cells producing predominantly IFN-γ (or IL-2), and Th2 cells producing IL-4 (or IL-10), respectively (28). In theory, Th1 and Th2 cells can be differentiated functionally and phenotypically by parameters other than IFN-γ and IL-4 production (13), but the two cytokines quintessentially classify the Th1/Th2 paradigm. Similarly, $CD8^+$ T cells can also develop into IFN-γ- or IL-4-producing cells that are called Tc1 and Tc2 cells, respectively (24). In the present review, we classify IFN-$\gamma^+CD4^+$ (or IL-$2^+CD4^+$), IFN-$\gamma^+CD8^+$ (or IL-$2^+CD8^+$), IL-$4^+CD4^+$, and IL-$4^+CD8^+$ cells as Th1, Tc1, Th2 and Tc2, respectively, and define IFN-$\gamma^+$ and IL-$4^+$ T cells as T1 and T2 cells, respectively. In addition, the concentration of these cytokines in T cell culture supernatants after stimulation is used as an indication of the functional changes of the polarised subsets.

## EFFECTS OF INFECTION FOLLOWING EXERCISE AND TRAINING ON T1/T2 CELL CONCENTRATION AND FUNCTION

Several studies have investigated the influence of viral infection such as herpes simplex virus type 1 (HSV-1) through an intranasal route, or lymphocytic choriomeningitis (LCMV) via injection following exercise on T1/T2 cells balance, using mice models (16-19). The results of these studies (table 1, see addendum) can be summarised in four points.

1) Exercise training with moderate intensity for 8 weeks up-regulated the function of both T1 and T2 cells in clearing HSV-1 in both young and older adult mice at different time points post training. This beneficial effect could last for at least 7 days.

2) Compared with the values measured 7 days after infection, both of T1 cell-derived IFN-γ, IL-2, and T2 cell-derived IL-10 concentrations increased 10 days after the infection in young adult mice, while the T1/T2 balance was still maintained.
3) In contrast, in older mice the cytokines associated with the balance of T1/T2 cells seemed heading to opposite directions at the two time points of tissue sampling after infection. The cytokine secretion patterns changed from type 1 phenotype at the 7$^{th}$ day post-infection to type 2 at the 10$^{th}$ day post-infection. In general, the change of T1/T2 balance appeared to be associated with ageing, and senescence might result in a defect in T1 cells development and a subsequent defect in cell-mediated immunity leading to an intracellular infection (1, 36). There was evidence that moderate intensity exercise training could prevent the defect from happening and maintain T1/T2 balance at the 7$^{th}$ day post-infection (17). However, excessive T1 cell response to viral infection might result in tissue damage (20). An elevation of T2 cell-like cytokines would then occur that would inhibit the differentiation of T1 cells through mutually inhibitory effect that would prevent further tissue damage at the 10$^{th}$ day post-infection.
4) High intensity exercises, either acute or chronic, could decrease the concentration of T1 cells or T1 cell-derived cytokines for clearing HSV-1 or LCMV in young adult mice. However, in older mice, high intensity exercise did not cause suppression of LCMV-specific T1 cell responses. The mechanisms underlying the differences observed in young and older mice are not clear and require further study.

## EFFECTS OF EXERCISE AND TRAINING ON T1/T2 CELL CONCENTRATION AND FUNCTION

### Effects of moderate intensity exercise

There have been several reports on T1/T2 balance in response to moderate intensity exercise in humans of different ages, including young adults who performed one bout of exercise and older adults who participated in exercise training (table 2, see addendum). One study demonstrated that T1 cell-derived IFN-γ increased significantly even just after a bout of moderate intensity exercise for 30 min, but none of T2 cell-derived cytokines were measured in that study (5). It was speculated that moderate intensity exercise might promote the T1-type cytokine response (5, 26).

It has been reported that long-term moderate intensity exercise training for 0.5, 2, or 4 years could all reverse age-associated reduction of T1 cells and T1 cell-derived cytokines (table 2, see addendum). There have been reports that moderate intensity exercise training was effective to improve immune function in older people, and it has been speculated that exercise-induced differentiation of naive T cells toward the type 1 phenotype is one of the mechanisms underlying the improvement (10, 30, 36).

However as demonstrated by Kohut et al. (2004), the function of both T1 and T2 cells in clearing HSV-1 were up-regulated in both young and older adult mice

after moderate intensity training (19). Therefore the T1/T2 balance may still be maintained when the T1 and T2 cells number and/or their cytokines both increase.

**Effects of high intensity exercise**

*Acute effects of high intensity exercise*

In this section, the acute effects of high intensity exercise in humans on T1 and T2 concentrations in peripheral blood (table 3, see addendum), and on the T1, T2-derived cytokine levels in T cell culture supernatants after stimulation (table 4, see addendum), are discussed, for the acute alteration of T1/ T2 distribution and function.

There has been consistent evidence that both of the distribution and function of T1 cells in the circulation are suppressed 1 h after one bout of prolonged (1 h and over) high intensity exercise, whereas no significant changes are found in the distribution and function of T2 cells (table 3, 4, see addendum). The suppression may last for more than 24 hours (5, 41). Furthermore, the trend of change in the cytokines produced by T1 and T2 cells is consistent with that of the number of the cells that produce these cytokines.

The suppression of the distribution and function of T1 cells following one bout of prolonged high intensity exercise may be associated with the changes of some hormones. In theory, glucocorticoids (GCs) act through their classic cytoplasmic/nuclear receptors on antigen presenting cells (APCs) to inhibit the production of IL-12 that is the main inducer of Th1 cells (7). IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells. Therefore, GCs' inhibition of IL-12 production may be a major mechanism by which GCs affect Th1/Th2 balance. Similarly, catecholamines (CAs) suppress the production of IL-12 and IFN-γ, and stimulate the production of type 2 cytokines (37). The correlation between stress hormones and T1-, T2-derived cytokines following exercise has been investigated using human models. It has been reported that the mean plasma adrenalin concentration correlated negatively with the percentage of circulating Tc1 cells at 2 h post-exercise (running at 75% $\dot{V}O_{2max}$ for 2.5 h), but plasma cortisol did not correlate with the percentage of circulating T1 cells (41). The relationship between cortisol and T1/T2 balance following exercise has not been reported and needs further investigation.

Interestingly, the changes of the polarised subsets of T cells are quite different from the changes of the cytokines secreted by these cells in the circulation within 5 min after a bout of prolonged high intensity exercise (table 3, 4, see addendum). The suppression of type 1 cytokines has been found immediately after an exercise and persisted for 24 h (22, 35, 40), but the concentration of type 1 T cells changed differently (increased, decreased or had no obvious change) immediately after or 5 min after similar or same exercise protocols (14, 21, 22, 35, 40). During a high intensity exercise, the circulating concentration of T1 cells can be affected more significantly by adrenalin and noradrenalin because there is a higher surface expression of β2-adrenergic receptors on T1 cells compared with T2 cells (27). The CAs mediate trafficking of circulating leukocytes from the spleen and lymphatic system to the circulation (positive effect for the circulating T1 cells) or

from the systemic circulation to the peripheral immune compartments such as skin, urogenital and gastrointestinal tracts (negative effect for the circulating T1 cells) (21). Therefore, the direction of the change in circulating T1 cells soon after high intensity exercise depends on the balance between the positive and negative effects under different exercise intensity and duration.

It is also interesting to find that high intensity exercise for a shorter duration (e.g. less than 30 min) did not decrease the distribution of T1 cells and inhibit the function of T1 cells in the circulation at 1 or 2 h post-exercise (40, 50). In these studies (40, 50), the changes in the distribution and function of T1 cells in the circulation immediately after exercise were also measured, but these changes were more likely having been affected by the stress hormones. With this consideration we just choose the time points at 1 or 2 hours post-exercise for discussion. Therefore, it seems that the number and function of the polarised subsets of T cells may also relate to the duration of high intensity exercise.

*Chronic effects of high intensity exercise*

In studies on both humans and animals, chronic and high intensity exercise training appeared to result in less T1 cell polarisation (table 5, see addendum). Furthermore, this inhibition of T1 cells could last for more than 7 days in mice (47). It is worthwhile to note that the values measured within one hour post-exercise would be more likely to reflect an acute response to the last exercise session, but not the cumulative effect of multiple bouts or days of exercise. Therefore, for a discussion of the chronic effects of exercise, the data collected at 1 h post last exercise session is not included, and the data collected next morning before exercise is used for discussion of the training effects (21) (table 5, see addendum).

To our knowledge, alteration in cytokines produced by the polarised subsets of T cells induced by chronic and high intensity exercise has not been investigated.

## CONTROVERSIES AND FUTURE DIRECTIONS

**The relationship between concentration and function of T1/ T2 cells**

As discussed above, alterations in T1 and T2 cell concentrations in peripheral blood are not consistent with T1- and T2-derived cytokine levels in T cell culture supernatants after stimulation, following a bout of intensive exercise. This disagreement was evident even under the same experimental conditions (40). A study (40) showed that high intensity exercise induced a promotion in the concentration of circulating T lymphocytes that produce IFN-γ ($P < 0.01$) and IL-2 ($P < 0.01$) immediately post-exercise. In contrast, exercise resulted in a decrease in the levels of IFN-γ ($P < 0.01$) and IL-2 ($P < 0.01$) produced by circulating lymphocytes at the same time point. Therefore, it is still inconclusive on whether an increased number of T1 cells in circulation is beneficial for cell-mediated immunity.

The suppression in the function of T1 cells, as indicated by decreased IFN-γ, has been associated with the expression of transcriptional factors such as signal trans-

ducer and activator of transcription 4 (STAT4), Th1-specific T box transcription factor T-bet, E twenty-six (ETS)-related transcription factor ERM, interferon regulatory factor-1 (IRF-1) which are crucial for the production of IFN-γ by T1 cells (32). Therefore, the number of T1 cells with lower expression of related transcriptional factors is not a meaningful indicator of cell immune response soon after high intensity exercise because these T1 cells can not produce IFN-γ effectively. In addition, in some other experimental reports included in the present review, exercise-induced changes in the concentration of circulating T1 and T2 cells were in parallel to that in the corresponding intracellular lymphocyte cytokines at other time points (21, 22, 35). Further research is needed to examine the relationship between the number of T1 and T2 cells and the level of related cytokines produced by these cells.

**The relationship between the amount of cytokines produced by circulating T1/T2 cells and the concentration of that in serum or plasma**

During and after exercise, the secretion of cytokines by T1/T2 cells into the circulation is a causative mediator of exercise-induced immune perturbation (43). Whether the changes in circulating cytokines can be used as an indication of the function of T1/T2 cells is an important question. Previous studies have shown that certain cytokines such as IL-6 and TNF-α are produced directly by exercising skeletal muscle or other tissues during exercise (39, 45). Other cytokines such as IFN-γ, IL-2 are produced primarily by T and natural killer (NK) lymphocytes in response to exercise (37). Considering the function of the pro-inflammatory cytokines (IFN-γ, IL-2) and anti-inflammatory cytokines (IL-4, IL-10), it seems reasonable to speculate that the up- or down-regulation in the circulation soon after exercise are supposed to be caused by promoting the trafficking of cytokines from the circulation to the vasculature of other immune compartments (e.g. skeletal muscle, urogenital and gastrointestinal tracts), instead of a reduction of the cytokines produced by circulating T1/T2 cells. Previous study have demonstrated that exercise-induced changes in the intracellular leukocyte cytokines (IFN-γ, IL-2, IL-4, IL-10) detected immediately after exercise are not necessarily in parallel to their changes in circulation (50). In another study, the plasma concentrations of IFN-γ, TNF-α, IL-2, IL-4 did not show significant changes immediately after a bout of exhaustive exercise (42). The relationship between the amount of cytokines produced by circulating T1/T2 cells and that in the serum or plasma at various time points post-exercise is still not clear.

**IFN-γ /IL-4 ratio and T1/T2 balance**

$CD4^+$ T cells and $CD8^+$ T cells are classified according to their cytokine profile as type 1 (Th1/Tc1) or type 2 (Th2/Tc2) (24). In order to more effectively evaluate the balance of T1 and T2 cells differentiation in response to physical activity, the ratio between $CD4^+$ T cells or $CD8^+$ T cells expressing intracellular IFN-γ and that expressing intracellular IL-4 has been used as an indication of Th1/Th2 or Tc1/Tc2 balance (14, 30). As mentioned before, the alteration in the concentration of T1/T2 cells are not always consistent with that in the corresponding intracellular cytokines after exercise (40). So the ratio might not be synchronous with the balance of cell-mediated immunity and humoral immunity.

Type 1 and Type 2 immune responses can be induced by T1- and T2-derived cytokines respectively, but that can't be induced directly by T1 and T2 cells. Obviously, it is more meaningful to evaluate T1/T2 balance using the concentration of cytokines than using the number of cells. Among these cytokines, IFN-γ and IL-4 are respectively signature cytokines of T1 and T2 cells (8). So we speculate that the ratio between IFN-γ and IL-4 produced by $CD4^+$ T cells and/or $CD8^+$ T cells may be an alternative indicator of Th1/Th2 or Tc1/Tc2. Likewise, to determine the IFN-γ/IL-4 ratio in plasma or serum might be more practical for evaluation of this balance at some particular time points after exercise if the two cytokines produced by circulating T1/T2 cells were in parallel to the concentrations of these cells in circulation. Furthermore, the ratio of IFN-γ/IL-4 in circulation and T cell culture supernatants after stimulation has been used as an indication of T1/T2 balance in the field of medical research (12, 13).

## CONCLUSIONS

Evidence accumulated over the past two decades indicates that: 1) high intensity exercise is in favour of type 2 phenotype T cells, which is one of the mechanisms underlying the down-regulation of host protection against viral infection post exercise; 2) moderate intensity exercise induces a shift of the type 1/type 2 T cell balance toward type 1 in older adults that is in the reverse of age-associated reduction of T1 cells or T1 cell-derived cytokines, and improves T1 cell function in young adults, but the effect of moderate intensity exercise on T2 cell function hasn't been clarified through experimental studies; 3) the alteration in cytokines produced by T1/T2 cells is not consistent with that in the number of cells expressing corresponding cytokines, and that of the same cytokines in the circulation in a short period of time after exercise, while the relationships in a long recovery period has not been examined; and 4) Th1/Th2 and Tc1/Tc2 ratios may be used as an indicator for T1/T2 cells differentiation but might not always represent their functional changes. The IFN-γ/IL-4 ratio in culture supernatant of stimulated T cells or in the circulation might be an effective indicator for monitoring the balance between cell-mediated immunity and humoral immunity.

# ADDENDUM

Table 1. Effects of infection following exercise on T1/T2 cells and related cytokines

| Reference | Subjects | Type of exercise | Level of exercise and protocol | Protocol of viral infection | Resource | post-infection |
|---|---|---|---|---|---|---|
| (17) | Young adult mice | Moderate, chronic, treadmill | Speed: 8 m/min during week one and gradually progressing to 18 m/min<br>Duration: gradually increased during week 1–4; from week 5–8, mice ran 40–45 min per day<br>Frequency: 5 days per week<br>Cycle: 8 weeks | Mice were infected with HSV-1 at 24 h post-exercise | Production by stimulated spleen cells | IFN-γ↑, IL-2↑; IL-10↑ (7 days post-infection) |
| | Older adult mice | | | | | IFN-γ↑*, IL-2↑*; IL-10↑ (7 days post-infection) |
| (19) | Young adult mice | Moderate, chronic, treadmill | Speed: 8 m/min during week one and gradually progressing to 18 m/min<br>Duration: gradually increased during week 1–4; from week 5–8, mice ran 40–45 min per day<br>Frequency: 5 days per week<br>Cycle: 8 weeks | Mice were infected with HSV-1 at 24 h post-exercise | Production by stimulated spleen cells | IFN-γ↑*, IL-2↑*; IL-10↑* (10 days post-infection) |
| | Older adult mice | | | | | IFN-γ–, IL-2–; IL-10↑* (10 days post-infection) |
| (16) | Young adult mice | Strenuous, acute, treadmill | Incremental exercise to exhaustion. Speed was increased from 4 m/min to 32 m/min by 4 m/min every minute; 13.1±4.6 min | Mice were infected with LCMV immediately post-exercise | Spleen cells | T1↓* (8 days post-infection) |
| | Older adult mice | | Incremental exercise to exhaustion. Speed was increased from 4 m/min to 17 m/min by 4 m/min every minute; 11.9±7.2 min | | | T1– (8 days post-infection) |
| (18) | Young adult mice | Strenuous, acute, treadmill | Gradually increasing speeds until fatigue. Speed began at 11.5 m/min and was increased by 4–6 m/min every 25 min. The maximum speed reached was 42 m/min<br>Duration: 2.5 h | Mice were infected with HSV-1 within 10-15 min post-exercise | Production by stimulated spleen cells | IFN-γ↓*, IL-2↓*; IL-10– (3 days post-infection) |

↑ higher than that in the control group; ↓ lower than that in the control group; – no obvious change; * significantly different from the control group.

Table 2. Effects of moderate intensity exercise on T1/T2 cells and related cytokines

| Reference | Subjects | Type of exercise | Level of exercise and protocol | Source | Post-exercise |
|---|---|---|---|---|---|
| (5) | Young adult human | Moderate, acute, cycling | 70% of their 4-mmol/L lactic acid threshold, 30 min | Production by stimulated peripheral blood lymphocytes | IFN-γ↑ (30 min post-exercise)<br>IFN-γ↑* (24 h post-exercise) |
| (10) | Older adult human | Moderate, chronic.<br>1.workout while marching and standing;<br>2. workout in low positions – in squat, on hands and knees, lying on your side, in prone position | Intensity: pulse rate ≦ 80% of the age-predicted maximum (200-age)<br>Duration: 50 min<br>Frequency: twice a week during 10 months of the year<br>Cycle: 2 years | Production by stimulated peripheral blood lymphocytes | IL-2↑*, IFN-γ–; IL-4– (At least the next day after the last exercise. The exact interval was not reported in this study) |
| (30) | Older adult human | Moderate, chronic, walking | Intensity: equivalent to 57% $\dot{V}O_2$ peak for 30 min every day, and for one and a half hours once a week<br>Duration and frequency: 3-5 km every day and 10 km once a week<br>Cycle: 4 years | Peripheral blood | Th1↑*, Tc1↑; Th2↑, Tc2↑; Th1/Th2 two-fold↑, Tc1/Tc2↑ (at least 24 hours after the last exercise) |
| (36) | Older adult human | Moderate, chronic, cycling and resistance training | Intensity and duration: part 1 (endurance training) was a cycle-ergometer exercise (30 min) at 80% work rate of the DPBP▲. Part 2 (endurance training) requires their muscles to work against gravity by moving their own weight up and down, which comprised three sets of seven exercises (10 repetitions).<br>Frequency: 5 days a week<br>Cycle: 6 months | Peripheral blood | Th1↑*; Th2↓ (at least 24 hours after the last exercise) |

↑ higher than pre-exercise or that in the control group; ↓ lower than that in the control group; – no obvious change; * significantly different from the control group.
▲DPBP (double-product break-point) is the point of accelerating double product (heart rate × systolic blood pressure), which has been shown to have strong positive correlations with the lactate and ventilatory thresholds.

Table 3. Acute effects of high intensity exercise on T1/T2 cells

| Reference | Subjects | Type of exercise | Intensity | Duration | Source | Immediately post-exercise | 1-2 h post-exercise | 1 d post-exercise |
|---|---|---|---|---|---|---|---|---|
| (41) | Endurance-trained male runners | Treadmill | 75% $\dot{V}O_{2max}$ | 2.5 h | Peripheral blood | Th1↓*, Tc1↓*; Th2–, Tc2– | Th1↓*, Tc1↓*; Th2–, Tc2– (2 h post-exercise) | Th1↓*, Tc–; Th2–, Tc2– |
| (22) | Moderately to well endurance-trained men | Cycle | 65% $\dot{V}O_{2max}$ | 2.5 h | Peripheral blood | Th1–, Tc1↑*; Th2–, Tc2– | Th1↓*, Tc1↓*; Th2–, Tc2– (2 h post-exercise) | |
| (21) | Endurance-trained male cyclists | Cycle | Exercise to exhaustion at ~63% Wmax (~74% $\dot{V}O_2$ max) | 107±7 min | Peripheral blood | T1↓*; T2– | T1↓*; T2– (1 h post-exercise) | |
| (14) | Healthy men | Treadmill, 5% downhill incline | 75% $\dot{V}O_{2max}$ | 1.5 h | Peripheral blood | Th1↑, Tc1↑; Th2↑, Tc2↑; Th1/Th2↑, Tc1/Tc2↑ | Th1↓, Tc1↓; Th2↑, Tc2↑; Th1/Th2↓, Tc1/Tc2↓ (2 h post-exercise) | |
| (35) ▲ | Male volunteers with several years of rowing experience | Rowing on a rowing ergometer | The highest possible power output (Average % of $\dot{V}O_2$ peak: 73.2±4.0) that could be maintained for 1 h | 1 h | Peripheral blood | Th1–, Tc1– (5 min post-exercise) | Th1↓*, Tc1↓ (1 h post-exercise) | |
| (40) | Endurance-trained men | Supine bicycle | 78 ± 3% $\dot{V}O_2$ peak | 19±1 min | Peripheral blood | T1↑* | T1– (2 h post-exercise) | |

↑ higher than pre-exercise; ↓ lower than pre-exercise; – no obvious change; * significantly different from pre-exercise.
▲ IL-2$^+$CD4$^+$ cells and IL-2$^+$CD8$^+$ cells were classified as Th1 and Tc1 respectively because IFN-γ$^+$CD4$^+$ cells and IFN-γ$^+$CD8$^+$ cells were not measured in this study.

