From The Department of Physiology and Pharmacology,
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# IMMUNOLOGICAL CHANGES IN HUMAN BLOOD AND SKELETAL MUSCLE IN RESPONSE TO PHYSICAL EXERCISE

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If you can talk with crowds – and keep your virtue,
Or walk with Kings – nor loose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds worth of distance run,
Yours is the Earth and everything