Table 4. Acute effects of high intensity exercise on T1, T2-derived cytokines

| Reference | Subjects | Type of exercise | Intensity | Duration | Source | 0-30 min post-exercise | 1-24 h post-exercise |
|---|---|---|---|---|---|---|---|
| (22) | Moderately to well endurance-trained men | Cycle | 65% $\dot{V}O_{2max}$ | 2.5 h | Production by stimulated peripheral blood CD4+ T cells | IFN-γ↓*, IL-4– (immediately after exercise) | IFN-γ↓*, IL-4– (2 h post-exercise) |
| | | | | | Production by stimulated peripheral blood CD8+ T cells | IFN-γ↓*; IL-4– (immediately after exercise) | IFN-γ↓*; IL-4– (2 h post-exercise) |
| (2) | Male cyclist | Cycle | 90% of the anaerobic threshold | 6 × 20 min | Production by stimulated peripheral blood cells | IFN-γ 16% ↓, TNF-a 26%↓, IL-2 35%↓; IL-4 35.5%↓ (immediately after exercise) | |
| (21) | Endurance-trained male cyclists | Cycle | Exercise to exhaustion at ~63% Wmax (~74% $\dot{V}O_{2max}$ ) | 107±7 min | Production by stimulated peripheral blood CD3+ T lymphocytes | IFN-γ↓*; IL-4– (immediately after exercise) | IFN-γ↓; IL-4– (1 h post-exercise) |
| (5) | Healthy people | Cycle | 100% of their 4-mmol/L lactic acid threshold | 90 min | Production by stimulated peripheral blood cells | IFN-γ↓ (30 min post-exercise) | IFN-γ↓ (24 h post-exercise) |
| (35) | Male volunteers with several years of rowing experience | Rowing on a rowing machine | The highest possible power output (Average % of $\dot{V}O_2$ peak: 73.2±4.0) that could be maintained for 1 h | 1 h | Production by stimulated peripheral blood cells | IFN-γ–, IL-2↓* (5 min post-exercise) | IFN-γ↓*, IL-2↓* (1 h post-exercise) |
| (3) | Elite male triathletes | Swimming, cycling, and running | São Paulo International Triathlon | ? | Production by stimulated peripheral blood cells | IFN-γ 35.1% ↓, TNF-a 16.9%↓ (15 min post-exercise) | |
| (4) | Elite male triathletes and marathoners | Running or swimming, cycling, and running | São Paulo International Triathlon or running 30 km in 2 h | ? | Production by stimulated peripheral blood cells | IFN-γ 27% ↓, TNF-a 19%↓; IL-4 18.7%↓ (15 min post-exercise) | |
| (50) | Healthy males | Cycle | 80% of the peak $\dot{V}O_2$ | 30 min | Production by stimulated peripheral blood cells | IFN-γ↑, IL-2↑*, TNF-a↑*; IL-4↑* , IL-10↑*, IL-6↑ (immediately after exercise) | IFN-γ↓, IL-2↑, TNF-a↓; IL-4↓, IL-10↓, IL-6↓ (1 h post-exercise) |
| (38) | Well trained competitive oarsmen | Rowing on a rowing machine | Incremental exercise to exhaustion. Male: $\dot{V}O_{2max}$ was 135.8±6.6% predicted; Females: $\dot{V}O_{2max}$ was 138.4±12.3% predicted | Male: 16.0±0.92 min; Females: 15.5±0.0 min | Production by stimulated peripheral blood cells | IFN-γ↓*, TNF-a↓*; IL-6↓*, IL-10↓ (20 min post-exercise) | |
| (40) | Endurance-trained men | Supine bicycle | 78 ± 3% $\dot{V}O_2$ peak | 19 ±1 min | Production by stimulated peripheral blood cells | IFN-γ↓*, IL-2↓* (immediately after exercise) | IFN-γ↓, IL-2↓ (2 h post-exercise) |

↑ higher than pre-exercise; ↓ lower than pre-exercise; – no obvious change; * significantly different from pre-exercise.
? duration was not reported in the study.

Table 5. T1/T2 cell changes after long-term high intensity exercise

| Reference | Subjects | Type of exercise | Level of exercise and protocol | Source | Within 36 h post-exercise | 7 d post-exercise |
|---|---|---|---|---|---|---|
| (21) ▲ | endurance-trained male cyclists | Cycle | Intensified training period for 7 days | Peripheral blood | T1↓*; T2– (next morning to the last exercise) | |
| (47) | Female rats | treadmill | Progressive load training period for 9 weeks (6 days a week)<br>week 1: 15 m/min × 40 min at 2% (grade)<br>week 2: 20 m/min × 60 min at 10% (grade)<br>week 3: 25 m/min × 90 min at 10% (grade)<br>week 4: 30 m/min × 120 min at 5% (grade)<br>week 5: 30 m/min × 120 min at 5% (grade)<br>week 6: 30 m/min × 120 min at 8% (grade)<br>week 7: 35 m/min × 120 min at 10% (grade)<br>week 8: 35 m/min × 120 min at 15% (grade)<br>week 9: 35 m/min × 120 min at 15% (grade) | Spleen cells | T1↓; T2↑ (36 h post-exercise) | T1↓*; T2↑* |

↑ higher than that in the control group; ↓ lower than pre-exercise or that in the control group; – no obvious change; * significantly different from pre-exercise or that in the control group.

▲ The blood samples were collected before, immediately after and 1 h after the first exercise and last exercise of 8 days training period which consists of 2-day of $VO_{2max}$ tests (exercise to exhaustion at ~74% $VO_2$ max) and 6-day intensified training period in the experiment. But we regard the samples collected at the time point of pre-exercise on the $8^{th}$ day training in order to differentiate from the acute effect of exercise in the present article.

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# EXERCISE IMMUNOLOGY REVIEW

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*Hinnak Northoff*
for the editorial team and all our readers

# *IL-17, neutrophil activation and muscle damage following endurance exercise*

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## ABSTRACT

*The T-cell subset Th17 is induced partly by interleukin (IL)-6 and activated by IL-23, and produces a proinflammatory cytokine IL-17. Since IL-6 increases dramatically following long-lasting endurance exercise, this response may also stimulate the induction of IL-17 and IL-23 after exercise. The aim of this study was to clarify the dynamics of IL-17 in association with endurance exercise-induced muscle damage and inflammatory responses. Fourteen male triathletes participated in a duathlon race consisting of 5 km of running, 40 km of cycling and 5 km of running. Venous blood and urine samples were collected before, immediately after, 1.5 h and 3 h after the race. Plasma and urine were analyzed using enzyme-linked immunosorbent assays (ELISA). Haematological and biochemical variables such as neutrophil activation marker (myeloperoxidase: MPO), muscle damage marker (myoglobin: Mb) and soluble receptor activator of nuclear factor (NF)-κB ligand (sRANKL) were also determined to estimate the biological and pathological significance. Plasma concentrations of IL-6 (+26.0×), MPO (+3.2×) and Mb (+4.9×) increased significantly immediately after the race and IL-17 and IL-23 tended to increase. Furthermore, plasma concentrations of IL-12p40 and sRANKL increased significantly after the race. The measured parameters related to Th17 cytokines in the urinary output were closely correlated with each other and muscle damage marker. These findings suggest that IL-17 induced by IL-6 and activated by IL-23 or other IL-17 producing-cells and IL-23 might promote neutrophil activation and muscle damage following prolonged endurance exercise.*

**Key words:** interleukin (IL)-17, helper T (Th) cells cytokine balance, neutrophil activation, muscle damage, urinary excretion

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## INTRODUCTION

Exercise induces peripheral blood neutrophilia (28, 29), and enhances the capacity of neutrophils and monocytes to produce reactive oxygen species (ROS) (22, 29). Furthermore, exercise induces neutrophil and cytokine accumulation in damaged muscle (25), which then releases myocellular proteins such as creatine kinase (CK) and myoglobin (Mb) into the circulation in a delayed-onset manner (30, 31). The involvement of neutrophils in muscle damage has also been demonstrated in animal experiments in which administration of anti-neutrophil antibody to deplete circulating neutrophils prevented post-exercise muscle proteolysis due to neutrophil infiltration (17). It was also observed that in myeloperoxidase (MPO) knockout mice, soleus muscles showed a significant 52% reduction in membrane lysis compared with wild-type mice (18), suggesting that MPO-containing neutrophils and their activation factors such as proinflammatory cytokines facilitate muscle damage following exercise.

Exhaustive exercise induces a systemic inflammatory response syndrome (SIRS), characterized by hypercytokinaemia (19, 22, 23, 32). It is apparent that the cytokine response to exercise differs from that elicited by severe infections. In sepsis, tumour necrosis factor (TNF)-α and interleukin (IL)-1 stimulate the production of IL-6, whereas IL-6 is the first cytokine present in the circulation during exercise (15, 20). The level of circulating IL-6 increases in an exponential fashion (up to 100-fold) in response to exercise (24). In these circumstances, it is suggested that stimulated production of proinflammatory cytokines by increased IL-6 might be related to muscle damage and neutrophil activation following exhaustive endurance exercise, but which proinflammatory cytokines are involved has not been determined yet.

IL-17 and IL-23 are also classified as proinflammatory cytokines. A subset of CD4+ T cells, helper T (Th) 17 cells and innate immune cells such as γδ T cells are major producers of IL-17 (4, 5, 37). It is clear that the Th17 subset has a role as a Th cell with a unique function distinct from Th1 and Th2 (7, 8, 12, 27, 35). IL-17 is believed to act primarily on parenchymal cells such as fibroblasts, epithelial cells, and endothelial cells. Signaling by IL-17 increases matrix metalloproteinase and proinflammatory cytokine expression (12, 36). IL-17 also acts to recruit neutrophils to peripheral sites through the induction of chemokines such as IL-8 (12). In addition, it has been reported that IL-17 promotes osteoclastogenesis through the induction of receptor activator of nuclear factor (NF)-κB ligand (RANKL) on osteoblasts (13). On the other hand, IL-23 is made by both dendritic cells (DCs) and macrophages (10). The receptor for IL-23 is expressed on activated/memory T cells (11). IL-23 has an important role in the regulation of the innate immune response, and also could serve to expand and stabilize Th17 responses (16, 34). The mRNA expression of RANKL correlates with that of IL-23 in the synovial tissues of patients with rheumatoid arthritis which develops by involvement of Th17 cells (26). Based on these findings, it might be hypothesized that neutrophil activation and inflammatory reactions via NF-κB induced by IL-17 and IL-23 are related to muscle damage following exhaustive endurance exercise.

As described above, Th17 cells are induced party by IL-6 and activated by IL-23, resulting in the production of the proinflammatory cytokine IL-17 (1, 2).

Since IL-6 increases dramatically during long-lasting endurance exercise, this response may also stimulate the induction of IL-17 and IL-23 after exercise. The aim of this study was to clarify the dynamics of IL-17 in association with endurance exercise-induced IL-6 release, neutrophil activation and muscle damage.

## METHODS

### Subjects

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

### Duathlon race

The present investigation was conducted at the 19$^{th}$ Kikunotsuyu duathlon race on March 16$^{th}$, 2008. It was held on the road course of Miyako Island, Okinawa, Japan. This 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 the same breakfast at 08:30. The breakfast contained 574 kcal, with 22.1 g protein, 13.7 g fat and 88.8 g carbohydrate. With the subjects resting quietly, the pre-race blood and urine samples (Pre) were collected at 10:30. They 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 (1.5 h) and 3 h (3 h) after the race. Peripheral blood samples were drawn by antecubital venepuncture with the subjects in the sitting position. They ate the same 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 after the race until 3 h after the race.

### Haematological and biochemical parameters

Approximately 7 ml of blood was drawn by a standard venepuncture technique from the antecubital vein using vacutainers containing no additive or 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 one 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. Complete blood cell counts, haemoglobin and haematocrit were determined on EDTA-treated venous blood using an automatic blood cell counter (pocH-100i, Sysmex, Kobe, Japan). Serum concentrations of creatinine (Cre), Mb and CK activity were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan).

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 Cre and Mb were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan).

**Assays for inflammatory substances**

Inflammation-related substances were measured in serum, EDTA-plasma and urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. We chose to measure the concentrations of IL-17 and IL-23 (Quantikine, R&D Systems, Minneapolis, MN, USA), IL-6 (Quantikine HS, R&D Systems, Minneapolis, MN, USA), IL-12p40 (OptEIA, Beckton Dickinson Biosciences, San Diego, CA, USA), MPO (Hbt ELISA test kit, Hycult biotechnology, Uden, The Netherlands), and sRANKL (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria) using ELISA. These concentrations were determined by comparison to a standard curve established in the same set of measurements using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA).

**Statistical analyses**

Data are presented as means ± SD. Statistical validation was made 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 (r). Statistical significance was evaluated at $p < 0.05$ or $p < 0.01$.

## RESULTS

**Haematological data**

Total neutrophil counts were significantly elevated immediately (+4.9×), 1.5 h (+4.4×) and 3 h (+4.1×) after exercise as compared to the pre-exercise values. Total lymphocyte counts were also elevated immediately after the race (+1.7×), but decreased 1.5 h and 3 h post-exercise compared with the pre-exercise values. Haemoglobin (+1.1×) and haematocrit (+1.1×) values increased significantly immediately after the race; thereafter both haemoglobin and haematocrit returned to pre-exercise levels, indicating that haemoconcentration occurred during exercise. Therefore, the post-exercise raw data were adjusted for alterations in plasma volume (Table 1).

**Biochemical data**

Serum concentrations of Cre (+1.4×) and Mb (+4.9×) and serum CK (+1.2×) activity increased significantly immediately after the race. Thereafter, CK

**Table 1.** Changes of haematological parameters following the duathlon race.

| | Unit | Pre | 0 h | 1.5 h | 3 h | Friedman test | Scheffe test |
|---|---|---|---|---|---|---|---|
| leucocyte | $\times 10^2/\mu l$ | 47.4±13.6 | 147.9±37.5 | 136.2±35.7 | 124.9±36.5 | ** | Pre-0 h**, Pre-1.5 h**, Pre-3 h* |
| neutrophil | $\times 10^2/\mu l$ | 25.4±10.9 | 113.0±33.0 | 113.4±31.3 | 102.6±32.9 | ** | Pre-0 h**, Pre-1.5 h** |
| lymphocyte | $\times 10^2/\mu ll$ | 16.9±4.8 | 25.6±8.2 | 13.6±5.0 | 14.6±5.1 | ** | Pre-0 h*, 0 h-1.5 h**, 0 h-3 h** |
| Hb | g/dl | 14.3±1.2 | 15.1±1.2 | 14.3±1.1 | 14.5±1.1 | ** | Pre-0 h*, 0 h-1.5 h** |
| Hct | % | 42.6±3.3 | 45.1±3.2 | 42.4±3.0 | 42.9±3.1 | ** | Pre-0 h**, 0 h-1.5 h**, 0 h-3 h* |

Values: means ± SD (n=14). Statistics: * $p < 0.05$ and ** $p < 0.01$.
leucocyte: leucocyte count, neutrophil: neutrophil count, lymphocyte: lymphocyte count, Hb: Haemoglobin concentration, Hct: haematocrit in the peripheral venous blood.

remained elevated by 3 h post-exercise; Cre and Mb decreased, but remained above pre-exercise values at 1.5 h (Cre: +1.3×, Mb: +5.7×) and 3 h (Cre: +1.2×, Mb: +4.9×) post-exercise.

The amount of urinary Mb decreased significantly immediately after the race and thereafter increased. Urinary Cre concentration was elevated significantly 1.5 h post-exercise (+2.2×) and then decreased. Because Cre clearance changed following exercise (3), the urinary concentrations of cytokines and other markers are reported as the gross amount (Table 2).

## Cytokines, MPO and sRANKL

The plasma concentrations of IL-6 (+26.0×), IL-12p40 (+1.3×) and MPO (+3.2×) increased significantly immediately after the race. Plasma concentrations of IL-6

**Table 2.** Changes of biochemical parameters following the duathlon race.

| | Unit | Pre | 0 h | 1.5 h | 3 h | Friedman test | Scheffe test |
|---|---|---|---|---|---|---|---|
| Cre-S | mg/dl | 0.81±0.08 | 1.11±0.21 | 1.08±0.19 | 1.00±0.16 | ** | Pre-0 h**, Pre-1.5 h**, 0 h-3 h* |
| Cre-U | g/l | 1.1±0.3 | 2.0±1.4 | 2.4±1.5 | 1.3±0.8 | ** | Pre-1.5 h* |
| Mb-S | ng/ml | 42.9±10.8 | 210.1±111.4 | 245.1±135.6 | 212.1±125.7 | ** | Pre-0 h*, Pre-1.5 h**, Pre-3 h* |
| Mb-U | ng | 2462.7±1187.1 | 681.2±490.0 | 1114.0±632.3 | 2018.5±2118.6 | ** | Pre-0 h** |
| CK-S | U/l | 357.9±264.8 | 437.7±290.9 | 495.4±291.6 | 528.3±299.1 | ** | Pre-1.5**, Pre-3 h**, 0 h-3 h* |

Values: means ± SD (n=14). Statistics: * $p < 0.05$ and ** $p < 0.01$.
Cre-S: serum creatinine concentration, Cre-U: urinary creatinine concentration, Mb-S: serum myoglobin concentration, Mb-U: urinary myoglobin amount, CK-S: serum creatine kinase activity.

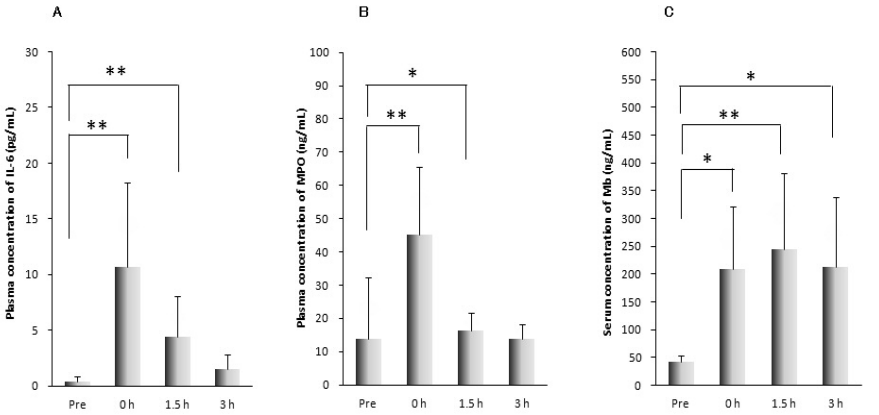


**Figure 1.** Changes in plasma concentrations of interleukin 6 (IL-6) and myeloperoxidase (MPO) and serum concentration of myoglobin (Mb) following the duathlon race.
A: plasma concentration of IL-6
B: plasma concentration of MPO
C: serum concentration of Mb
Values: means + SD.
Statistics: *$p < 0.05$ and **$p < 0.01$.

(+10.9×) and MPO (+1.2×) were also significantly higher 1.5 h after the race compared with the pre-exercise values. Plasma concentrations of IL-17 and IL-23 decreased significantly immediately after the race, but were significantly higher at 1.5 h and 3 h compared with values at 0 h post-exercise. There was a trend for serum sRANKL concentration to increase following exercise.

In contrast, the urinary amounts of IL-6, IL-17, IL-23 and IL-12p40 decreased significantly immediately after the race. Thereafter, the urinary amounts of IL-6 (+3.4×), IL-17 (+2.6×) and IL-12p40 (+5.3×) increased significantly at 3 h after the race compared with immediately after the race. The urinary amount of sRANKL changed significantly following exercise. There was a trend for urinary MPO to change following exercise (Fig. 1) (Table 3).

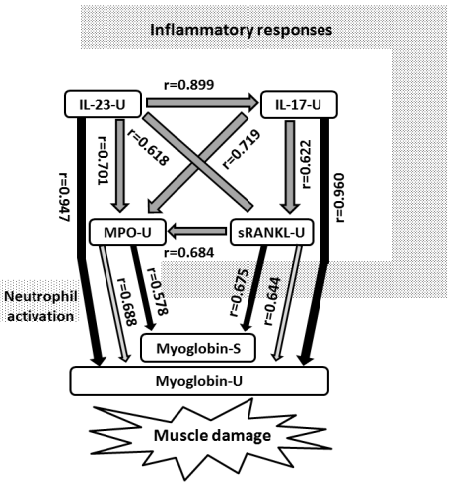


**Figure 2.** Correlations of inflammatory responses, neutrophil activation and muscle damage markers.
MPO: neutrophil activation marker
sRANKL: NF-κB activation factor
IL-17: proinflammatory cytokine
IL-23: promoter of the differentiation of Th17 cells

## Associations between measured parameters

The area under the curve (AUC) for

**Table 3.** Changes in plasma and urinary cytokines following the duathlon race.

| | Unit | Pre | 0 h | 1.5 h | 3 h | Friedman test | Scheffe test |
|---|---|---|---|---|---|---|---|
| MPO-P | ng/ml | 13.9±18.5 | 45.2±20.5 | 16.3±5.2 | 14.0±4.4 | ** | Pre-0 h**, Pre-1.5 h*, 0 h-3 h** |
| MPO-U | ng | 33.3±15.6 | 14.7±10.1 | 20.6±12.1 | 28.8±29.0 | † | - |
| IL-17-P | pg/ml | 2.4±1.0 | 2.0±0.7 | 2.5±0.8 | 2.6±0.8 | ** | Pre-0 h**, 0 h-1.5 h**, 0 h-3 h** |
| IL-17-U | pg | 845.9±349.7 | 280.0±171.6 | 340.8±189.4 | 735.3±751.2 | ** | Pre-0 h**, 0 h-3 h* |
| IL-23-P | pg/ml | 5.0±2.9 | 4.1±2.3 | 4.9±3.0 | 5.4±3.2 | ** | Pre-0 h*, 0 h-1.5 h*, 0 h-3 h** |
| IL-23-U | pg | 966.4±463.9 | 319.8±209.1 | 407.5±247.3 | 1169.0±1204.2 | ** | Pre-0 h** |
| sRANKL-S | pmol/l | 0.14±0.10 | 0.15±0.11 | 0.16±0.13 | 0.18±0.14 | † | - |
| sRANKL-U | pmol | 0.22±0.16 | 0.07±0.07 | 0.08±0.07 | 0.20±0.23 | * | - |
| IL-12p40-P | pg/ml | 91.0±43.9 | 118.7±51.4 | 118.4±53.1 | 98.7±35.7 | ** | Pre-0 h* |
| IL-12p40-U | pg | 18815.5±14747.8 | 5494.1±4524.7 | 16808.3±16851.0 | 28872.4±14574.4 | ** | Pre-0 h**, 0 h-3 h** |

Values: means ± SD (n=14). Statistics: * $p < 0.05$, ** $p < 0.01$, † $p < 0.1$.
- P & -S: Data are adjusted for alterations in plasma volume.
- U: Data are the gross amount in the volume of urinary excretion.
Abbreviations: interleukin (IL)-17, 23, 12p40, myeloperoxidase (MPO), soluble receptor activator of nuclear factor (NF)-κB ligand (sRANKL).

Pre-, 0 h, 1.5 h and 3 h of plasma concentrations of IL-17 (IL-17-P) was correlated with that of IL-12p40-P ($r = 0.613$, $p < 0.05$). The AUC of urinary amounts of IL-17 (IL-17-U) was correlated with MPO-U ($r = 0.719$, $p < 0.01$), sRANKL-U ($r = 0.622$, $p < 0.05$), Mb-U ($r = 0.960$, $p < 0.01$), IL-23-U ($r = 0.899$, $p < 0.01$) and that of serum CK (CK-S) activity ($r = 0.543$, $p < 0.05$). The AUC of IL-23-P was correlated with IL-12p40-P ($r = 0.622$, $p < 0.05$). The AUC of IL-23-U was correlated with MPO-U ($r = 0.701$, $p < 0.01$), sRANKL-U ($r = 0.618$, $p < 0.05$), Mb-U ($r = 0.947$, $p < 0.01$) and CK-S ($r = 0.587$, $p < 0.05$). The AUC of sRANKL-U was correlated with MPO-U ($r = 0.684$, $p < 0.01$), Mb-U ($r = 0.644$, $p < 0.05$) and Mb-S ($r = 0.675$, $p < 0.01$). The AUC of Mb-S was correlated with that of MPO-U ($r = 0.578$, $p < 0.05$). The AUC in Mb-U was positively correlated with MPO-U ($r = 0.688$, $p < 0.01$) and CK-S ($r = 0.582$, $p < 0.05$) (Table 4) (Fig. 2).

## DISCUSSION

The Th1/Th2 cytokine balance is an important paradigm from an immunomodulatory viewpoint, and the balance of cellular and humoral immunity regulated by Th1 (IL-12, IL-2, interferon (IFN)-γ and TNF-α) and Th2 (IL-4 and IL-10) cytokines is increasingly recognized to be important in the maintenance of health

**Table 4.** Spearman's rank correlation coefficient matrix of inflammatory mediators, neutrophil activation and muscle damage markers.

| | IL-17-P | IL-17-U | IL-23-P | IL-23-U | sRANKL-P | sRANKL-U | Mb-S | Mb-U | CK | MPO-P | MPO-U | IL-12p40-P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-17-P | | 0.015 | 0.288 | 0.068 | 0.064 | −0.275 | −0.090 | 0.046 | 0.336 | −0.222 | −0.336 | 0.613* |
| IL-17-U | 0.015 | | 0.262 | 0.899** | −0.077 | 0.622* | 0.451 | 0.960** | 0.543* | −0.121 | 0.719** | 0.143 |
| IL-23-P | 0.288 | 0.262 | | 0.051 | 0.213 | 0.147 | 0.112 | 0.138 | 0.429 | −0.169 | 0.130 | 0.622* |
| IL-23-U | 0.068 | 0.899** | 0.051 | | −0.138 | 0.618* | 0.587* | 0.947** | 0.587* | −0.160 | 0.701** | 0.156 |
| sRANKL-P | 0.064 | −0.077 | 0.213 | −0.138 | | −0.033 | 0.099 | −0.112 | 0.130 | 0.398 | −0.244 | −0.112 |
| sRANKL-U | −0.275 | 0.622* | 0.147 | 0.618* | −0.033 | | 0.675** | 0.644* | 0.297 | 0.143 | 0.684** | −0.077 |
| Mb-S | −0.090 | 0.451 | 0.112 | 0.587* | 0.099 | 0.675** | | 0.534* | 0.125 | 0.262 | 0.578* | 0.213 |
| Mb-U | 0.046 | 0.960** | 0.138 | 0.947** | −0.112 | 0.644* | 0.534* | | 0.582* | −0.095 | 0.688** | 0.152 |
| CK | 0.336 | 0.543* | 0.429 | 0.587* | 0.130 | 0.297 | 0.125 | 0.582* | | −0.147 | 0.196 | 0.455 |
| MPO-P | −0.222 | −0.121 | −0.169 | −0.160 | 0.398 | 0.143 | 0.262 | −0.095 | −0.147 | | −0.073 | −0.327 |
| MPO-U | −0.336 | 0.719** | 0.130 | 0.701** | −0.244 | 0.684** | 0.578* | 0.688** | 0.196 | −0.073 | | 0.051 |
| IL-12p40-P | 0.613* | 0.143 | 0.622* | 0.156 | −0.112 | −0.077 | 0.213 | 0.152 | 0.455 | −0.327 | 0.051 | |

All data were calculated as area under the curve (AUC).
Statistics: * $p < 0.05$, ** $p < 0.01$.
AUC: total value of Pre, 0 h, 1.5 h and 3 h.
– P & -S: plasma and serum data were adjusted for alteration in plasma volume.
– U: urinary data were the gross amount into the volume of urinary excretion.
Myoglobin (Mb), creatine kinase (CK), interleukin (IL)-17, 23, 12p40, myeloperoxidase (MPO), soluble receptor activator of nuclear factor (NF)-κB ligand (sRANKL).

and the development of immune-based diseases including infections, autoimmune, allergic and asthmatic diseases (21). Several studies have reported that blood concentrations of Th1 cytokines show no change or decrease, and that peripheral blood production of Th1 cytokines by lymphocytes decreases following exhaustive exercise (32, 36). In this study, the plasma concentrations of the Th1 cytokines IL-12 (0.27 ± 0.16 pg/ml), IL-2 (0.24 ± 0.27 pg/ml), IFN-γ (0.12 ± 0.23 pg/ml) and TNF-α (0.09 ± 0.04 pg/ml) were low immediately after the race. On the other hand, several studies have reported that blood concentrations of Th2 cytokines (IL-4 and IL-10) and anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1ra) increase following exhaustive exercise (13, 19, 26). In the present study, the plasma concentration of IL-10 increased significantly immediately after the race and IL-1ra also increased markedly 1.5 h after the race. It has been reported that plasma IL-4 increased several hours after exhaustive exercise (32). In the present study, the plasma concentration of IL-4 was low immediately (0.33 ± 0.15 pg/ml), 1.5 h (0.30 ± 0.10 pg/ml) and 3 h (0.24 ± 0.08pg/ml) after the race. Th1 and Th2 cell differentiations depend on their respective effector cytokines (i.e., IFN-γ and IL-4, respectively) (4). Therefore, it might be difficult to state that Th2 cytokine responses occurred during exercise at least in the circulation.

Aside from Th1 and Th2 cytokines, a third family of effector T-cells, Th17 cells are also important to induce inflammation. Th17 cell development can occur in the presence of IL-6 and moreover in the absence of IFN-γ and IL-4 (1, 36). IL-23 is not involved in the initial differentiation of Th17 cells (14), however, IL-23 promotes the production of IL-17 from Th17 cells (16). In this study, the plasma concentrations of IFN-γ (0.12 ± 0.23 pg/ml) and IL-4 (0.33 ± 0.15 pg/ml) were lower than IL-17 (2.0 ± 0.7 pg/ml) immediately after the race, whereas the plasma concentration of IL-6 was markedly elevated immediately after the race (+26.0×), and this response occurred earlier than the IL-1β and TNF-α responses. Moreover, IL-12 (IL-12p70) is a heterodimeric molecule formed by the subunits p35 and p40 and classified as a major immunomodulatory cytokine promot-

ing the differentiation of Th1 cells, whereas IL-12p40 is a homodimer and acts as an antagonist of IL-12 (6) and has recently been recognized as having some homology with IL-23 (8). IL-23 is a heterodimeric molecule formed by subunits p40 and p19, and IL-12p40 is a monodimeric molecule formed by subunit p40. Hence, IL-12p40 and IL-23 might share p40 to induce production of IL-17. The plasma concentration of IL-12 (0.27 ± 0.16 pg/ml) was much lower than IL-12p40 (118.7 ± 51.4 pg/ml) immediately after the race in this study. Therefore, we examined the associations between these cytokines. Positive correlations were found for changes in the plasma concentrations of IL-12p40 and IL-17 and IL-23. That is, it might be possible that released IL-6 induced IL-17, IL-23 and IL-12p40, activated neutrophils and/or monocytes and was related to inflammation.

We observed more close associations in the urinary analyses. IL-17 was correlated with IL-23, MPO, Mb and sRANKL. IL-23 was also correlated with MPO, Mb and sRANKL. MPO was correlated with Mb and sRANKL. Mb was correlated with sRANKL. MPO is an activation marker of neutrophils, Mb is a marker of muscle damage and sRANKL is a factor of activated NF-κB, and these markers were closely correlated. Taken together, these findings suggest that IL-17 induced by IL-6 and IL-23 activates sRANKL. Moreover, our results suggest that IL-17 and IL-23 might promote neutrophil activation and muscle damage following prolonged endurance exercise (Fig. 2).

As mentioned earlier, we observed that plasma concentrations of IFN-γ and IL-4 were low, whereas the urinary excretion of these cytokines were large following exhaustive exercise in this study which implies that IFN-γ and IL-4 production was increased during exercise. Therefore, it might be possible that the differentiation of naive CD4+ T cells to Th17 cells was suppressed in the presence of IFN-γ and IL-4 during and following exhaustive endurance exercise (9, 36). Moreover, because there were trends for plasma IL-17 and IL-23 concentrations to increase following exercise and urinary excretion of IL-6 was not correlated with IL-17, IL-23, MPO, Mb and sRANKL, it might be possible that IL-17 was produced by either Th17 cells or by other cells (2, 4, 5, 37). Further research is needed to determine the mechanisms influencing the plasma concentrations and urinary excretion of each cytokine 3 h after exhaustive endurance exercise, and to clarify which cells produce IL-17 in relation to neutrophil activation and muscle damage during and following prolonged endurance exercise.

In conclusion, it is suggested that IL-17 induced by IL-6 and activated by IL-23 might promote neutrophil activation and muscle damage in a different way from the classical proinflammatory cytokines IL-1β and TNF-α following prolonged endurance exercise. However, further research is needed to clarify the cells that produce IL-17 in relation to neutrophil activation and muscle damage during and following prolonged endurance exercise.

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# *A soy-based supplement alters energy metabolism but not the exercise-induced stress response*

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## ABSTRACT

***Objective***
*To determine the changes in endurance capacity as well as in metabolic, hormonal and inflammatory markers induced by endurance training combined with a soy protein based supplement.*

***Design***
*Randomized controlled study consisting of moderate endurance training without (G0) or with (G1) a soy protein based supplement.*

***Subjects***
*Two groups of 15 subjects (10 males and 5 females in each group): healthy sports students aged 23.6 ± 1.9 years.*

***Measurements***
*Body composition (body mass (BM), body density (BD) by air displacement) and physical fitness (determined by treadmill ergometry) were measured at baseline and after 6 weeks of the intervention; changes in circulating metabolic and hormonal parameters (glucose, lactate, urea, uric acid, ammonia, cortisol, insulin, IGF-1), and exercise-induced stress and inflammatory markers (CK, LDH, myoglobin, hs-CRP, IL-6, IL-10, blood cell counts) were determined after the intervention period in a field test (11.5 km running on hilly ground).*

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***Results***
*30 participants completed the 6-week study; 28 students were able to perform the field test. No significant changes in BM and BD were noted after intervention with only slight increases in running performance and maximum aerobic capacity in the total group (2%, p=0.016). Subjects in the G1 group showed significant improvements in running velocity and lower lactate values following the intervention (-12%, p=0,003). In addition, the G1 group showed significantly lower differences in the exercise-induced increase of metabolic parameters (triglycerides, uric acid) and insulin in the post-exercise recovery period.*

***Conclusions***
*Our data suggest that moderate endurance training in combination with a soy-based protein supplement improves aerobic energy supply and metabolic function in healthy sports students, even without changes in body composition and without changes in the exercise-induced stress and inflammatory reaction.*

## INTRODUCTION

The ratio of the different dietary macronutrients has a distinct influence on body composition and metabolic functions. However, the exact amount and the underlying mechanisms are still under discussion for both physically inactive and overweight as well as active and normal-weight subjects (22). Recently, it has been shown that protein rich diets may not only increase thermogenesis, but improve glycaemic control, induce weight loss, help to stabilize weight following weight reduction and limit the loss of muscle mass (11, 12). These facts may be of special interest for overweight and insulin resistant subjects (10, 20), but also normal weight and physically active subjects may benefit from an improved carbohydrate metabolism and insulin sensitivity known to be of high relevance for fuel selection and in particular for the utilization of fatty acids (19, 41). Therefore, a protein-rich diet could help to improve aerobic capacity and metabolic flexibility in combination with an endurance-training programme.

So far, ingestion of protein or essential amino acids has been predominantly linked to an increased muscular protein synthesis. Although results are equivocal, studies in humans suggest that protein ingestion can enhance skeletal muscle hypertrophy in response to chronic resistance training. The evidence linking protein intake to an enhanced endurance capacity is still scarce (34). Recently, some authors suggested that protein intake during or after exercise may induce an ergogenic effect on endurance performance (7, 14, 30). Nevertheless, there is no consensus whether the ergogenic effect of protein is only an effect of adding calories, or may actually be a unique and specific benefit of the protein intake itself (8, 28). Therefore, we performed an intervention study in which the combination of endurance training and a soy-based protein supplement in healthy sports students was tested. The food supplement was a commercially available soy-yoghurt-honey product (Almased®) which has been recently tested for its significant benefits in body composition and metabolism control (11, 12, 20).

The aim of the study was to answer the following questions:

1. Is there an association between the intake of a protein-rich supplement over 6 weeks and changes in body composition?
2. Does the intake of the supplement influence endurance performance and energy supply during exercise?
3. Does the intake of the supplement induce changes in circulating hormone levels?
4. Does the intake of the supplement influence the exercise-induced muscular or systemic stress reactions?

## METHODS

The study was designed as a RCT (Randomized Controlled Trial) according to GCP (Good Clinical Practice). 30 clinically healthy sports students of both genders were randomized to a verum (G1; N=15, 10 males, 5 females) and control (G0; N=15, 10 males, 5 females) group. Both groups were instructed to perform a moderate endurance training (60 minutes per day, 5 times a week) at the aerobic threshold over a period of 6 weeks. In addition, the subjects of the verum group (G1) were instructed to take two 50 g servings of the supplement (Almased® containing 53.3 g protein, 30.5 g carbohydrates, 2.0 g fat, 354 kcal per 100 g) solubilized in 200 ml water every day for 6 weeks, until the day of the field test. During the study period one participant dropped out due to a viral infection in each group resulting in 2 x 14 completers according to the study protocol.

Three examinations were carried out: U-1 as pre-intervention examination including health control, anthropometric measurements, blood drawing (blood specimen "a") and performance diagnostics (5); U-2 as post-intervention examination after the 6-week training period (both groups) and intake of the protein-rich supplement (verum group) with the same conditions as in U-1; and finally U-3 as a post-intervention running stress test (cross-country race on hilly ground with a distance of 11.5 km) starting at 11:00 a.m., three hours after breakfast. At U-3 blood samples were drawn at 8:00 a.m. at rest in a fasting state ("b") as well as one ("c") and four hours ("d") after performing the field test; an additional blood sample was drawn at 8:00 a.m. at rest in a fasting state the morning after the field test ("e").

The following parameters concerning muscular energy supply and exercise-induced stress reaction were measured: blood glucose, blood lactate, serum urea, uric acid, ammonia; creatine kinase (CK), lactate dehydrogenase (LDH), myoglobin, cortisol, insulin, IGF-1, hs-CRP, blood cell count, IL-6, IL-10; all variables were measured with standardized methods of clinical chemistry.

In addition, body composition was analyzed by air displacement plethysmography with the Bod-Pod technology (13) before and after intervention (U-1, U-2). Individual data regarding health status, training and dietary behavior were collected by weekly protocols.

For statistical evaluation the SPSS 18.0.2 program was used. For intra- and inter-individual comparisons the groups were examined with the Friedman test (non-parametric equivalent of ANOVA), intra-individually over all time points as well as inter-individually with the principle of the repeated measurements. To reject the null hypotheses, a significance level of $p < 0.05$ was used.

The study protocol was approved by the Ethical Commission of the University of Freiburg. All participants were educated by an informational conversation supported by written information and gave their written consent to participate in the study.

## RESULTS

Both groups were comparable in gender, age, body composition and physical fitness at the beginning of the study (Table 1). After the 6-week intervention, in both groups, no changes in body mass or body density were detectable (Table 2). An increase in body mass by the additionally supplied calories could not be established in the verum group. In addition, there were no differences in health status, training or dietary behaviour between the groups.

**Tab. 1:** Personal and anthropometric data of the subjects (N=30) examined

| | G0 (N=15) | G1 (N=15) |
|---|---|---|
| **Sex [male/female]** | 10/5 | 10/5 |
| **Age [yrs]** | 24.0 ± 2.1 | 23.3 ± 1.6 |
| **Weight [kg]** | 67.5 ± 10.6 | 68.3 ± 12.3 |
| **Height [m]** | 1.76 ± 0.10 | 1.73 ± 0.11 |
| **Body mass [kg/m$^2$]** | 21.8 ± 2.21 | 22.5 ± 2.15 |
| **$VO_2$max [ml/kg/min]** | 50.8 ± 6.43 | 51.1 ± 5.66 |

The performance capacity of the total group of students, described as maximum running velocity (km/h) in the treadmill test, showed a minor increase of +2% after the training programme of 6 weeks; however, these changes were statistically significant ($p=0.022$ and $p=0.016$, respectively) (Table 2). In the verum group, running velocity at the defined lactate thresholds (at 2 mmol/l and 4 mmol/l blood lactate) increased significantly by about 15% ($p=0.011$, Table 2).

Only in the verum group blood lactate values in the stress test after intervention were significantly reduced (Table 2): at the aerobic threshold -20% ($p=0.009$), at the anaerobic threshold -11% ($p=0.009$), and for total lactate production (calculated as sum of lactate of each stress step) -12% ($p=0.003$). In contrast to the changed lactate kinetics of the stress test, there were no differences with respect to the results of the field test (G0: 60.2 ± 8.8 min versus G1: 61.4 ± 9.9 min) or

**Tab. 2:** Anthropometric and performance data before and after intervention in both groups; intra-group significance (before/after intervention) given as index * ($p<0.05$) and ** ($p<0.01$)

| | G0 (before) | G0 (after) | G1 (before) | G1 (after) |
|---|---|---|---|---|
| **Weight [kg]** | 67.5 ± 10,6 | 68.7 ± 11,2 | 68.3 ± 12.3 | 68.2 ± 12.1 |
| **Density [kg/l]** | 1.067 ± 0.019 | 1.066 ± 0.017 | 1.065 ± 0.016 | 1.064 ± 0.018 |
| **Running velocity max [km/h]** | 16.1 ± 2.07 | 16.4 ± 1.65 | 16.2 ± 1.80 | 16.4 ± 1.83 |
| **Running velocity 2mmol L [km/h]** | 10.1 ± 2.04 | 10.4 ± 2.22 | 9.28 ± 2.75 | 10.6 ± 2.45 ** |
| **Running velocity 4mmol L [km/h]** | 13.2 ± 1.49 | 13.5 ± 1.58 | 12.8 ± 2.00 | 13.3 ± 2.16 ** |
| **Lactate [mmol/l] resting state** | 1.38 ± 0.22 | 1.48 ± 0.25 | 1.44 ± 0.29 | 1.26 ± 0.23 * |
| **Lactate [mmol/l] aer threshold** | 1.52 ± 0.45 | 1.55 ± 0.48 | 1.81 ± 0.60 | 1.44 ± 0.50 ** |
| **Lactate [mmol/l] anaer threshold** | 3.06 ± 0.49 | 3.06 ± 0.48 | 3.31 ± 0.60 | 2.94 ± 0.49 ** |
| **Lactate [mmol/l] sum over all** | 22.9 ± 8.26 | 21.4 ± 8.77 | 25.9 ± 6.12 | 22.7 ± 6.17 ** |

the training amount (G0: 250 ± 54.5 min/week versus G1: 231 ± 58.2 min/week) between both groups.

Blood glucose metabolism was not influenced by the intervention; as expected, exercise induced a significant fall in blood glucose concentration of about approximately -15 mg/dl 1 hour after strain ($p=0.001$) in both groups (Table 3). The production of urea during and after the field test was significantly influenced by the intervention. In both groups there was a significant increase of urea production after exercise (Table 3); however, the increase in urea during endurance exercise was more pronounced in the verum group ($p=0.005$). Within one day after the field test, urea values comparable to pre-stress levels were reached in both groups again (Table 3). In contrast, there was a more pronounced increase in uric acid in the control group (Table 3) as an end metabolite of the purine nucleotide cycle (PNC); particularly in the morning after the field test ($p=0.028$) (Table 3). In both groups a stress-induced increase in blood ammonia was seen ($p=0.001$); this increase was also more pronounced in the control group ($p=0.06$ for inter-individual comparison) (Table 3).

The regulation of the triglycerides in the recovery phase showed a correlation to the time as well as the assigned group (Table 3). In intra-individual comparison,

the triglycerides showed a stronger increase (p=0.016) in the control group compared with the verum group; this led to significantly different triglyceride values 4 hours after the field test between the groups (p=0.018) (Table 3 and Figure 1b).

Markers of muscular (Table 4), systemic (Table 4), and immunological stress (Table 5) showed significant changes after exercise in both groups (all p=0.001 for CK, LDH and myoglobin, for the blood cell counts as well as for IL-6 and IL-10); hs-CRP was significantly elevated in both groups the day after the field test (p=0.013, Table 5). However, regarding these parameters there was no evidence for significant effects of the intervention.

**Tab. 3:** Metabolic substrates before and after intervention in the course of the field test; n.e. (not estimated); intra-group significance (before/after field test) given as index * (p<0.05), ** (p<0.01) and *** (p<0.001)

| Glucose [mg/dl] | G0 | G1 |
|---|---|---|
| **a** | 80.5 ± 8.58 | 81.6 ± 9.24 |
| **b** | 90.6 ± 5.51 | 90.33 ± 5.17 |
| **c** | 75.1 ± 16.6 *** | 74.3 ± 11.2 *** |
| **d** | 90.7 ± 14.7 | 90.4 ± 10.9 |
| **e** | 86.73 ± 9.20 | 86.6 ± 3.70 |

| Urea [mg/dl] | G0 | G1 |
|---|---|---|
| **a** | 34.0 ± 6.94 | 33.0 ± 7.71 |
| **b** | 30.7 ± 5.69 | 34.8 ± 7.14 |
| **c** | 34.8 ± 6.69 *** | 43.4 ± 7.23 *** |
| **d** | 36.3 ± 6.66 *** | 48.4 ± 9.25 *** |
| **e** | 33.6 ± 8.11 *** | 35.8 ± 5.62 * |

| Uric acid [mg/dl] | G0 | G1 |
|---|---|---|
| **a** | 4.86 ± 0.99 | 4.89 ± 0.94 |
| **b** | 4.49 ± 0.65 | 4.66 ± 0.85 |
| **c** | 5.53 ± 0.79 *** | 5.53 ± 0.85 *** |
| **d** | 5.37 ± 0.85 *** | 5.31 ± 0.89 *** |
| **e** | 5.11 ± 0.82 *** | 4.88 ± 0.87 |

| Ammonia [µmol/l] | G0 | G1 |
|---|---|---|
| **a** | n.e. | n.e. |
| **b** | 29.9 ± 6.70 | 27.1 ± 4.22 |
| **c** | 42.0 ± 13.7 *** | 35.5 ± 6.94 *** |
| **d** | 37.2 ± 9.12 *** | 31.9 ± 7.55 *** |
| **e** | n.e. | n.e. |

| Triglycerides [mg/dl] | G0 | G1 |
|---|---|---|
| **a** | 77.3 ± 38.1 | 88.9 ± 45.2 |
| **b** | 90.9 ± 33.8 | 88.0 ± 36.3 |
| **c** | 126 ± 71.3 * | 90.1 ± 39.4 |
| **d** | 150 ± 85.0 * | 87.2 ± 39.6 |
| **e** | 87.1 ± 57.5 | 77.5 ± 45.4 |

**Tab. 4:** Muscular stress indicators (CK, LDH activity, myoglobin concentration) and blood cell counts (Tsd, thousands of cells) before and after intervention in the course of the field test¸ intra-group significance (before/after field test) given as index * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$)

| CK [U/l] | G0 | G1 |
|---|---|---|
| **a** | 199 ± 141 | 243 ± 258 |
| **b** | 152 ± 104 | 146 ± 151 |
| **c** | 253 ± 145 *** | 258 ± 236 *** |
| **d** | 460 ± 475 *** | 409 ± 329 *** |
| **e** | 749 ± 426 ** | 441 ± 395 *** |

| LDH [U/l] | G0 | G1 |
|---|---|---|
| **a** | 181 ± 23.8 | 188 ± 52.2 |
| **b** | 173 ± 29.1 | 179 ± 42.7 |
| **c** | 230 ± 39.2 *** | 225 ± 47.1 *** |
| **d** | 216 ± 41.2 *** | 233 ± 50.6 *** |
| **e** | 200 ± 54.3 *** | 200 ± 39.3 *** |

| Myoglobin [µg/l] | G0 | G1 |
|---|---|---|
| **a** | 71.4 ± 15.6 | 72.3 ± 22.7 |
| **b** | 64.6 ± 9.07 | 64.5 ± 5.79 |
| **c** | 255 ± 147 *** | 297 ± 161 *** |
| **d** | 199 ± 123 *** | 243 ± 140 *** |
| **e** | 82.1 ± 24.6 *** | 80.4 ± 45.0 *** |

| Leucocytes [Tsd/µl] | G0 | G1 |
|---|---|---|
| **a** | 6.63 ± 1.12 | 6.25 ± 0.94 |
| **b** | 6.29 ± 1.27 | 6.68 ± 1.56 |
| **c** | 11.7 ± 2.46 *** | 12.3 ± 2.71 *** |
| **d** | 12.0 ± 2.62 *** | 12.4 ± 1.57 *** |
| **e** | 6.56 ± 1.17 | 6.39 ± 1.32 |

| Lymphocytes[Tsd/µl] | G0 | G1 |
|---|---|---|
| **a** | 2.55 ± 0.34 | 2.66 ± 0.31 |
| **b** | 2.50 ± 0.47 | 2.53 ± 0.64 |
| **c** | 1.61 ± 0.33 *** | 1.46 ± 0.47 *** |
| **d** | 1.99 ± 0.55 * | 2.03 ± 0.46 * |
| **e** | 2.41 ± 0.39 | 2.22 ± 0.48 |

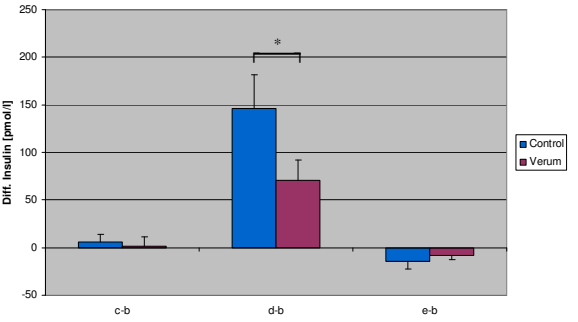
| Neutrophiles [Tsd/µl] | G0 | G1 |
|---|---|---|
| **a** | 3.28 ± 1.15 | 2.88 ± 0.56 |
| **b** | 3.03 ± 1.01 | 3.31 ± 1.34 |
| **c** | 9.01 ± 2.15 *** | 9.37 ± 2.51 *** |
| **d** | 9.07 ± 2.39 *** | 9.10 ± 1.25 *** |
| **e** | 3.39 ± 1.01 | 3.33 ± 1.16 |

**Tab. 5:** Systemic and immunological stress indicators (serum concentration of hs-CRP, IL-6, and IL-10) before and after intervention in the course of the field test; intra-group significance (before/after field test) given as index * (p<0.05), ** (p<0.01) and *** (p<0.001)

| hs-CRP [mg/dl] | G0 | G1 |
|---|---|---|
| **a** | 0.76 ± 0.63 | 0.80 ± 0.72 |
| **b** | 0.75 ± 0.59 | 0.69 ± 0.63 |
| **c** | 0.97 ± 0.69 | 1.10 ± 1.59 |
| **d** | 1.01 ± 0.69 | 1.40 ± 2.25 |
| **e** | 1.74 ± 1.10 * | 2.49 ± 3.66 * |

| IL6 [pg/ml] | G0 | G1 |
|---|---|---|
| **a** | 0.86 ± 0,80 | 0.51 ± 0.37 |
| **b** | 0.81 ± 0,62 | 0.97 ± 1.27 |
| **c** | 2.33 ± 1,44 *** | 2.96 ± 1.13 *** |
| **d** | 1.20 ± 1,39 | 1.35 ± 0.96 |
| **e** | 0.82 ± 0,71 | 1.51 ± 1.83 |

| IL10 [pg/ml] | G0 | G1 |
|---|---|---|
| **a** | 0.45 ± 0.48 | 0.42 ± 0.30 |
| **b** | 0.51 ± 0.57 | 0.59 ± 0.56 |
| **c** | 16.2 ± 12.6 *** | 13.8 ± 11.0 *** |
| **d** | 1.52 ± 1.59 * | 6.16 ± 11.5 * |
| **e** | 3.13 ± 6.78 * | 2.23 ± 3.45 * |



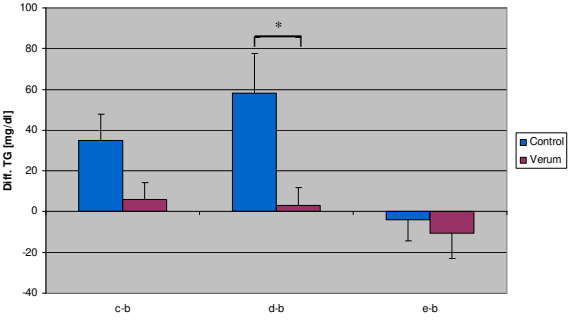




**Fig. 1a and 1b:** Insulin (1a) and triglyceride (1b) response after the endurance stress test (11.5 km on hilly ground) in the control and verum group (* p<0.05)

**Tab. 6:** Blood hormones (serum concentration of insulin, cortisol, IGF-1 and HGH) before and after intervention in the course of the field test; intra-group significance (before/after field test) given as index * (p<0.05), ** (p<0.01) and *** (p<0.001)

| Insulin [pmol/l] | G0 | G1 |
|---|---|---|
| **a** | 46.5 ± 23.6 | 40.5 ± 19.8 |
| **b** | 57.0 ± 33.2 | 51.6 ± 12.3 |
| **c** | 63.4 ± 36.5 | 53.1 ± 34.4 |
| **d** | 203 ± 154 *** | 122 ± 84.3 ** |
| **e** | 42.6 ± 21.5 | 43 ± 16.9 |

| Cortisol [nmol/l] | G0 | G1 |
|---|---|---|
| **a** | 621 ± 209 | 683 ± 205 |
| **b** | 709 ± 170 | 746 ± 194 |
| **c** | 736 ± 233 | 723 ± 278 |
| **d** | 426 ± 199 *** | 404 ± 225 *** |
| **e** | 631 ± 161 * | 696 ± 189 ** |

| IGF-1 [ng/ml] | G0 | G1 |
|---|---|---|
| **a** | 220 ± 77.0 | 231 ± 63.1 |
| **b** | 253 ± 63.8 | 293 ± 58.4 |
| **c** | 265 ± 66.7 | 288 ± 54.4 |
| **d** | 261 ± 68.8 | 296 ± 55.1 |
| **e** | 259 ± 78.3 | 304 ± 69.8 |

| HGH [ng/ml] | G0 | G1 |
|---|---|---|
| **a** | 2.56 ± 3.58 | 3.00 ± 5.94 |
| **b** | 3.15 ± 4.55 | 2.27 ± 3.29 |
| **c** | 2.84 ± 2.72 | 2.66 ± 2.18 |
| **d** | 1.24 ± 2.21 * | 1.14 ± 1.78 * |
| **e** | 4.45 ± 6.95 | 1.58 ± 2.31 |

In both groups a significant increase in serum insulin after the field test was detectable in the recovery phase (4 hours after field test: p=0.001) (Table 6). Being specified for the group factor, this increase was more pronounced in the control group (p=0.09; Figure 1a). The IGF-1 levels of both groups showed neither stress-induced nor intervention related changes (Table 6). The cortisol levels showed a stress-induced decrease in the recovery phase in both groups (4 hours after field test: p=0.001); however, group-specific differences were not detectable (Table 6).

## DISCUSSION

The main result of the present study was that a 6-week soy-based protein supplementation promotes an increased aerobic energy provision during endurance exercise of moderate intensity. It was shown that the concentration of lactic acid at both aerobic and anaerobic threshold and also the total lactate production during the treadmill test to exhaustion was lower following the soy protein supplementation.

With respect to anaerobic energy supply, a reduced ammonia and uric acid production was found following the 11.5 km running test. Increased concentrations of ammonia and uric acid indicate adenine nucleotide breakdown and the utilization of the anaerobic purine nucleotide cycle (3, 5). It has been shown previously that endurance exercise leads to an increased utilization of some free amino acids for energy provision (15). As the exercise-induced changes in urea were more pronounced in the verum group, it can be assumed that there was an additional energy supply from protein in this group. Finally, the significant differences in the post-exercise regulation of triglyceride and insulin metabolism suggest an improved use of fatty acids for energy metabolism (1, 18, 32) induced by the regular intake of the soy-based dietary supplement. This effect could only be shown in the verum group, whereas in the control group an increased hepatic synthesis of triglycerides by the unutilized free fatty acids has to be assumed (19). This observation is in accordance with the results of studies investigating molecular effects of soy protein in rats (27)(24).

The results are also consistent with experimental data of a recently published trial in which the specific effects of this soy protein supplement on postprandial fuel selection and appetite regulation were investigated (21). The glycaemic and insulinaemic responses were considerably higher after a standardized breakfast with a high GI than following the soy protein supplement. In addition, the postprandial decrease in fat oxidation was significantly less pronounced after intake of the supplement; this effect was also detectable after lunch as a "second-meal" effect. It has been demonstrated by several groups that a lower GI of a pre-exercise meal was associated with a higher fat oxidation both before and during exercise (9, 35, 36, 38, 42). This effect can mainly be attributed to lower pre-exercise insulin concentrations which will lead to enhanced peripheral lipolysis, increased plasma FFA and increased β-oxidation in skeletal muscles. On the one hand, this effect could be used to improve training gain in endurance type sports, on the other hand, it has a glycogen sparing effect, thereby minimizing the ergolytic effects of carbohydrate depletion. Glycogen sparing means that an increased fat oxidation during intense, prolonged endurance exercise reduces the relative proportion of carbohydrate oxidation. The lower rate of carbohydrate oxidation will preserve the intramuscular and intrahepatic glycogen stores. These stores can be used in the later stages of exercise and prevent premature fatigue (35, 42).

Considerable research has been done during the last decades to elucidate the effect of macronutrient composition on physical performance, particularly endurance performance (25). For endurance athletes, a high-carbohydrate diet (6, 16) has been recommended by sports nutritionists. The diet should contain 6 to 8 grams of carbohydrates per kilogram of body mass (31). However, it should be mentioned that this amount of carbohydrates can easily reach up to 2,400 kilocalories of carbohydrate per day and may eventually interfere with an adequate intake of protein (4, 17, 23, 37). In addition, dietary carbohydrates, particularly with a high glycaemic load, generate high blood glucose and insulin levels which could impair fat metabolism (2). A higher intake of protein may be a feasible way to burn more fat (10, 21). Furthermore, there is experimental evidence that soy protein influences cellular energy metabolism by molecular mechanisms. Soy

protein improves insulin resistance and lipid levels by activating peroxisome-proliferator activated receptors (PPARs) (39). PPARs are known as nuclear receptors which control metabolic processes, particularly affecting energy metabolism, by regulating the expression of genes involved in glucose homeostasis, lipid metabolism, and fatty acid oxidation (26, 27, 33, 43). It has been shown (26) that consumption of isoflavone-rich soy protein improves glucose tolerance, insulin resistance and hepatic triglyceride concentrations in rats. In addition, these investigators showed that isoflavone-rich soy extracts increased the gene expression of PPARs in cell culture studies, suggesting that the beneficial effects of soy protein on glucose and lipid metabolism may be mediated by PPAR activation.

More recently, it was also demonstrated (27) that soy protein feeding in rats increased the activity and mRNA levels of several skeletal muscle enzymes involved in fatty acid oxidation, including carnitine palmitoyltransferase (CPT1) activity and beta-hydroxyacyl-CoA dehydrogenase (HAD), acyl-CoA oxidase, and medium-chain acyl-CoA dehydrogenase. Moreover, PPAR gamma coactivator-1 (PGC1)- alpha and PPAR-alpha mRNA levels were also found to be elevated, suggesting that soy protein intake stimulates skeletal muscle fatty acid oxidation by activating PPAR pathways leading to a reduced accumulation of body fat (24). It may be assumed that soy protein works in the same or a similar manner in human organisms, however, comparable results from experimental studies in humans are still lacking. Therefore, further research is needed to confirm this assumption.

In the present study, it was further investigated if the intake of the supplement could also prevent muscle soreness and exercise-induced inflammatory stress. It has been assumed that an improvement in these parameters may enhance regeneration and help to achieve a stable fitness level (29)(40). However, there is no consensus whether an additional protein intake could prevent or reduce post-exercise muscular or systemic stress (4, 28, 34).

Apart from an increased post-exercise glycogen resynthesis, many other mechanisms have been discussed which could improve the immune response during and following exercise (28). These include an increased central drive, a blunting of exercise-induced muscle damage (8), and a modification in the pattern of exercise-related cytokine production. However, in our study we found no indices to assume such mechanisms. It could be possible that the study protocol (moderately endurance-trained subjects, 6 weeks of supplementation, duration of stress test 60 minutes) was not suitable to induce respective changes. Either the supplementation period was too short or the stress test was not appropriate. In addition, it has to be critically remarked that many studies so far have failed to demonstrate improved stress tolerance or altered immune function by measuring stress markers in the blood.

The intake of the protein-rich food supplement was not associated with changes in body composition within the 6-week period. The combined effects of protein supplementation and physical training on body composition and particularly muscle mass in healthy and trained subjects have been equivocal. It can be assumed that in

the present design, the length of supplementation and training volume (frequency x intensity x duration) were not sufficient to induce alterations in body composition.

In conclusion, the results support the hypothesis that the soy-based food supplement promotes aerobic energy supply during moderate endurance training. In addition, in these healthy and normal-weight sports students, the intervention led to an increased endurance performance. It can be assumed that the supplement significantly influences the supply of fat as a source of energy during exercise. The group-specific behaviour in the post-exercise triglyceride and insulin kinetics, which were evident in the regeneration phase, suggests an altered mitochondrial metabolism of the muscle cells and an improved use of fatty acids for energy metabolism following additional soy protein intake.

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# *Immune Cell Inflammatory Cytokine Responses Differ Between Central and Systemic Compartments in Response to Acute Exercise in Mice*

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## ABSTRACT

***Background:*** *Exhaustive exercise induces apoptosis and oxidative stress in systemic organs and tissues and is associated with increased levels of pro-inflammatory cytokines. The effects of acute exercise on cytokine expression and apoptosis of immune cells in the central nervous system (CNS) have not been well characterized.*
***Purpose****: We investigated the effects of a single bout of strenuous exercise on the expression of TNF-α, IL-6, and IL-β, as well as the apoptotic status of cells in the hippocampus of healthy mice. To compare central vs. systemic differences, cytokine expression in the intestinal lymphocytes of a subset of mice were also assessed.*
***Methods:*** *Female C57BL/6 mice were divided into three groups: sedentary controls (NOTREAD) (n = 22), treadmill exercise with immediate sacrifice (TREAD-Imm) (n = 21), or treadmill exercise with sacrifice after 2 hours (TREAD-2h). TNF-α, IL-6, and IL-1β expression in the hippocampus and intestinal lymphocytes were measured by Western blot analysis. Percentages of hippocampal cells undergoing apoptosis (Annexin$^+$) or necrosis (Propidium Iodide$^+$) were determined through flow cytometry. Plasma levels of 8-isoprostane and corticosterone were measured using commercially available EIA kits.*
***Results:*** *Acute treadmill exercise led to significant decreases in TNF-α ($p<0.05$) and increases in IL-6 ($p<0.05$) expression in the hippocampus of healthy mice. No effects of acute exercise on the apoptotic status of hippocampal cells were observed. In intestinal lymphocytes, the exercise bout led to significant increases in TNF-α ($p<0.05$), IL-6 ($p<0.05$), and IL-1β ($p<0.05$). Acute exercise was associated with a significant increase in both plasma 8-isoprostane ($p<0.05$) and corticosterone ($p<0.05$) levels.*
***Conclusion:*** *Acute exercise differentially affects the pattern of pro-inflammatory cytokine expression in the hippocampus compared to intestinal lymphocytes and, further, does not induce apoptosis in hippocampal cells.*

**Key words:** Acute exercise, hippocampus, intestinal lymphocytes, cytokines, apoptosis, oxidative stress

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## INTRODUCTION

Exercise can have a positive and a negative impact on the immune system depending on its duration and intensity. Acute exercise is associated with reduced immune function and increased risk of infection (22; 27; 34). Moreover, exhaustive exercise leads to leukocytosis immediately followed by lymphocytopenia (40), which may be due in part to exercise-induced DNA fragmentation and apoptosis (26; 29; 31). Many of these effects are thought to be the result of increased oxidative stress in the affected tissues. Oxidative stress is an imbalance between endogenous antioxidants, such as superoxide dismutase and glutathione peroxidase, and reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radical (48). Acute exercise leads to increases in oxygen consumption, which then results in the formation of ROS and consumption of intracellular antioxidant levels, alterations in mitochondrial membrane potential, and DNA damage leading to apoptosis (29; 42). Essential components of apoptotic cell death are caspase-3 and caspase-7 (2; 15) both of which are significantly elevated after prolonged exhaustive exercise (18; 19; 20). In addition to changes in oxygen metabolism, acute exercise alters the cytokine balance systemically. For example, physical activity at intensities greater than 70% $VO_{2max}$ increases the levels of TNF-$\alpha$ (a cytokine that can activate apoptosis) in plasma (36; 51; 53; 57) and colonic lymphocytes (19; 20). Similar exercise-induced increases were also observed for the cytokines IL-1$\beta$ and IL-6 (36; 51; 57) suggesting that strenuous exercise (and especially eccentric exercise with muscle damage) results in inflammation.

Few studies, however, have determined if acute exercise affects pro-inflammatory cytokine status in the central nervous system (CNS) and none have examined its effects on apoptosis of immune cells in the brain. Scopel et al. (47) found that in rats two weeks of treadmill exercise at 60% $VO_{2max}$ worsens existing damage to hippocampal mitochondria induced by in vitro oxygen and glucose depletion. In contrast, Ackigoz et al. (1) reported that exhaustive treadmill running (25 m/min, 5° slope) in Wistar rats did not change superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities or thiobarbituric acid reactive susbstance (TBARS) levels in the hippocampus, prefrontal cortex, or striatum; this exercise protocol was therefore not associated with significant lipid peroxidation in these brain compartments. In an older study, Radak et al. (45) reported that exhaustive treadmill exercise at 24 m/min and 15% incline did not alter levels of SOD, catalase, GPx or TBARS in the hippocampus and cerebellum of rats. Somani et al. (49) reported no changes in SOD activity in the cortex, striatum, cerebellum, medulla, and hypothalamus of rats following treadmill exercise at 100% $VO_{2max}$ for 40 min. Together these results imply that the brain is protected from the systemic inflammatory damage related to oxidative stress which occurs with high-intensity aerobic exercise.

However, a recent review by Packer et al. (38) suggests that acute exercise may still pose an inflammatory "threat" to the CNS. Steensberg et al. (52) found that cerebrospinal fluid (CSF) levels of HSP72 (an indicator of oxidative stress) were increased 5-fold in healthy men who underwent 2 hours of cycle ergometry; no changes in CSF IL-6 concentrations were noted and TNF-$\alpha$ levels remained undetectable before and after the exercise bout. In contrast, cerebral IL-6 levels (as

measured by internal jugular venous to arterial differences) were significantly elevated in men who participated in two successive 60 min bouts of cycle ergometry (35). Animal studies (8; 9; 10) also indicate that muscle-damaging downhill treadmill exercise in mice leads to elevations in IL-1β in the cortex and cerebellum through activation of perivascular and meningeal macrophages. Thus, the direction of effects of exhaustive exercise on CNS brain inflammatory cytokine responses is unclear and some of this variation may be due to inter-species differences. This is in contrast to the generally pro-inflammatory and apoptotic responses observed in the peripheral compartments after acute exercise. Moreover, the issue of exercise-induced apoptosis in brain immune cells has gone unexplored.

The purpose of this study was to examine the effects of a single bout of acute, strenuous exercise on the expression of classical pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and apoptotic status in the hippocampus of healthy mice. This brain region was chosen because it is involved in cognition, memory, and the stress response (12; 33). In addition, plasma levels of 8-isoprostaglandin $F2_{\alpha}$ and corticosterone were measured to determine whether the exercise protocol was sufficient to elicit a stress response. A second purpose was to compare these pro-inflammatory cytokine responses centrally vs. peripherally following the acute exercise challenge. We hypothesized that exposure to a strenuous bout of aerobic exercise would lead to increases in the expression of hippocampal TNF-α, IL-1β, and IL-6, coupled with increases in the apoptotic status of hippocampus cells, in a manner similar to intestinal lymphocytes (i.e., a peripheral lymphoid compartment). Through this investigation, the relationship between indicators of exercise-induced oxidative stress in the plasma and apoptosis and central inflammatory processes in hippocampal immune cells was explored.

## MATERIALS & METHODS

### Animals

Female C57BL/6 mice (n = 63) (Harlan Indianapolis, IN, USA), 4-5 months of age, were housed in individual cages at 21 ± 1 °C, on a 12/12 h reversed light/dark cycle. *Ad libitum* access to a standard rodent diet (Lab Rodent Chow, PMI Feeds, Richmond, IN, USA) and tap water were provided. The experimental procedures adhered to the guidelines established by the Canadian Council on Animal Care and were approved by the University Animal Research Ethics Committee.

### Exercise protocol

Mice were matched on weight and randomly assigned to one of three treadmill exercise conditions: (1) treadmill running (90 min, 2° slope) with sacrifice immediately after exercise (TREAD-Imm; n = 21), treadmill running (same duration, speed and grade) with sacrifice 2 h after exercise (TREAD-2h; n = 20), and control animals that were exposed to treadmill noise and vibrations for 90 min, without running, before sacrifice (NOTREAD; n = 22). The running protocol consisted of a 10 min warm-up, 30 min at 22m/min, 30 min at 25 m/min, 30 min at 28 m/min, and a 5 min deceleration to 0 m/min on an Omni-Max metabolic treadmill (Omni Tech Electronics, Columbus, OH, USA). All running took place at the beginning of the

dark cycle (between 7 and 9 am). Mice were motivated to run by gentle prodding using a nylon brush and were fasted overnight prior to the start of exercise.

**Plasma collection**

Mice were sacrificed by sodium pentobarbital overdose (0.6-0.8 cc per mouse, i.p.). After confirmation of a negative toe pinch response, skin was grasped at the mid-ventral position of the body and an incision was made across the chest to expose the rib cage. This was cut to expose the heart, and blood was collected immediately using a heparinized syringe. Blood was centrifuged at 1500 *g* for 6 min and plasma was collected and stored at -80 °C until analysis of corticosterone and 8-isoprostaglandin $F2_{\alpha}$.

**Hippocampus removal and single cell suspensions**

Excision of mouse hippocampi was performed according to Hassan et al. (16). All brain dissections took place on an ice-mounted stage. Decapitation was completed immediately following sacrifice. A midline incision was made along the skull, granting access to underlying structures, and the brain was excised and washed in cold PBS (0.5% BSA/PBS), transferred to the dissection stage and bisected at the midline. A clean number-1 paintbrush was inserted into the fissure beneath the dorsal cerebral cortex, and the hippocampi from both hemispheres were visualized, isolated, and placed in 1.5 mL RPMI (1640, 2.5% FCS), pressed through a 70 μm cell strainer, and centrifuged at 1500 RPM for 5 min. Cells were resuspended in 5 mL RPMI at room temperature, layered over 5 mL of Lympholyte M (Cedarlane Laboratories, Hornby, ON, Canada), and centrifuged at 1250 *g* for 20 min. Cells at the interface were recovered, washed, suspended in 300 μL PBS and counted by microscopy. Cell samples were stored at -80 °C until analysis.

**Assessments of apoptosis of hippocampal cells**

Immediately following the preparation of hippocampal single cell suspensions, 1 x $10^5$ hippocampal cells were incubated for 15 min in the dark with 2.5 μl of Annexin V-FITC (Pharmingen, San Diego, CA, USA), 2.5 μl of Propidium Iodide (PI) (Sigma Chemical, St. Louis, MO, USA), and 100 μl of Annexin binding buffer, in order to obtain percentages of apoptotic and necrotic cells as has been previously described (18).

**Protein determination and Western blot analysis of hippocampal cell and intestinal lymphocyte TNF-α, IL-1β, and IL-6**

Hippocampal cells were lysed, placed on ice for 45 min, and the lysates centrifuged (10,000 *g*, 15 min) for protein determination by bicinchoninic acid (BCA) assay. Protein supernatant (40 μg) and molecular weight markers (Full Range Rainbow, Amersham Biosciences, Buckinghamshire, UK) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 12-15%), transferred to a polyvinyldiene fluoride (PVDF) membrane, and stained with Ponceau S to confirm quality of transfer and equal loading. Membranes were then incubated with primary antibody for 1 h (1:200 in 10% FBS-TBST): TNF-α (sc-1350), IL-1β (sc-71435), or IL-6 (sc-1265) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and subsequent incubation with secondary antibody for 1 h: horseradish peroxidase-conjugated anti-goat (TNF-α) or anti-mouse (IL-1β, IL-6)

at 1:2000 in FBS-TBST. Protein quantity was determined using ECL Plus Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK) and the ChemiGenius 2 Bio-imaging system (Cambridge, UK). Intestinal lymphocytes were collected as described (20) from a subset of mice (n = 30) for comparison with hippocampal cells and to be used as internal controls to document whether the acute exercise protocol led to previously observed systemic cytokine changes in TNF-α, IL-1β, and IL-6. Western blot analysis of intestinal lymphocytes was performed as described above for hippocampus.

**Corticosterone assessment**

Plasma samples were assessed for corticosterone levels using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Purification requirements of the samples were determined using the cold spike protocol, and concentration of corticosterone was measured at 412 nm using a PowerWave 340 microplate spectrophotometer (Biotek Instruments, Vermont, USA), according to the manufacturer's protocol. All samples were run in duplicate. The intra-assay % CV was 6.3% and the lower detection limit was 30 pg/ml.

**8-isoprostaglandin $F2_\alpha$ assessment**

Plasma 8-isoprostaglandin $F2_\alpha$ (8-isoprostane) levels were quantified using a direct EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Samples were hydrolyzed (25 µl 10 N NaOH: 100 µl sample) at 45 °C for 2 h, neutralized with 12 N HCl, centrifuged (5 min, 14,000 *g*), and supernatant incubated with 8-isoprostane antibody for 24 h at 4 °C. Absorbance was measured at 405 nm at room temperature (PowerWave 340 microplate spectrophotometer, Biotek Instruments, Vermont, USA). All samples were run in duplicate. The intra-assay % CV was 8.6% and the lower limit of detection was 2.7 pg/ml.

**Statistical analysis**

Cytokine concentrations and measures of apoptosis and necrosis were analyzed using one-way ANOVA with acute treadmill exercise challenge (3 levels: NOTREAD, TREAD-Imm, TREAD-2h) as the independent factor and cytokine protein expression, % $Annexin^+$, and % $PI^+$ as the dependent factors (SPSS Version 18; Chicago, IL, USA). Corticosterone and 8-isoprostane levels were analyzed using one-way ANOVA with acute treadmill exercise challenge (2 levels: NOTREAD, TREAD-Imm) as the independent factor and corticosterone and 8-isoprostane concentrations as the dependent factor (SPSS Version 18; Chicago, IL, USA). Post hoc analysis was determined with Tukey's HSD test and all ANOVAs results were checked for homogeneity of variance with Levene's test). Significant difference from chance alone was accepted if $p < 0.05$; all values are expressed as group means ± 1 SEM for respective units.

## RESULTS

**Body Mass**

At sacrifice, the mean body mass of mice was 26.7 ± 0.8 g (NOTREAD: 27.7 ± 0.7 g; TREAD-Imm: 26.1 ± 0.8 g; TREAD-2h: 26.2 ± 0.8 g) and these groups did not differ ($F$ (2, 62) = 1.309, n.s.).

## Apoptosis

No differences were observed with respect to the percentage of Annexin$^+$ ($F$ (2, 62) = 0.231, n.s.) or PI$^+$ ($F$ (2, 62) = 0.696, n.s.) hippocampal cells between the NOTREAD (Annexin$^+$: 4.0 ± 0.3 %; PI$^+$: 3.6 ± 0.3 %), TREAD-Imm (Annexin$^+$: 4.2 ± 0.3 %; PI$^+$: 4.0 ± 0.3 %), and TREAD-2h (Annexin$^+$: 3.9 ± 0.3 %; PI$^+$: 3.8 ± 0.3 %) mice.

## Hippocampal Cytokines

**Figure 1** shows the effects of acute treadmill exercise on the expression of pro-inflammatory cytokines (in Arbitrary Units [AU]) in the hippocampus. There was a significant effect of acute treadmill exercise on TNF-α expression ($F$ (2, 58) = 3.31, $p < 0.05$) and expression of this cytokine was lower in TREAD-Imm (1.1 ± 0.1 AU) and TREAD-2h (1.1 ± 0.1 AU) compared to NOTREAD (1.4 ± 0.1 AU) mice. Acute exercise significantly affected IL-6 expression in mouse hippocampus ($F$ (2, 58) = 6.23, $p < 0.05$) with this cytokine being higher in TREAD-2h (1.2 ± 0.06 AU) compared to NOTREAD (0.9 ± 0.06 AU) and TREAD-Imm (1.0 ±

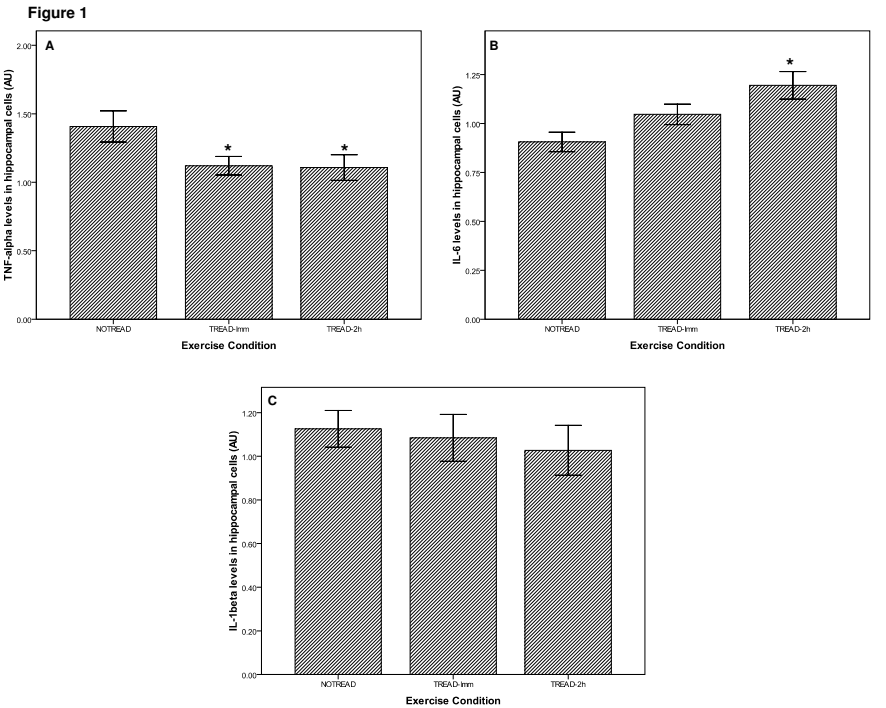


**Figure 1**

Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in hippocampal cells of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A:* TNF-α expression. *Panel B:* IL-6 expression. *Panel C:* IL-1β expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

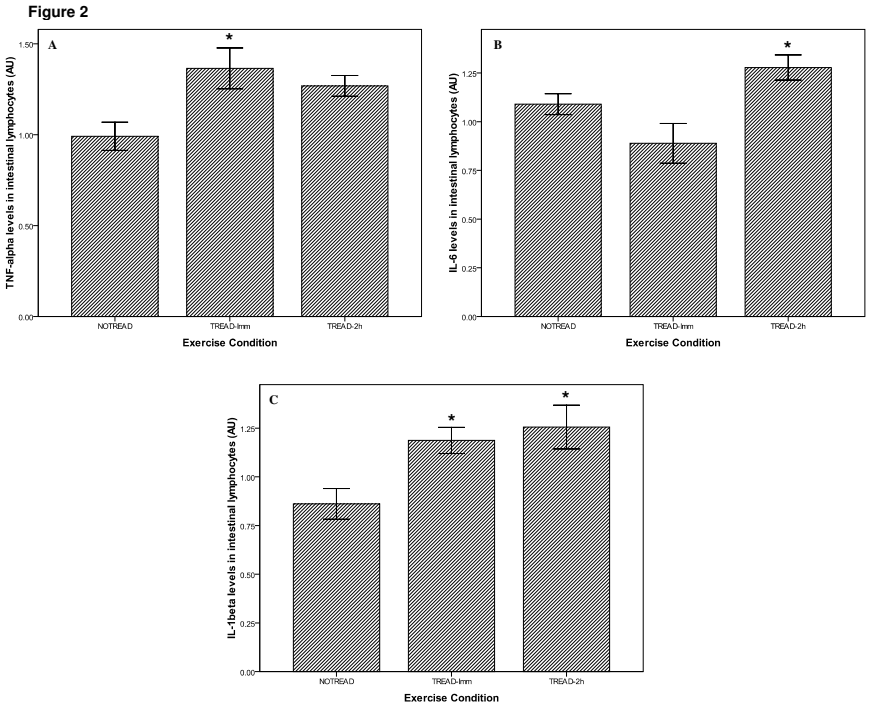


**Figure 2**
Cytokine (TNF-α, IL-6, IL-1β) expression [AU] in intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A:* TNF-α expression. *Panel B:* IL-6 expression. *Panel C:* IL-1β expression. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

0.06 AU) animals. Expression of IL-1β in mouse hippocampus did not differ as a function of acute treadmill exercise ($F$ (2, 58) = 0.23, n.s.).

## Intestinal Lymphocyte Cytokines

**Figure 2** shows the effects of acute treadmill exercise on the expression of pro-inflammatory cytokines in intestinal lymphocytes. There was a significant effect of acute treadmill exercise on TNF-α expression ($F$ (2, 28) = 5.21, $p < 0.05$) due to higher expression in TREAD-Imm (1.4 ± 0.1 AU) compared to the NOTREAD (1.0 ± 0.1 AU) mice. TNF-α expression in intestinal lymphocytes was also elevated in TREAD-2h (1.3 ± 0.1 AU) compared to NOTREAD animals, but this difference only approached significance ($p = 0.06$). A significant effect of acute exercise on intestinal IL-6 was found ($F$ (2, 28) = 6.60, $p < 0.05$). A small and non-significant decrease in IL-6 expression occurred in the intestinal lymphocytes from TREAD-Imm (0.9 ± 0.1 AU) mice compared to NOTREAD (1.1 ± 0.1 AU) mice. The TREAD-2h (1.3 ± 0.1 AU) mice, however, had significantly higher expression of IL-6 in intestinal lymphocytes to the NOTREAD animals. Intestinal

lymphocyte IL-1β expression was affected by acute exercise ($F$ (2, 29) = 5.13, $p < 0.05$) with this cytokine elevated in the TREAD-IMM (1.2 ± 0.1 AU) and TREAD-2h (1.3 ± 0.1 AU) compared to the NOTREAD (0.9 ± 0.1) mice.

Representative immunoblots for TNF-α expression in hippocampal cells and intestinal lymphocytes for NOTREAD, TREAD-Imm, and TREAD-2h mice are shown in **Figure 3.** Note that only the smaller (17 kDa) cleaved form of the cytokine was analyzed and presented in Figure 1 Panel A and Figure 2 Panel A. The larger pro-form (28 kDa) was not analyzed.

**Figure 3**

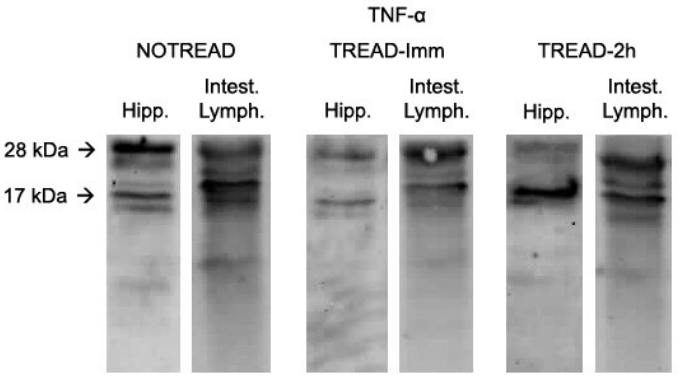


**Figure 3**
Representative immunoblots for TNF-α in hippocampal cells and intestinal lymphocytes of mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) vs. sedentary controls (NOTREAD). Arrows indicate molecular weight (kDa) for the two forms of TNF-α: a larger pro-form at 28 kDa and a smaller cleaved form at ~17 kDa. Only the smaller molecular weight cytokine was analyzed in the experiments.

### Cortisterone and 8-isoprostaglandin F2α (8-isoprostane)

**Figure 4** shows the plasma corticosterone (Panel A) and 8-isoprostane (Panel B) responses to acute treadmill exercise. The treadmill running led to a significant increase in plasma corticosterone concentration ($F$ (1, 39) = 60.25, $p < 0.05$) in TREAD-Imm (99.8 ± 5.7 ng/ml) compared to NOTREAD (37.5 ± 5.7 ng/ml) mice. Acute treadmill exercise also was associated with a significant increase in plasma 8-isoprostane levels ($F$ (1, 32) = 7.53, $p < 0.05$) in TREAD-Imm (97.3 ± 6.2 pg/ml) compared to NOTREAD (73.5 ± 6.0 pg/ml) mice. Plasma corticosterone and 8-isoprostane levels were not assessed in the TREAD-2h group as previous studies (17; 20) have shown that differences in these measures are no longer significant after a 2 hour rest period.

**Figure 4**

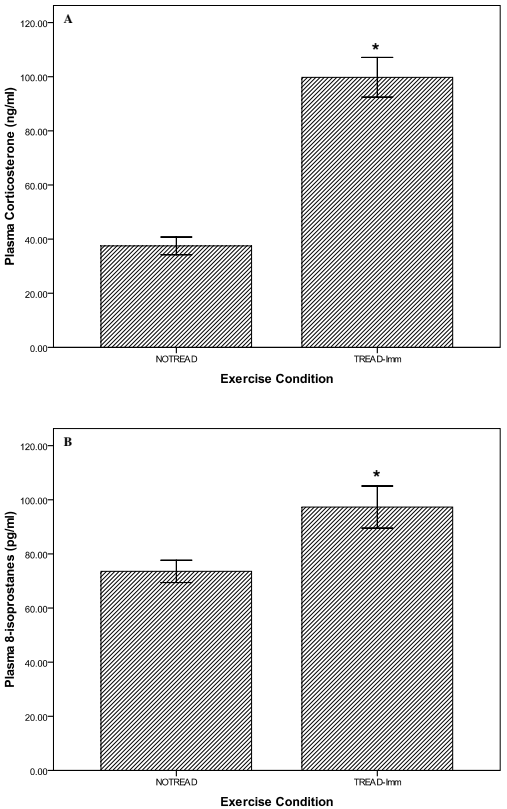


**Figure 4**
Plasma corticosterone and 8-isoprostane concentrations in mice given a single acute exercise bout and sacrificed immediately (TREAD-Imm) or after 2 hours (TREAD-2h) versus sedentary controls (NOTREAD). *Panel A:* Plasma corticosterone concentrations [ng/ml]. *Panel B:* Plasma 8-isoprostane concentrations [pg/ml]. Values are means ± one standard error. Significance compared to NOTREAD control indicated by an asterisk (*). See text for details of analysis.

## DISCUSSION

We determined the effects of a single bout of strenuous aerobic exercise on cellular apoptosis and necrosis, and on the expression of pro-inflammatory cytokines in the hippocampi of healthy mice. A secondary objective was to evaluate intestinal lymphocyte pro-inflammatory cytokine expression in a subset of the experimental groups, along with physiological markers of stress, to determine that the exercise protocol was sufficient to elicit inflammatory cytokine changes as we previously reported (19; 20).

Although several studies have investigated the phenomenon of acute exercise-induced apoptosis in systemic lymphoid compartments, no previous studies, to our knowledge, have explored such effects in the healthy brain. Our novel results suggest that the hippocampus may be protected against the loss of cells incurred as a result of intense physical activity. This perspective is suggested by related findings in the literature. For example, Kim et al. (23) investigated the hippocampi obtained from rats undergoing 10 days of treadmill exercise following induced traumatic brain injury (TBI). TBI was found to impair short-term memory, increase DNA fragmentation, elevate caspase-3 and Bax expression, and decrease Bcl-2 protein expression in the hippocampus. However, in the exercised animals, there was less memory impairment, DNA fragmentation, and caspase-3 and Bax expression, indicating that physical activity reduces the apoptosis associated with central trauma. Although this study examined repeated bouts of treadmill running over a 10 day period, each "session" of forced exercise did not exacerbate traumatic damage. Um et al. (54) found that long-term treadmill running inhibits the apoptotic cascade in the brain by reducing cytochrome c, caspase-9, and caspase-3 protein levels, while inducing the expression of superoxide dismutase-1, catalase, and Bcl-2 to combat the effects of oxidative stress. Radak et al. (46) examined the effects of a prolonged acute exercise bout on markers of oxidative damage in the hippocampus of rats following a period of stress; immobilization increased lipid peroxidation, carbonylated protein concentration, DNA damage, and reduced glutamine synthetase activity. A single bout of acute exercise was able to restore levels to those observed in control animals. We suggest that intense aerobic exercise (at least at the intensity and duration used in this study) may not be sufficient to generate apoptotic conditions in the brain, and instead initiates the generation of conditions that may even be anti-apoptotic. Nevertheless, the literature in this area is limited (38), and caution must be used when comparing these studies: some utilized training (54), others repeated acute exercise (23), and still others a single-bout of acute exercise (46).

In contrast to our initial hypothesis, high-intensity exercise decreases the expression of TNF-$\alpha$ and increases the expression of IL-6 in the hippocampus. IL-6 has both pro- and anti- inflammatory functions, depending on the surrounding cytokine milieu, and elevated plasma IL-6 inhibits circulating levels of TNF-$\alpha$ both directly and through up-regulation of the soluble TNF receptor and IL-1ra (39; 41; 50). Thus, the higher IL-6 and lower TNF-$\alpha$ expression observed in our study may be a "mechanism" not only where acute exercise leads to decreased immunity to infection, but also preserves cognitive capacity, immediately after the physical stressor. TNF-$\alpha$ has a largely anti-pathogenic activity and is found throughout many areas of the central nervous system. It is responsible for MHC I and II expression in the glia and is highly involved in nitric oxide production in the CNS as a means of eliminating infectious agents. In contrast, IL-6 is primarily localized within the hippocampus and prefrontal cortex, promotes neuron survival factor, protects against excitotoxic brain damage, and has stress modulating effects on cognition (56). Acute exercise-induced increases in central IL-6 expression may also be responsible for greater cognitive task performance during, and immediately after, exercise bouts of varying intensity (5; 24).

We also found that the pattern of cytokine expression in the CNS differs from that of intestinal (systemic) tissues after an acute exercise challenge. In the hippocampus there were decreases in TNF-α, increases in IL-6, and no change in IL-1β expression after an acute exercise challenge in mice. In the intestine, however, all of the pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) showed increased expression. The intestinal lymphocyte cytokine pattern confirms earlier research that acute exercise leads to increases in TNF-α, IL-6, and IL-1β expression in intestinal lymphocytes, blood, and muscle (20; 25; 32). The acute exercise-induced increase in intestinal TNF-α expression is accompanied by elevations in pro-apoptotic proteins and lymphocytosis, which are thought to be a result of the oxidant stress generated by the stressor (20). Administration of an anti-oxidant prevented exercise-induced lymphocyte apoptosis (44), establishing the fact that acute exercise can have damaging effects in the intestine (and likely other systemic immune compartments) as a result of oxidative stress. The mechanism of oxidative stress in the periphery is also indicated by the lack of apoptotic responses in intestinal lymphocytes following injection of corticosterone at concentrations observed with intense treadmill exercise (43).

Acute high-intensity treadmill exercise leads to systemic elevations in markers of oxidative stress, including plasma 8-isoprostanes and corticosterone concentrations (20; 21; 52). Glucocorticoids, in particular, are modulators of cytokine activity (37) and cortisol secretion is correlated with IL-6 release and TNF-α suppression in blood obtained from major depression patients after an endotoxin challenge (55). Audet et al. (3) examined these phenomena in male mice, utilizing an acute psychosocial stressor (pairing of submissive and dominant mice) to evaluate corticosterone and cytokine responses. Mice with high plasma corticosterone responses to stress also had elevated expression of IL-6 (but not IL-1β) and of mRNA for IL-6 and IL-1β in the hippocampus. The authors suggested that IL-1β protein expression increases at a later time-point not considered in their study. In addition, hippocampal TNF-α mRNA in response to stress was unchanged, but pre-frontal cortex TNF-α mRNA production was lower in corticosterone high responders. Circulating levels of corticosterone are thus increased under immunological or psychological stress (14) and in response to intense exercise (7); this is coupled with increases in brain IL-6 in rats in response to chronic mild stress (30).

In addition, glucocorticoid administration inhibits plasma TNF-α increases that occur following an endotoxin challenge in healthy humans (4; 6). LPS-induced serum corticosterone levels are positively correlated with brain IL-6 and IL-1β concentrations 2 hours after endotoxin challenge (11). Central TNF-α levels were found to be elevated 16 hours after LPS stimulation whereas plasma (peripheral) corticosterone, IL-6, and IL-1β levels had already returned to baseline concentrations. Moreover, corticosterone administered intraperitoneally to adrenalectomized rats crosses the blood-brain-barrier with uptake and retention of the hormone in the hippocampus (28). These studies suggest that systemic glucocorticoids (whether from endogenous or exogenous sources), affect central and peripheral cytokine expression. Thus, it may be the case that exercise-induced changes in central pro-inflammatory cytokine synthesis or balance are influenced by corticosterone rather than oxidative responses to the exercise.

This study is not without limitations. Firstly, we did not separate cell subsets in the hippocampus in order to determine whether or not there were differential effects of acute exercise depending on cell type. Instead, our hippocampal single cell suspensions consisted of a mixture of microglia and other non-immune cells, making it difficult to interpret the source of cytokines observed, as well as impact of apoptotic changes in specific cell populations. Another limitation is that only a single bout of exercise was given prior to sacrifice. Human studies have shown that successive exercise bouts can lead to major increases in central IL-6 levels (35) and this must also be addressed in future animal investigations. It is unclear whether repeated exercise will lead to similar cytokine changes in the mouse hippocampus. Furthermore, this study was cross sectional, as we only measured changes immediately and two hours after the exercise bout. It cannot be determined from our results whether the differences in cytokine concentrations observed in the hippocampus and intestine were transient or more long-lasting. Studies will be needed with additional post-exercise time points to provide this clarification and to assess the kinetics of central vs. systemic cytokine expression in immune cells. As such, future experiments should include timed resting controls to address potential temporal effects. In addition, we report on levels of corticosterone in the plasma, but did not measure brain glucocorticoid expression. If corticosterone is affecting the concentration of central pro-inflammatory cytokines, it is essential to determine if this is a result of systemic or central sources of this stress hormone. Furthermore, it needs to be clarified whether corticosterone is actually responsible for the observed cytokine changes. Future investigations may involve repeating the exercise protocol with adrenalectomized mice, or with administration of cortisol receptor antagonists, to test this hypothesis. Studies must also include testing of other stress hormones, such as catecholamines, and additional markers of oxidative stress, including dichlorofluorescein diacetate. We did not measure apoptotic status (i.e., Annexin V positive) in intestinal lymphocytes because of limited tissue availability. However, we have shown elsewhere that aerobic exercise leads to a loss of intestinal lymphocytes and accompanying increases in apoptotic cells (17). Our experiments were conducted only with female C57BL/6 mice, which did not allow for the determination of any gender-specific effects in the hippocampus or intestine due to acute exercise. Females were chosen in order to 1) allow comparison with earlier studies from our lab (17; 19; 20), and 2) because they are better runners and show less bout-length attrition than males of this strain (13).

In conclusion, a single bout of intense aerobic treadmill running in healthy female C57BL/6 mice does not affect the percentage of apoptotic hippocampal cells, but alters the expression of pro-inflammatory cytokines by decreasing TNF-$\alpha$ and increasing IL-6 in the hippocampus immediately and 2 hours after cessation of exercise. These changes in the brain do not mirror the cytokine changes observed in intestinal lymphocytes. In the intestine, the expression of pro-inflammatory cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6) is increased after intense exercise. We suggest that the oxidative stress that accompanies acute exercise is not sufficient to generate damaging (apoptotic) effects in the central nervous system, as this compartment may be protected to preserve cognitive function during physical activity. We also tentatively propose that exercise-induced elevations in circulating corti-

costerone levels may be one mechanism to explain the pattern of hippocampal cytokine expression. Glucocorticoids are known to increase and reduce central levels of IL-6 and TNF-α, respectively. Future studies on physical activity and the central expression of specific pro- (e.g., caspases, Bax) and anti- (e.g., Bcl-2) apoptotic proteins and other pro-inflammatory cytokines will be necessary. Whether reducing or blocking glucocorticoid release (e.g., adrenalectomy) affects brain pro-inflammatory cytokine response to acute exercise stress remains to be determined.

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# *Exercise, Physical Activity and Breast Cancer: The Role of Tumor-Associated Macrophages*

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## ABSTRACT

*Regular exercise and physical activity provide many health benefits and are encouraged by medical professionals for the primary prevention of, and adjuvant treatment of breast cancer. Current consensus in the discipline of exercise oncology is that both regular physical activity and exercise training exert some protective effect against breast cancer risk, and may reduce morbidity in some advanced cases. While there is growing interest in the role of exercise and physical activity in breast cancer prevention, it is currently unclear how exercise may modulate tumor behavior. The tumor microenvironment is populated by stromal cells such as fibroblasts and adipocytes, as well as macrophages. Termed tumor-associated macrophages (TAMs), these immune cells are highly plastic and respond to different signals from the cancer microenvironment, causing them to either display tumor-promoting or tumor-suppressing phenotypes. Because of such plasticity, there has been considerable interest by immunologists to develop immunotherapies based on skewing the behavior of TAMs to become cancer-suppressive. Previous studies have indirectly shown the ability of exercise training to induce an anti-tumor effect of macrophages, although the studies did not address this in the tumor microenvironment. Nevertheless, this opens up the possibility that regular exercise training may exert a protective innate immune effect against breast cancer, potentially by inducing a cancer-suppressing phenotype of TAMs. This review will describe potential mechanisms through which exercise may modulate the behavior of TAMs.*

**Key words:** Exercise, physical activity, breast cancer, microenvironment, tumor-associated macrophages.

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## INTRODUCTION

Breast cancer is the primary type of cancer afflicting women in the United States of America (51). The American Cancer Society estimated up to 226,000 American women to be newly diagnosed with breast cancer in 2012 (51). Importantly, this disease is the second leading cause of deaths among different cancer types in American women, with an expected 40,000 deaths in 2012 (51). Breast cancer is a disease of the mammary gland. The normal mammary gland is comprised of branching mammary milk ducts, containing ductal epithelial cells, that terminate in the lobule with luminal epithelial cells forming an inner lining in the lobular lumen (Figure 1). Surrounding these cells are the extracellular matrix and stromal cells (fibroblasts, endothelial cells, leukocytes, adipocytes) of the microenvironment. Similar to other types of cancer, the progression of breast cancer follows a sequential series of events: initiation, promotion and progression (35). Initiation occurs when DNA in mammary epithelial cells encounters some form of deleterious interaction with a carcinogen. A DNA adduct is formed and results in the erroneous insertion of the complementary nucleotide during DNA transcription. At this stage, without a supportive microenvironment, the initiated epithelial cells remain latent and will not develop into tumors. In the promotion stage, initiated epithelial cells are exposed to promoters that increase their proliferation. The proliferation of these epithelial cells is not permanent, as removal of the promoters would reverse this process. In the progression state, initiated cells become tumors when a second genetic event allows the initiated cells to become permanently altered. Some of these cells acquire a selective growth advantage and become malignant. Malignant cells proliferate uncontrollably and in advanced stages, spread to distant organs (metastasis), resulting in death. In recent years, the role of the tumor microenvironment in cancer biology has been better understood. It is apparent that tumor cells communicate with stromal cells in the microenvironment in a complicated, bi-directional crosstalk. The outcome of this crosstalk then influences the response of the tumor cells.

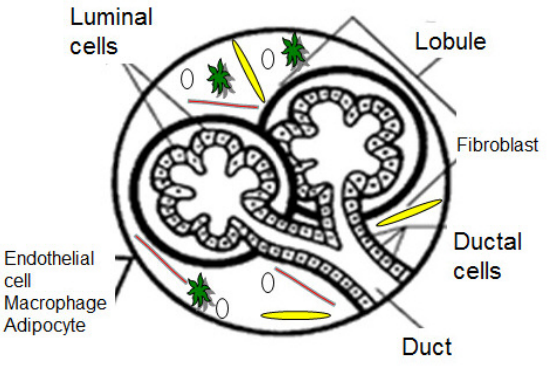


**Figure 1.** The normal mammary gland is comprised of a branching duct, containing ductal epithelial cells, that leads to the lobule. In the lumen of the lobule are the luminal epithelial cells. Different stromal cells reside in the mammary gland microenvironment, such as fibroblasts (yellow), macrophages (green), endothelial cells (red) and adipocytes (white). During tumorigenesis, the interaction of these stromal cells with the epithelial cells influences the progression of the disease.

The terms tumor and cancer have been used interchangeably, but it is important to differentiate the two. A tumor is an amalgamation of cell mass, and can be

benign or malignant. A benign tumor grows slowly, seldom divides and has morphological characteristics similar to the tissue it arose from (35). In contrast, cancer is a malignant tumor that has lost the regulatory control mechanisms for cell proliferation and division (35). In this review, the term tumor will be used when describing a cancer phenotype and the tumor microenvironment. This is pertinent when describing i) the primary tumor, localized to the site of origin, and ii) the secondary tumor, when the primary tumor has breached the basement membrane and seeded individual tumor cells into the blood circulation that metastasize to distant locations. Metastasis is the final stage in the development of breast cancer and ends with the death of the host.

The risk factors of breast cancer have been identified and are attributed to i) genetic heritability; carriers of mutated BRCA1 and BRCA2 gene have increased risk of breast cancer (46) and ii) environmental influences, such as diet and physical activity (53). Thus, the combination of both susceptible genes and poor lifestyle behavior can contribute to increased breast cancer risk, suggesting that lifestyle behavior is one modifiable risk factor for cancer. Physical activity is one such modifiable risk factor. It is defined as skeletal muscle contraction that results in increased energy expenditure above basal levels (4). It includes activity that is related to home maintenance (e.g. gardening), occupation (e.g. construction), commuting (e.g. biking to work) or recreation (e.g. sports, dance etc) (4). Exercise is a subset of physical activity, where it is planned, repetitive and structured, with the goal of improving or maintaining physical fitness (4). Exercise can be further categorized as “acute” or “chronic”, where the former typically refers to one bout of activity, and the latter refers to regular, periodic bouts of activity.

Throughout this text, the distinction between physical activity and exercise are made when referring to the pertinent studies. This distinction is important for several reasons. First, in epidemiological studies, physical activity is an independent variable that is observed but not manipulated, whereas exercise is an independent variable that is manipulated in randomized controlled trials or other forms of interventional studies. This suggests that the degree of experimental control is different, with the "dose" (frequency, intensity, time, type) of physical activity more variable amongst subjects in physical activity studies, compared with exercise studies. Second, outcomes from observational studies depict correlations between physical activity and disease outcome. Experimentally, it is difficult to elucidate the mechanistic effects of physical activity on cancer outcomes because physical activity usually spans a broad definition and the amount of physical activity performed is neither uniform nor controlled, leading to inter-subject variation. Finally, manipulation of the independent variable (exercise) is necessary in order to determine cause and effect.

Exercise training or physical activity could be protective against cancer by regulating the behavior of macrophages in the tumor microenvironment. Research has shown that exercise exerts modulatory effects on macrophage metabolism, phagocytosis, chemotaxis, and anti-tumor activity (66). Therefore, it is relevant to understand how their beneficial effects against breast cancer can be harnessed with exercise training or regular physical activity. A paradox in breast cancer and tumor-associated macrophages (TAMs) exists, whereby the presence of the TAMs in the breast microenvironment is usually correlated with poor prognosis. Yet, experimental models have often shown that macrophages are capable of destroy-

ing tumors (22). It may be possible that the paradox depends on the phenotype of the macrophages present, which will be the focus of this review. It is acknowledged that other cellular mechanisms such as anti-oxidative effects and metabolic alteration on tumor cells may contribute to the exercise-induced effects on carcinogenesis and metastasis, but they are beyond the scope of this review.

**Physical Activity Attenuates Breast Cancer Risk and Improves Survival in Human Epidemiology Studies**

In order to define the "dose" of physical activity in epidemiological studies, scientists typically report the weekly caloric expenditure of their subjects. Caloric expenditure in this case, is measured in terms of metabolic equivalents (MET)s, which is the oxygen cost of a physical activity expressed as a ratio to oxygen cost at rest. These MET values are used widely and obtained from the compendium of physical activity (1). It has been well described that regular physical activity is associated with decreased incidence of some cancers. A five-year prospective follow up of a cohort of post-menopausal women showed, after controlling for confounding factors, that women with the highest baseline levels of physical activity had a 29% lower incidence of breast cancer compared to women who were least physically active (38). The most physically active women expended 42 metabolic equivalents (MET) hours per week, whereas the most sedentary women expended between 1-7 MET hours per week (38). In a systematic literature review (15), a total of 87 cohort studies and case-control studies specific to different types of physical activity (recreational, occupational, transport, household) and breast cancer were retrieved and studied. The overall finding was a 25% risk reduction for cancer risk amongst women in the most physically active group, compared with the least physically active women. In addition, the authors reported a dose-response relationship, where participation in vigorous intensity physical activity was associated with a greater decrease in breast cancer risk, compared with moderate intensity physical activity (mean decrease of 26% versus 22%). Agreeing with these findings, another study showed that American women between the ages of 35 and 64 years, who participated in recreational physical activity throughout their lifetime, had a 35% reduced risk of developing invasive breast carcinoma, compared with women that were sedentary (39).

In July 2010, an expert panel from the American College of Sports Medicine reviewed current studies of exercise training and cancer survivorship and released a roundtable consensus statement, concluding that exercise training is "safe during and after cancer treatments and results in improvements in physical functioning, quality of life, and cancer-related fatigue" (49). The panel also stated that exercise training before and after breast cancer diagnosis is associated with a decrease in the risk of recurrence and/ or death from breast cancer. In this regard, Schmidt (48) reported that four of six cohort studies have shown a protective effect of pre-diagnosis physical activity on breast cancer survivorship, whereas two studies did not. In another prospective cohort study that recruited women diagnosed with either *in situ* or regional cancer (27), participation in physical activity after breast cancer diagnosis had a stronger protective effect compared with pre-diagnostic physical activity. In this study, compared with inactive women, physically active women who expended a minimum of 9 METs per week prior to diagnosis, had a hazard ratio for total deaths of 0.69 (95% CI, 0.45 to

1.06, P=0.045), compared with a hazard ratio for total deaths of 0.33 (95%CI, 0.15 to 0.73, P=0.046), for women that were physically active 2 years after diagnosis. These results have been corroborated by similar findings by other studies (24, 25). In the Holick study (24), women between the ages of 20 and 79 years and diagnosed with invasive breast cancer were recruited into a prospective study and followed for an average of 6 years. The authors reported that compared with women that were sedentary, women expending 21 or more MET hours per week had a lower risk of breast cancer mortality (hazard ratio, 0.51; 95% CI: 0.29-0.89; P for trend =0.05). Finally, in the Nurses Health Study (25), women aged 30-55 years and diagnosed with breast cancer (stages I-III) were enrolled in a prospective observational study. During the follow up period, it was observed that postmenopausal women that participated in moderate physical activity (greater than 9 METS hours per week) had a reduced risk of breast cancer mortality (relative risk, 0.73; 95% CI: 0.54, 0.98), compared with women that expended less than 9 METS hours per week. In addition, the hormonal levels of breast cancer also appeared to be influenced by physical activity. Moderate physical activity was shown to exert a more protective effect in women that were physically active and had estrogen receptor (ER)- positive and progesterone receptor (PR)-positive breast cancers than women with ER-negative and PR-negative breast cancers (odds ratio 0.50; 95% CI: 0.34-0.74 versus odds ratio 0.91; 95% CI: 0.43-1.96).

It is concluded that epidemiological studies generally support the use of physical activity and exercise training after diagnosis of breast cancer, suggesting that this type of life style change may slow the progression of breast cancer and perhaps also reduce the risk of recurrence and hence improve survivorship. However, unresolved questions remain regarding the effect on immunity. The only clinical studies that investigated the role of the immune system in cancer and exercise intervention in human subjects, have thus far have involved NK cells (11) and lymphocytes (26) in the blood circulation. We speculate that TAMs represent an under-studied cell population in the tumor microenvironment, particularly as it relates to exercise oncology. It is unknown whether exercise training or physical activity modulates the immune response in the tumor microenvironment, and if so, what mechanisms are involved. Elucidating these mechanisms can identify how macrophages and their secreted factors can play a role in reduced metastasis and explain the improved survivorship for physically active women with breast cancer.

**Macrophages in the Tumor Microenvironment Modulate Tumor Behavior**

The "seed and soil" hypothesis suggests that for tumor cells ("seeds") to propagate and advance to malignancy, the tumor microenvironment ("soil") has to be permissive and supportive of their growth (38). In other words, stromal cells secrete factors and cross-talk with tumor cells to display the phenotypic hallmarks of cancer, such as self-sufficiency in growth and increased invasiveness and metastatic potential. Macrophages in the tumor microenvironment, referred to as TAMs, are stromal cells that can influence tumor behavior. Macrophages are recruited into the tumor microenvironment where they differentiate to become TAMs. In general, the presence of TAMs is associated with poor prognosis in cancer survivors (42). This clinical outcome is likely attributed to TAMs' role in supporting tumor progression (increased tumor proliferation, vascularization, tissue

invasion and metastasis). In paraffin embedded, archived samples of human mammary carcinoma, a higher count of macrophages in random high powered fields, shown by positive cluster of differentiation (CD) immunostaining was correlated with less than 5 years of survival, compared with samples that stained for a lower count of macrophages (19).

The role of macrophages in malignancy was well characterized in a murine model of cancer, where knock out of the gene encoding the macrophage growth factor, colony-stimulating factor (CSF)-1, resulted in the growth of benign mammary cancers with a reduction in pulmonary metastasis (31). In breast cancer, CSF-1 expressed by epithelial carcinomas promotes the recruitment of macrophages to the tumor microenvironment (42). Once these macrophages arrive, they produce epithelial growth factor (EGF) that in turn, enhances the migration and invasion capabilities of mammary carcinomas in a CSF-1-dependent manner (20). Furthermore, primary tumors induce the upregulation of inflammatory chemokines, S100A8 and S100A9, which recruit macrophage antigen (MAC)-1 myeloid cells in the pre-metastatic tumor microenvironment (23). In this study, administration of S100A8 and S100A9 antibodies prevented the development of pseudopodia in the primary tumor cells, as well as the migration of primary tumor cells and MAC-1 myeloid cells to the pre-metastatic sites, suggesting that certain sub-populations of macrophages are responsible for promoting tumor metastasis.

Even though increased populations of TAMs in the tumor microenvironment have been associated with a poor clinical prognosis, it must be noted that TAMs are phenotypically diverse, reflecting their plasticity within different tissue microenvironments. Two different sub-populations of activated macrophages have been described, namely, "classically activated," or M1 macrophages, or "alternatively activated," or M2 macrophages (47). This nomenclature is a simplistic view of the complicated functions and behavior of macrophages, but is used to functionally distinguish the cytokine signals that induce their differential polarization. The main phenotypic characteristics of M1- and M2 tumor-associated macrophages are listed in Figure 2.

M1 macrophages are activated in response to bacterial lipopolysaccaride (LPS) and interferon (IFN)-$\gamma$. In turn, they secrete tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-12, reactive oxygen species (ROS) and reactive nitrogen species, as evidenced by the up-regulation of inducible nitric oxide synthase (iNOS) (47). Secretory products such as TNF-$\alpha$ and ROS can destroy cancers (47) while iNOS has been demonstrated to enhance the anti-tumor effects of doxorubicin (8). As well, IL-12, a heterodimeric cytokine is secreted by macrophages to activate natural killer (NK) cells (21) and also activate T-helper 1 (Th1) cells to elicit anti-tumor immune responses (10). Nuclear Factor-kappa B (NF-$\kappa$B) activation by the binding of the p50 and p65 subunits is also another characteristic of M1 macrophage activation (50). Although tumor cells down-regulate major histocompatibility complex (MHC)-I molecules to escape immune detection, dying primary tumor cells express extracellular damage-associated molecular patterns (DAMPs), such as high mobility group box protein (HMGB)-1 and heat shock proteins (HSPs) (16). These are detectable by macrophages *via* toll-like receptors (TLRs) (16). Purified HSP70 from mice with Dalton's Lymphoma was able to reverse the immunosuppressive macrophage phenotype induced by the tumor,

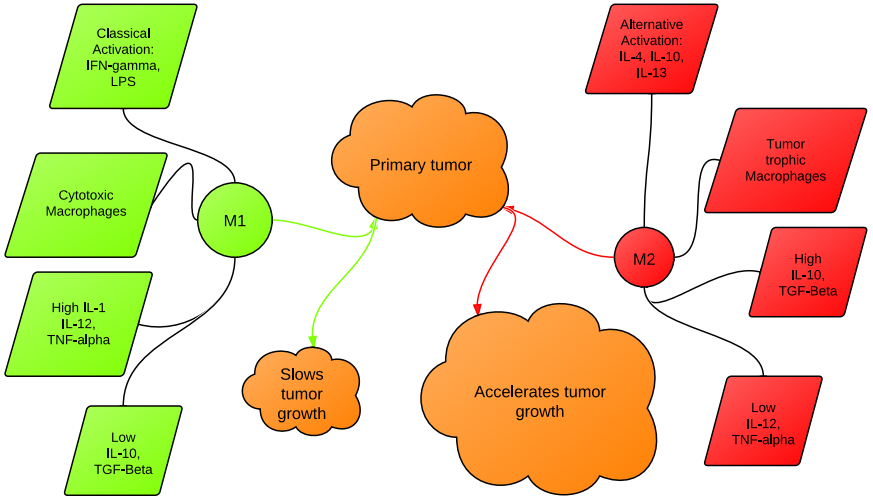


**Figure 2:** Characteristics of M1 and M2 macrophages. M1 macrophages produce high amounts of TNF-alpha, IL-1, IL-12 and low amounts of IL-10 and TGF-beta. Conversely M2 macrophages produce high amounts of IL-10 and TGF-beta, and low amounts of TNF-alpha, IL-1 and IL-12. M1 macrophages are cytotoxic and pro-inflammatory, whereas M2 macrophages support tumor growth and are associated with wound repair and tissue remodeling.

suggesting that HSP70 can change the polarization status of M2 macrophages to that of M1 (30).

There is practical rationale for investigating the effects of M1 macrophages in breast cancer because they play a role in tumor regression. These anti-tumor abilities of macrophages were reported in a study conducted by Hicks and colleagues (22). The authors generated a line of mice that displayed resistance against experimental tumor induction. These mice were named spontaneous regression/ complete resistance (SR/ CR) mice because they were able to either completely eradicate injected cancers, or to prevent the cancers from growing. Intriguingly, when the macrophages from these mice were injected into wild-type mice, the latter also developed resistance to the experimental cancer. This study suggests that macrophages are capable of recognizing and destroying certain cancers and hence useful for clinical immunotherapy. Although the polarization state of the macrophages was not investigated in that study, it is probable that they may share characteristics of M1 macrophages.

Unlike M1 macrophages, M2 macrophages are activated by the cytokines IL-4, IL-10, and IL-13 as well as glucocorticoids, while secreting factors and cytokines such as vascular endothelial growth factor A (VEGFA) (pro-angiogenic), IL-10 (inhibits dendritic cell maturation and promotes Th2 response) and matrix metalloproteinases (MMPs-2, -7, -9, -12) (47). Additionally, the balance of L-arginine metabolism in macrophages is also indicative of the direction of polarization to either M1 or M2 macrophages in the tumor microenvironment. M1

macrophages catalyze L-arginine to synthesize nitric oxide and L-citrulline, whereas M2 macrophages catalyze the hydrolysis of L-arginine to form L-ornithine and urea (50). A depletion of L-arginine in the tumor microenvironment can then inhibit T lymphocyte function and induce immunotolerance (47).

A certain sub-population of immature myeloid cells, termed myeloid-derived suppressor cells (MDSCs), further influences macrophage polarization in the tumor microenvironment (37). The presence of MDSCs in the tumor microenvironment has been reported in many cancers, including breast tumor and evidence suggests that MDSCs suppress immunosurveillance, and promote cancer progression and metastasis (54). MDSCs express the surface receptors CD11b and granulocyte differentiation antigen (Gr)-1 (57), originate from the bone marrow and are found in the tumor microenvironment. This is where they cross-talk with macrophages via cell-to-cell contact to induce the M2 phenotype, with an increase in IL-10 production that cause a corresponding decrease in IL-12 production by macrophages. The reduced macrophage production of IL-12 is particularly significant, since it dampens natural killer (NK) cell activity and also polarizes M1 macrophages toward the M2 phenotype (52). As well, increased MDSC production of IL-10 skews $CD4^+$ and $CD8^+$ T cells toward a cancer-promoting program and also inhibits dendritic cell (DC) maturation (52). Thus, macrophage polarization in the tumor microenvironment is influenced by complex cross-talk with MDSCs.

The macrophage phenotype is typically M2 in the tumor microenvironment. However, recent research also suggests that the phenotype of TAMs might not simply be M2, but a more progressive transition from M1 to M2, as the tumor becomes malignant and induces a different array of molecular signaling (47). Thus, it is conceivable that M1 macrophages are first polarized within an initiated tumor. With progressive growth and acquisition of malignancy, M1 macrophages might then be polarized to differentiate towards M2 macrophages, which then become pro-tumor and become the "tumor-educated" macrophages that Pollard hypothesized (43).

**Physical Activity or Exercise Modulates Macrophage Anti-tumor Activity**

Exercise or physical activity has a profound effect on macrophage physiology, including phagocytosis, chemotaxis, metabolism and anti-tumor activity (66). In murine models of acute exercise, peritoneal macrophage phagocytosis (12) was increased *in vitro*, relative to sedentary conditions. In young, healthy humans subjected to strenuous interval training (running and cycling), an exercise-induced decrease in monocyte chemotactic protein (MCP)-1 induced monocyte chemotaxis was observed (7). This contrasts with the increase in macrophage recruitment in the murine models described above. However, it must be noted that the murine studies utilized acute bouts of exercise, whereas the human study utilized a three-week exercise training protocol. It would be interesting to compare the effects of macrophage recruitment in murine models after exercise training, compared with acute exercise. The physiological implication of this study suggests that exercise training may be "anti-inflammatory", in that there may be a decrease in monocytes being recruited into a pre-malignant tumor microenvironment. Perhaps one anti-tumor mechanism induced by exercise could involve a reduction in macrophage presence in the tumor microenvironment. What is clearly needed is to determine whether polarized phenotypes are different with exercise training.

Woods and colleagues reported other macrophage functions that were modulated with acute exercise (61, 62, 63, 64, 65). In one of their studies, male C3H/HeN mice pre-assigned to either three days of moderate-intensity or exhaustive treadmill running were injected subcutaneously with SCA-1 adenocarcinomas. Subsequently, the mice were exercised for an additional 14 days (64). Moderate-intensity exercise resulted in greater numbers of highly phagocytic cancer-infiltrating macrophages compared with either controls or exhaustive exercise. Tumor incidence, defined as the onset of palpable cancers, was delayed in the control group on the 7th day after implant compared with either of the exercise groups. However, final tumor weights were not different between groups. This suggests that short-term exercise training in C3H/HeN mice slowed the early onset of tumor growth, but was ineffective in reducing the final tumor burden. This lack of a robust effect may be due to the "dose" of the exercise given, which was a few days of treadmill running. A long-term exercise protocol greater than two weeks may be needed to stimulate a stronger anti-tumor effect.

To address the mechanistic effects of acute exercise on macrophage activation (64) and inflammatory macrophage response (65) against cancers, male C3H/HeN mice were injected intraperitoneally with thioglycollate (64, 65) or propionibacterium acnes (64) to induce peritoneal inflammation and macrophage influx. The mice were then subjected to acute moderate-intensity treadmill running or exhaustive treadmill running for three consecutive days post-injection before they were sacrificed. The significant finding from both studies was that compared with controls, moderate-intensity and exhaustive treadmill running resulted in enhanced macrophage cytotoxicity against spinocerebellar ataxia (SCA)-1 cancer cells *in vitro*, as measured by the reduced [$^3$H] Thymidine incorporation by the cancer cells, a marker of cell proliferation. Acute exercise had neither effect on the percentages of macrophages in peritoneal cells nor the number of macrophages that adhered to culture dishes, suggesting that quantitative changes in macrophage numbers may not be responsible for the phenotypes observed with acute exercise. These two studies also suggest that peritoneal macrophage anti-cancer cytotoxicity may be modulated with acute exercise *in vitro,* but do not give any indication of TAM function nor the types of macrophages (M1 or M2) that are recruited into the cancer microenvironment. As discussed earlier, TAMs either inhibit or stimulate cancer growth and metastasis, depending on their polarized phenotype. Zielinksi and colleagues (67) reported that in female BALB/c mice that ran on treadmills for two weeks after implantation of allogeneic lymphoid cancers, macrophage infiltration into the cancers were significantly lower than control sedentary mice. Whether such an effect is seen in other cancer models, strains of mice, or the phenotype of macrophages that were reduced is unclear, but is an important issue to address.

Not all acute or short-term exercise-induced changes in macrophage functions are necessarily beneficial. Antigen presentation by macrophages may be down-regulated. To illustrate this, male BALB/c mice were injected with thioglycollate and then subjected to moderate-intensity or exhaustive treadmill running for four consecutive days (5). Upon sacrifice, peritoneal exudate cells were harvested, washed to remove non-adherent cells, and incubated with T-hybridoma cells and chicken ovalbumin. Chicken ovalbumin was used as an antigen for macrophage antigen presentation to the T-hybridoma cells, and the resultant pro-

duction of IL-2 by the hybridoma cells was a direct measure of macrophage antigen presentation. Exercised mice showed decreased IL-2 concentrations, as measured by an enzyme linked immunosorbent assay (ELISA) kit at different concentrations of ovalbumin, thus suggesting a suppression of macrophage antigen presentation, allowing the cancer to escape immune detection and cytolysis. An exercise training study that was of longer duration was also conducted. It involved young (6 months) and old (22 months) BALB/c male mice made to run on treadmills for four months (32), the investigators observed that compared with sedentary controls, exercise training increased macrophage cytolysis of P815 cancer cell lines, although the effect was stronger in the young mice. In addition, macrophage production of nitric oxide was also increased in exercised mice, with an increased gene expression of iNOS in the young exercised mice, but not old exercised mice, suggesting that the cytotoxic effects may not be mediated *via* iNOS.

The conclusion drawn from these studies is that exercise training in mice generally enhances the anti-tumor effect of macrophages *in vitro.* Discrepancies in the findings from the various studies may be due to differences in exercise duration and/or intensity, length of exercise training, diet protocol, dosage or timing of tumor cells or carcinogens injected and strain of rodents studied. In some cases, discrepant results may stem from the fact that some unidentified subsets of dendritic cells, which play a bigger role in antigen presentation than macrophages, may influence the immune response in cooperation with, or independent of macrophages after exercise.

**Can Physical Activity or Exercise Training Shift Macrophage Polarization?**

Exercise training in mice appears to shift macrophage polarization, at least as extrapolated indirectly from the cytokine milieu of three animal studies. In the first study (58), 10 days of treadmill running in male BALB/c mice transplanted intraperitoneally with Dalton's lymphoma resulted in reduced vascularization around the peritoneal region, compared with sedentary control mice. This observation was accompanied by the reduction of VEGF expression, decrease in the number of erythrocytes in peritoneal fluid, and increase in oxygen concentration in Dalton's lymphoma cell-free ascitic fluid. Finally, the authors reported that the peritoneal fluid from exercised mice had a higher concentration of Th1 cytokines, compared with Th2 cytokines, such that there was an increase in IFN-$\gamma$ and a decrease in IL-4 and IL-10. In the second study (29), three weeks of treadmill running increased LPS-stimulated NO, IFN-$\gamma$ and TNF-$\alpha$ production in peritoneal macrophages of male BALB/c mice, compared with control, sedentary mice. On the other hand, the production of IL-10, a cytokine that is commonly associated with M2 macrophages, was lower in trained mice versus control mice. Finally, in the third study (32), exercise training increased macrophage production of nitric oxide, concomitant with increased iNOS gene expression. This effect was however, attenuated in old mice.

The first study suggests that exercise training in cancer-bearing BALB/c male mice may shift the cytokine balance from a Th2 to a Th1 phenotype, at least in the cancer microenvironment. The second study indirectly corroborates the first, and suggests that biomarkers of M1 macrophages appeared to be increased in peritoneal macrophages of healthy, exercise-trained BALB/c male mice. Although the first study was conducted using Dalton's lymphoma, there is a pos-

sibility that exercise training or physical activity may result in a similar outcome in mammary carcinoma. The exercise-induced phenotypes in BALB/c mice from both studies suggest a shift in macrophage polarization, although whether these phenotypes extend to mice of other strains is unclear. For example, bronchoalveolar macrophages obtained from C57BL/6 mice and BALB/c mice were reported to respond differently to acute treadmill running. C57BL/6 mice are prototypical Th1 strains, whereas BALB/c mice are Th2 strains. In this study, unlike M2 bronchoalveolar macrophages from BALB/c mice, M1 bronchoalveolar macrophages from C57BL/6 mice did not increase phagocytosis of unopsonized particles after an acute bout of treadmill exercise, nor did they increase expression of macrophage receptor with collagenous structure (MARCO). The studies cited above suggest that exercise training in mouse models may shift the cytokine milieu to be representative of M1 macrophages.

**Exercise-Induced Macrophage Signaling Triggers Specific Anti-Tumor Mechanisms**

It is known that macrophages and MDSCs cross-talk in the cancer microenvironment. It is possible that cytokines specific to both cell types, and that are responsive to acute or chronic bouts of exercise, may represent an "immune" signature for exercise-induced immunomodulation in the cancer microenvironment. That is, the balance of these cytokines may indirectly reflect changes in the macrophage phenotype in the tumor microenvironment. For example, it was reported that acute exercise increases serum IL-12 in elite female soccer players, when blood was drawn 15-20 minutes after a soccer match (2). It appears that to elicit increases in this cytokine, the exercise must be done at an intensity that could be considered vigorous. Increases in serum IL-12 were observed 24 hours after cycle ergometry was performed at a high intensity (70% of $VO_2$max), but were not observed when exercise was performed at moderate intensity (55% of $VO_2$max) (17). Therefore, these human studies suggest that vigorous exercise may elicit an increase in serum IL-12. While the source of this cytokine is unknown, it is possible that it may be produced by macrophages. While speculative, regular exercise training may induce IL-12 production in the tumor microenvironment, which enhances the release of IL-15 in TAMs and subsequently, recruits NK and $CD8^+$ cells to aid in cancer regression (59).

Exercise also increases the release of extracellular HSP70 from liver into the circulation (45). The secretion of this molecular chaperone has immunomodulatory implication, for it is known that HSP70 binds to human monocytes and upregulates the expression of TNF-$\alpha$, IL-1 and IL-6 (3). We hypothesize that exercise may: i) activate the heat shock response as a means to enhance macrophage surveillance against potential danger, which in this case are the transformed epithelial cells or ii) induce a DAMP response in stressed tumor cells, potentially by increasing HSP70, which can recruit and activate M1 macrophages for phagocytosis. The anti-cancer response involving DAMP could involve toll-like receptor (TLR) signaling. TLR-4 is a transmembrane protein expressed on monocytes, macrophages and dendritic cells, that functions as a pattern recognition receptor in response to recognition of DAMPs, such as those expressed by bacterial lipoproteins, or other "danger signals" (18). One such "danger signal" would include cancer cells. Indeed, the innate immune system is capable of recognizing

cancer cells *via* TLR activation and the subsequent production of anti-cancer molecules, such as IFN-γ (9). The activation of TLRs *via* ligand binding then results in their binding to intracellular adaptor proteins such as MyD88, and recruits other proteins involved in the inflammatory process, such as IL-1R-associated kinase (IRAK)-1, as well as inducing the production of inflammatory cytokines such as IL-1β and TNF-α, both of which are transcriptional upregulators of iNOS and also products of M1 macrophages (47). However, cancer cells can induce immune tolerance in monocytes by down-regulating the expression of IL-1β and TNF-α by activation of IRAK-M, a negative regulator of the inflammatory response (9). This activation of IRAK-M appeared to be dependent on TLR4 signaling as well, since pre-incubation of human monocytes with TLR4-specific antibodies reduced IRAK-M induction in a dose-dependent manner (9).

In addition to its role in cancer cytotoxicity, TLR4 acts as a functional receptor for serum amyloid A3 (SAA3) on lung endothelial cells and macrophages during the pre-metastatic phase, suggesting that TLR4 expression is up-regulated in TAMs such that they are then recruited to pre-metastatic sites (23). These studies illustrate a mechanistic role for TLR4 in mediating anti-cancer response in innate immune cells, as well as in the chemoattractant response for TAMs to condition the pre-metastatic site for eventual metastasis. These two roles appear to be juxtaposed to each other, such that TLR4 signaling may be detrimental in terms of priming the pre-metastatic site for eventual metastatic colonization, and yet, TLR4 signaling is involved in the activation of the M1 phenotype. To address this dichotomy, it may be required to consider whether TLR4 signaling in a pro-inflammatory cancer microenvironment is associated with a better or poorer clinical prognosis. From a clinical perspective, it was found that physically active and exercise-trained individuals have lower monocyte expression of TLR4 (18), suggesting that physical activity may exert an anti-inflammatory response *via* TLR4 downregulation in monocytes. Curiously, physically active individuals also have lower blood concentrations of inflammatory cytokines such as IL-1β and TNF-α (34). In addition, Timmerman and colleagues (55) also reported that combined resistance and endurance training resulted in a reduction in percentage of $CD14^+CD16^+$ inflammatory monocytes in circulation as well as reduced LPS-stimulated TNF-α production in whole blood cultures of elderly men and women.

These reports of exercise-induced down-regulation of TLR4 expression and inflammatory cytokine production are not incompatible with the prevailing view that exercise or physical activity improves innate immunity and reduces inflammation (40). In the context of breast cancer, it may mean that in the pre-initiation phase of carcinogenesis, macrophages present in the breast microenvironment should be of the M2 phenotype. This assertion is supported by findings that macrophages are involved in the remodeling of the mammary tissue during development, lactation and involution (6). A healthy mammary microenvironment likely has an influx of both M1 and M2 macrophages to clear the apoptotic epithelial cell and assist in the branching of the terminal milk ducts (41). The balance between M1 and M2 in the mammary microenvironment may favor the development of pre-cancer. Adipocytes in the mammary microenvironment may secrete pro-inflammatory cytokines to recruit M1 macrophages and increase the susceptibility of mammary cancer risk. To illustrate this point, a chronic inflammatory state associated with increased expression of M1 macrophages in adipose tissues

has been reported in diet-induced obesity in mice (33). Conversely, exercise training reduced gene expression of the M1 macrophage marker, CD11c, in adipose tissue as well as inhibited adipose tissue TLR4 expression in C57BL/6 mice fed a high fat diet (28). These studies were not reported in the context of cancer cytotoxicity. Since inflammation is observed in the tumor microenvironment, it may

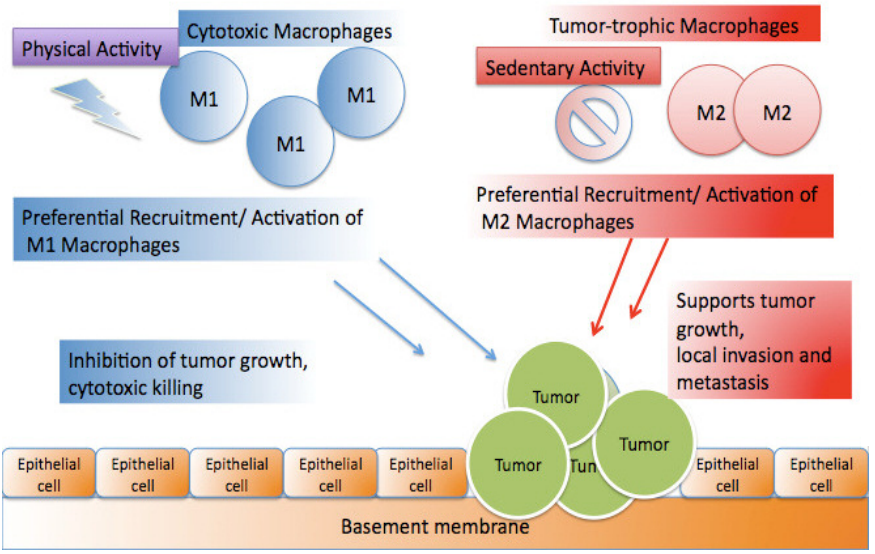


**Figure 3:** Proposed role of physical activity and exercise on the polarization of macrophages in the tumor microenvironment. Physical activity preferentially polarizes tumor-associated macrophages (TAMs) to an M1 phenotype with anti-tumor affects. Lack of physical activity results in the preferential polarization of TAMs to the M2 phenotype, which supports tumor growth, local invasion and metastasis.

be possible that each specific tissue microenvironment affects the plasticity of TAMs differently. Whereas an M1 cytotoxic macrophage polarization is desirable for the host in the context of cancer cytotoxicity, excessive inflammation, such as in the case of chronic inflammation, may lead to tissue destruction, DNA damage, and oxidative stress, which can paradoxically accelerate carcinogenesis and metastasis (56). Thus, the balance of M1 to M2 macrophages in a normal mammary microenvironment is tightly regulated by their interactions with the epithelial cells and other stromal cells.

Whether an exercise- or physical activity-induced polarization is seen in TAMs within the breast tumor microenvironment is unclear, but indirect evidence from the studies described earlier (29, 32,58) suggest that this may be probable,

as illustrated in Figure 3. In this scenario, physical activity would preferentially polarize tumor-associated macrophages (TAMs) to an M1 phenotype with anti-tumor affects, while lack of physical activity would result in the preferential polarization of TAMs to the M2 phenotype resulting in tumor growth, local invasion and metastasis. It is unclear whether physical activity/ exercise training reduces macrophage infiltration of the tumor microenvironment. According to Czepluch *et al.* (7), young, healthy human subjects undergoing interval training comprising bouts of running and cycling were shown to have attenuated MCP-1 induced migration of monocytes *in vitro*. When the subjects were allowed to recover for 4 weeks after the exercise training period, their serum concentrations of MCP-1 protein remained depressed. Whether this suggests a global attenuation of reduced monocyte trafficking is unclear, and the question then, is whether this outcome is desirable in terms of overall immune function. Certainly, the case for having reduction in macrophage infiltration of the tumor microenvironment is desirable, but only when these macrophage become polarized to that of the M2 phenotype. It is possible that individuals that are endurance-trained or physically active may have reduced monocyte trafficking, which may be concomittant with lower pro-inflammatory cytokines in circulation. In the event that such individuals are diagnosed with breast cancer, their long-term training status may result in a reduction of macrophage infiltration of the tumor microenvironment, and therefore, may also result in a reduction in the quantity of M2 macrophages being polarized. Alternatively, trained individuals may simply have a better ability to resolve M1-type inflammation during the different stages of mammary development, and this ability to down-regulate inflammation could be a protective factor in itself.

## SUMMARY

This review has discussed the effects of physical activity and aerobic exercise on the biology of breast cancer and the possible modulatory effects on TAMs. Not much is known about other forms of physical activity and exercise training, such as the impact of occupational and household physical activity, swimming, weight lifting etc. For individuals with limited access to recreational physical activity, it may be more applicable to determine whether being physically active at work or doing household chores could provide improved immuno-modulation of TAMs.

Some crucial questions remain in order to elucidate the role of physical activity/or exercise on TAMs: i) does exercise training and/ or physical activity reduce the number of monocytes recruited to the cancer microenvironment, ii) does exercise training and/ or physical activity alter the phenotype of macrophages within the cancer microenvironment, but not the trafficking/ recruitment of monocytes to the specific cancer microenvironment? iii) What is the optimal "dose" of physical activity or exercise training in eliciting a beneficial macrophage polarization response? iv) Are there differences in macrophage polarization in the pre-cancer and cancer microenvironment? Addressing these questions would allow investigators to enhance the knowledge of clinically relevant markers of prognosis, and determine whether physical activity and exercise training can be used routinely as primary or adjunctive prevention methods to modulate these markers.

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# *Impact of endotoxin exposure after exhausting exercise on the immune system in solid organ transplant recipients*

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## ABSTRACT

*Subsequent to prolonged exhausting exercise a transient immunosuppression is often observed in athletes. This so-called "open window" results in a reduced resistance of the athletes to viral and bacterial infections after an exhaustive exercise bout. Concerning the effect of bacterial endotoxin contact after exhausting exercise in transplant recipients, who are innately immunosuppressed by their medication, no data exists at present. After performing 81 km cycling, including ascending more than 1800 m in altitude, peripheral blood from 10 male kidney transplant recipients and from 10 healthy controls matched for age and gender, was obtained. Simulating contact of the athletes with a pathogen post-exercise, the blood samples were incubated with Lipopolysaccharides (LPS). Thereafter, microarray analysis was performed. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation. Especially immune response genes were significantly over-represented in controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. Merging our previous data with these recent findings it should be discussed if transplant recipients need to reduce their immunosuppressive medication before performing exhaustive exercise.*

**Key words:** Exhaustive Exercise, Transplant recipients, Cycling, Immune system

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## INTRODUCTION

It is well known that moderate exercise has a positive effect on the immune system, while exhausting exercise can lead to a transient immunodepression, a so-called "open window" resulting in a reduced resistance of the athlete to viral and bacterial infections after exhausting exercise (9; 11). Several conditions are suspected to be responsible to induce such an "open window" situation after an exhaustive exercise bout. For instance, white blood cells are activated and regulated following exhausting exercise. In regard to innate immunity, neutrophils are initially increased but after prolonged exercise a reduced degranulation and oxidative burst in response to bacterial stimulation has been observed (15). The number of circulating NK cells, which are important cells in first line of defence against infections, is also known to be reduced in blood following prolonged exercise (15). Concerning adaptive immunity, T helper (CD4+) type1 (Th1) cells play an important role in defence against intracellular pathogens (e.g. viruses) by cytokine release and stimulation of effector cells (15). Suzuki et al. were able to show that exhausting exercise can lead to the suppression of Th1 activity, by shifting the cytokine profile towards Th2 cells, inducing humoral immunity including allergic reactions (14). Furthermore, exhaustive exercise also leads to generation of reactive oxygen and nitrogen species (ROS, RNS) (10), with damaging potential and promotion of lymphocyte apoptosis (6).
All in all these conditions can lead to depression of immune function in athletes following exhaustive exercise. In the last edition of "Exercise Immunology Review" we had the opportunity to present data on the impact of exhaustive exercise on the immune response in transplant recipients (8). In regard to our previous findings and knowing about the "open window" after exhaustive exercise in healthy athletes it is of great interest for the transplant community, particularly since the immune system is strongly affected by the life-long required immunosuppressive medication after organ transplantation, to know what happens in transplant recipients after pathogen contact and exhausting exercise. In regard to simulating contact with a pathogen shortly after exhaustive exercise in transplant recipients, who are innately immunosuppressed by their medication, no data exists at present. We are delighted to present additional results from our previous study obtained during the „Euregio cycling tour 2009" in brief.

## METHODS

As already described, the *"Euregio cycling tour"* is completed over 3 days and involves cycling more than 300 km (day 1: 110 km, day 2: 90 km, day 3: 102 km). Blood samples (2.5 ml whole blood) were drawn from seated subjects into PAXgene™ Blood RNA Tubes (Qiagen, Hilden, Germany) at rest before (t0) and immediately (up to 15 minutes) after cycling 81 km with an ascent of more than 1800 m in altitude from the starting point (t1). The whole blood samples in EDTA at (t1) were additionally incubated with Lipopolysaccharide (LPS) for 24 hours at room temperature. The final concentration of LPS used for stimulation of the whole blood samples was 1 ng·ml-1 (Sigma-Aldrich, St. Louis, USA). As already

mentioned the detailed candidate selection was described previously and is only summarized herein in brief. Out of approximately 30 solid organ transplant recipients, 10 male patients, all of whom had received a kidney transplant in their past were selected with a healthy control group, consisting of 10 cyclists, matched for age and gender. Only BMI showed a significant difference between groups (p=0.006, comtrols mean 78.0kg, transplant recipients mean 71.3kg) (8).
Immunosuppressive medication used daily by the transplant recipients included FK 506 (Tacrolimus), Cyclosporine, Mycophenolate-Mofetil or Azathioprine. Each subject gave written informed consent prior to participation in the study. The experimental protocols were approved by the Institute's Human Ethics Committee according to the principles set forth in the Declaration of Helsinki of the World Medical Association (449/2008BO2).

**Transcriptome profiling**

For expression profiling 400 ng of total RNA were linearly amplified and biotinylated using the Illumina® TotalPrep™ RNA Amplification Kits (Ambion) according to the manufacturer's instructions. Human HT-12v3 bead arrays (Illumina, San Diego, CA) were hybridized with 750ng cRNA for 18h at 58°C according to the Illumina® Whole-Genome Gene Expression with IntelliHyb Seal System Manual. Arrays were washed three times with buffer E1BC, High-Temp Wash Buffer and 100% ethanol, respectively, stained with streptavidine-Cy3 and again washed with buffer E1BC. Raw fluorescence intensities were recorded on a BeadArray Reader GX (Illumina). Average signal intensities, background correction, quantile normalization and quality control were performed with BeadStudio 3.1 software (Illumina).
All subsequent data analysis steps were performed on the software platform R 2.10.0 and Bioconductor 2.6.1 (5) with the packages "beadarray" (2; 4), "limma" (12; 13), "GOstats" (3). Initially, the expression data from all chips were normalized with VSN (7). The signal values were then averaged for the individual subgroups and differences in expression level were calculated. Differences between subgroups were extracted as contrasts and analyzed with the moderated F-test (empirical Bayes method) including a correction step for multiple testing with the 5%-FDR-based method of Benjamini and Hochberg. To attribute significant regulations to individual genes, a decision matrix was generated based on the function decide tests within the Limma option nestedF, where significant up- or down-regulations are represented by values of 1 or -1, respectively.
Due to the factorial design of the experiment, two parameters (patient group and time/treatment) have an impact on gene expression, while the influence of inter individual differences has to be taken into account. For both groups the factors time/treatment and donor was used to design a linear model capturing the influence of the different factors on gene expression levels. A non specific filter based on detection p-values was applied to remove non informative genes before the fitting of the linear models was performed. The coefficients describing the expression profiles of the remaining probe sets were calculated and the standard errors were moderated using an empirical bayesian approach. From the F statistic the resulting p-values were established and corrected for multiple testing with „Benjamini-Hochberg" (1).

Resulting gene lists were analyzed for over representation of gene ontology terms (www.geneontology.org) in the branches "biological process" and "molecular function" and KEGG pathways (www.kegg.jp) with conditional hypergeometric tests. Categories and pathways with a p-value less than 0.01 were considered significantly enriched. (8)

## RESULTS

All cyclists, consisting of the 10 transplant recipients and the 10 corresponding healthy control athletes finished the tour safely and successfully. Both groups were comparable in their performance and none of the athletes fell ill during the tour. Simulating pathogen contact of the athletes post exercise, the blood samples were incubated with LPS. Thereafter, microarray analysis was performed. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation, directly after exhausting exercise (t1). Whereas 86 significantly up-regulated and 4 down-regulated genes were detected in transplant recipients (LPS treated vs untreated), we found an up-regulation of 151 and a downregulation of 18 significantly differentiated genes in the corresponding controls. Only 79 genes were regulated in common between these two groups.
Especially immune response genes were significantly over-represented in the controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. Significantly over-represented gene ontology terms and KEGG pathways of the differentially expressed transcripts in transplant recipients and their corresponding controls are given in table 1.

## DISCUSSION

Following prolonged exhaustive exercise a number of peripheral immunological parameters have been demonstrated to change significantly (16). These changes comprise induction of cytokines and hormones, ROS and RNS, changes in NK cell and in Th1 cell activity, leading so to a transient immunodepression, resulting in a reduced resistance of the athlete to viral and bacterial infections after exhausting exercise (9 – 11; 14; 16). Being aware of the fact, that this transient immunodepression ("open window") exists in healthy athletes after exhaustive exercise, the question has arisen in the transplant community as to what happens to transplant recipient athletes, who are innately immunosuppressed by their medication, after pathogen contact and exhausting exercise. We recently showed that the relative increase of neutrophils in transplant recipients was significantly smaller than in their corresponding controls after exhausting exercise and that concerning expression regulations the control athletes demonstrated a higher immune response regulation than the transplant recipients (8). Transplant recipients showed after exhaustive exercise a significant activation of genes related to cell metabolism, but genes related to the immune response were missing (8).
To further elucidate the answer to the question of what happens after pathogen

**Table 1:** Ontology terms significantly over-represented exclusively in transplant recipients and corresponding healthy control subjects, respectively, as well as collectively over-represented ontology terms in both groups, after the exhaustive exercise bout with LPS stimulation.

| Over-represented ontologies in transplant recipients | p-value |
|---|---|
| regulation of apoptosis | < 0.001 |
| regulation of cell death | < 0.001 |
| negative regulation of apoptosis | < 0.001 |
| regulation of cellular metabolic process | 0.001 |
| cell death | 0.001 |
| response to wounding | 0.004 |
| response to hyperoxia | 0.004 |
| induction of apoptosis | 0.007 |
| removal of superoxide radicals | 0.008 |
| recognition of apoptotic cell | 0.008 |
| cellular macromolecule biosynthetic process | 0.009 |

| Over-represented ontologies in corresponding healthy controls | p-value |
|---|---|
| toll-like-receptor 4 signaling pathway | < 0.001 |
| negative regulation of Notch signalling pathway | < 0.001 |
| lipopolysaccharide mediated signalling pathway | 0.003 |
| activation of innate immune response | 0.004 |
| negative regulation of transcription factor import into nucleus | 0.005 |
| regulation of NF-kappaB | 0.006 |

| Over-represented ontologies in both groups | p-value |
|---|---|
| cyclooxygenase pathway | 0.001 |
| adult somatic muscle development | 0.001 |
| oxidoreductase activity | 0.002 |
| prostaglandin-endoperoxide synthase activity | 0.002 |
| procollagen-proline 4-dioxygenase activity | 0.004 |
| prostaglandin E receptor activity | 0.005 |

contact and exhausting exercise, we incubated peripheral blood samples from transplant recipients and their corresponding controls with LPS, followed by microarray analysis and are now delighted to present the results. Microarray analysis revealed a markedly oppositional pattern of gene expression in transplant recipients compared with their controls after LPS incubation, directly after exhausting exercise. Interestingly especially immune response genes were significantly over-represented in controls immediately after the exhaustive exercise bout with LPS stimulation, whereas numerous apoptotic genes were over-represented in transplant recipients. From our point of view this is a very disconcerting finding. We are aware of the fact that LPS incubation of peripheral blood is not exactly comparable to a real infection of the athlete, but nevertheless at least it gives a likely indication of how the immune system might respond to an infection. Con-

cerning the notable expression of apoptotic genes in transplant recipients, exhaustive exercise followed by pathogen contact, could lead to an increased risk of infection and cell damage in transplant recipients. Patients with long-life immunosuppressive medication are, however, known to be more prone to develop neoplasia over the course of time. Accordingly, additional apoptotic gene expression after exhaustive exercise may harbour an increased risk of cell failure with the potential consequence of promoting neoplasia. It seems possible that the immune system in transplant recipients is impaired by the effect of exhausting exercise, pathogen contact and additionally by the immunosuppressive medication. This could lead to an increased risk of infection in transplant recipients with potential cell damage and its consequences after exhaustive exercise. Whether a reduction of the immunosuppressive medication before exhaustive exercise could be advantageous remains unclear. From our point of view certain topics need to be urgently further elucidated. At first, investigations need to be done concerning the effect of moderate and regular exercise on the immune system in transplant recipients. It is possible that in transplant recipients, since inflammatory mechanisms are curbed by their medication, exercise has no pro-inflammatory effects and consequently no anti-inflammatory counter regulation and is therefore simply neutralized in its interaction with the immune system. On the other hand if regular moderate exercise shows an anti-inflammatory effect, could this be a possibility means of reducing the amount of immunosuppressive medication? Furthermore, the consequence of exhaustive exercise on its own and with pathogen contact after exhaustive exercise needs to be investigated in detail by immune function assays and expression and protein assays in transplant recipients.

Altogether our findings are the first to show these exciting gene expression alterations in transplant recipients after pathogen contact post-exhaustive exercise. Nevertheless our results raise new questions that are more than ever of great interest and impact for the transplant community particularly for those that do sports. Hence, this topic should be further investigated in the near future.

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# *Obituary for Professor Eric Arthur Newsholme, MA, DSc, (PhD, ScD Camb)*

Eric Newsholme died peacefully in hospital from heart failure a few hours after slipping into a coma on 17th March 2011. Although those of us who were close to him were well aware of his heart and circulation problems over several years, it was nevertheless a shock and, as many have observed, untimely.

He had not completed his final project, which was a book on the scientific basis for outstanding physical performance in football. Football was a very appropriate topic for Eric, since he was born in Liverpool, England in May 1935 and was a lifelong devotee of Liverpool Football Club. He used to express surprise that so many premier league football clubs showed little, if any, interest in therapeutic nutrition when an expensive player was hors d'combat. Those of us who acted as occasional amanuenses, due to Eric's intense distrust of computers, were aware of his current interests. Eric would request reprints which we could obtain via Pubmed: he initially tried his hand at the vernacular, asking one of us to achieve this by "surfing the internet, or whatever it is that you do!"

Eric read Natural Science at Cambridge and this was followed by a PhD in Biochemistry. In 1964 he moved to Oxford University to work with Sir Hans Krebs: he became a Fellow of Merton College and a Lecturer at the Department of Biochemistry in 1973. Eric was a rare example of a talented researcher who was also an outstanding teacher. He certainly captured the attention of undergraduates. The usual trend is for numbers to decline during a term's series of lectures: Eric's lectures were exceptional, in that the reverse occurred, and numbers of students attending actually increased as word got round. Dr Chris Milne (Australasian College of Sports Physicians) who encountered him as a visiting lecturer in London says:

"I was fortunate to be exposed to Eric's superb teaching whilst completing the Diploma Course in Sports Medicine at the London Hospital Medical College in the 1980s.

"I had experienced some teaching from exercise physiologists during my undergraduate and postgraduate years, but none from an exercise biochemist. It was great to hear about the Krebs cycle from someone who had worked alongside Hans Krebs himself. For most of us, a study of the Krebs cycle is only palatable following a small libation with $C_2H_5OH$, but Eric made it possible without any such premedication! He had the knack of making complex scientific ideas simple to understand and, in my view, was second only to the famous science fiction (and fact) writer Isaac Asimov in that regard.

"On hearing of Eric's untimely death, I pulled out an article he co-wrote with Tony Leech entitled 'Fatigue Stops Play' (New Scientist, 22 September 1988 p39 43). It is a masterpiece in communication of complex scientific principles in layman's terms, such that coaches can make good use of the material. It even anticipates some aspects of Tim Noakes' 'Central Governor' model by several years."

More than 50 PhD students and a similar number of postdoctoral scientists received research training in Eric's lab. He was regarded by them as a gifted and

inspirational supervisor. As one of his former DPhil students said: "Eric was an excellent supervisor who taught me how to be a scientist, always probing and questioning and never rushing, always stressing the importance of paying attention to detail. These are qualities I am trying to pass on to my students, both undergraduate and postgraduate." A frequent tribute to Eric from graduate students and colleagues alike was his kindness and courtesy. He would spend time discussing an individual's research ideas regardless of the quality: equally, he enjoyed coming into the lab and saying to whoever was around "I have this crazy idea....!" to prompt what he hoped would be a lively discussion, involving challenges to his concepts.

He frequently advised us on, and tried to adhere to, the principle which Krebs instilled in him, viz. that you should only make one point per sentence. We were not always successful! His use of red pen on first, second, third and more of his and our drafts was legendary. In pre-computer days, his secretary (upon presenting him with a 5$^{th}$ draft of a paper) said: "You can read it but sit on your hands!" It says much for his teaching that, out of more than 40 Oxford DPhil theses which he supervised, there was only one referral.

Eric published over 300 research papers and reviews: his textbooks *(Regulation in Metabolism* by Newsholme & Start (1973) and *Biochemistry for the Medical Sciences* by Newsholme & Leech (1983) provided many biochemists with much knowledge of intermediary metabolism and metabolic control. In particular, Newsholme & Start (1973) was regarded by many as a classic. Eric recently updated his 1983 textbook to become *'Functional Biochemistry in Health and Disease'* with Tony Leech (2010).

His first full paper (with his PhD supervisor , Sir Philip Randle, in Biochem. J. 1961) was on the regulation of glucose uptake by muscle. Four of his papers on muscle metabolism of fatty acids, ketone bodies, glucose and pyruvate, (two of which were in Nature) expanded on the "glucose-fatty acid cycle" data first published by Randle, Garland, Hales and Newsholme in the Lancet (1963). About 20 of the 100 papers that Eric published in the Biochemical Journal are recognized as seminal papers by the research community.

Eric took up marathon running in his mid-30s and successfully completed around 40 marathons: this gave him a considerable appreciation of energy metabolism in exercise. However, his work on the biochemistry of exercise and potential nutritional therapy initially stemmed from an unusual occurrence: his 6-yr-old daughter suffered from a particularly aggressive form of Guillain-Barre syndrome (inflammatory demyelinating polyneuropathy), and was paralysed for 6 months. She rapidly recovered to full health after receiving daily doses of sunflower seed oil and home-based physiotherapy. Eric recognized that, in order to understand the possible mechanisms for this outcome, he would need to "get his feet wet in immunology". He was fortunate to receive advice and generous help with facilities from immunologists Simon Hunt and Siamon Gordon at the Dunn School of Pathology in Oxford.

Work in the late 70s, early 80s with a DPhil student, MSM Ardawi, resulted in the discovery (among others) that the amino acid, glutamine, was used as a fuel at a surprisingly high level in resting, unstimulated lymphocytes. In vitro work in Eric's lab by another student, Mark Parry Billings, demonstrated that, despite the presence of all other essential nutritional components in cell culture medium,

only when glutamine was decreased did a decrease in the proliferative ability of lymphocytes occur. Philip Newsholme (Eric's son) and, later, Philip Calder (also students) showed that glutamine was essential for macrophage function. On becoming aware of a decrease in some key immune cell numbers after prolonged, exhaustive exercise, Eric hypothesized that a lack of glutamine might be responsible.

Glutamine is a metabolic fuel for many cells and a nitrogen donor for purine and pyrmidine nucleotide synthesis, and thus for DNA synthesis: there is an extensive literature on the role of glutamine in clinical situations, to which Eric's group has contributed, showing that burns and major trauma are associated with very low glutamine levels in the blood. Several field studies confirmed that the plasma concentration of glutamine was indeed low (by 20-25%) in endurance runners after an event, as well as in overtrained athletes at rest. Subsequent studies on more than 150 marathon runners showed that the provision of glutamine within the recovery period reduced self-reported illness (mostly upper respiratory tract infections) by around 43% compared with placebo. Five other published studies unequivocally show similar decreases.

Disappointingly, it has not so far been possible to demonstrate which aspects of the immune system are affected by restoring plasma glutamine to normal physiological levels. However, this situation is similar to some other, more recent studies which have looked at different supplements and self-reported illness.

Eric's work in the biochemistry of exercise extended to the central fatigue hypothesis, fatigue emanating from the brain rather than from muscle. This is based upon the effects of a surge of unbound tryptophan, uniquely splitting from albumin in the blood, crossing the blood-brain barrier (in competition with branched chain amino acids, BCAA) to increase the synthesis of the neurotransmitter 5-HT which is involved in fatigue. Eric suggested that the provision of BCAA might combat the surge in tryptophan (a precursor for 5-HT, also known as serotonin) and thus reduce premature fatigue. Some studies with BCAA vs placebo have shown some effect on physical performance, others have not. Several central fatigue studies have shown an increase in mental performance. Most of the studies showing no effect of BCAA did not measure mental performance: this is an unfortunate omission, since the mental component required to maintain a given power output is clearly of considerable importance.

The third major aspect of Eric's input into exercise biochemistry concerns fatty acids and the immune system. This work was carried out largely with his former student, Philip Calder, whose excellent research in this area is widely reported and has certainly made an impact on exercise nutrition. It is hoped that their contribution to sports science in this area will be reviewed elsewhere.

Eric's book with Tony Leech and Glenda Duester (Eric's daughter) *"Keep on Running"* (1994) included his hypotheses. It is an extremely practical book, including running schedules from Bruce Tulloh and high carbohydrate/low fat recipes for athletes (not just runners), but the biochemistry is also there if the reader is interested.

Eric's key contributions almost certainly emanated from his desire to provide quantitative descriptions of complex metabolic pathways, and to consider whole body metabolism, not just the cell or tissue in which the study was con-

ducted. All students and members of the Newsholme laboratory were made familiar with this approach! Another principle which he liked to instil in us was: "you can never prove or disprove a hypothesis: you can only add to the evidence for or against it." A senior academic colleague from the 1980s recalls "He was such a stalwart seeker after truth, and possessed an inspiringly independent mind".

What is unquestionable is that many scientists, all over the world, have worked on Eric's novel ideas for years, adding to the evidence for or against the hypotheses. This is true not just of past members of the Newsholme group, who now have their own successful laboratories in the UK, Europe, North America, South America, Asia and Australia.

Eric was also a man of integrity and never expected nor received royalties from firms producing the amino acids on which he worked. He did receive research funding from pharmaceutical companies (there are few who do not!) but, true to form, always maintained an intransigent view about independence.

Eric was once described to me (by an innovator in sports science) as being "the greatest metabolist this country has ever produced". In an earlier appreciation of his work (The Biochemist, 2006) it was said: "Throughout the 1970s and 1980s, the University of Oxford was the base of….key researchers [who] have shaped our understanding of the regulation of energy metabolism in health and diseases. Hans Krebs, Philip Randle, Derek Williamson and Eric Newsholme all pursued their own research within areas they each considered to be the most important aspects of metabolic regulation….Of all these great names, Eric arguably has had the most impact at 'grassroots' level…."

It seems fitting to close with Eric's own words, speaking of his retirement, as part of a message to the inaugural meeting of the revised Metabolic Discussion Group this March:

"....I find biochemistry and especially Metabolism impossible to neglect, and I am delighted that recently it seems to be close to being restored to a central core of Biochemistry, at least in relation to research related to clinical problems and medicine!" *[EAN, 7 March 2011]*

Eric passed on his enthusiasm for running marathons to his wife, Pauline, who completed even more than he did, including the New York marathon to celebrate her 60th birthday! He is survived by Pauline, whom he met while undertaking his National Service and married in 1959; his son Philip, daughters Glenda and Clare; six grandchildren and one great-grandchild.

*[The material on Eric's publications is reproduced in part from Past Times (by Philip Newsholme, John Challiss and Greg Cooney; published in The Biochemist (2006). I am grateful to Chris Milne, Simon Hunt and Elizabeth Opara for permission to use their tributes, and to Philip Newsholme for commenting on this obituary. Lindy Castell, April 2011]*

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The general format of the review is somewhat flexible. A review must however have an abstract, an introduction and a conclusion around the main sections. Reviews with three or more sections should list the headings of the sections in form of a bullet point table at the end of the introduction. Longer sections should also give a short interim summary at their end.
If substantial amounts of the authors‘ own new data are to be shown, a section on methods and on results must be included. Data will only be accepted, if methods are stated clearly and appropriate statistical evaluation of results is given.

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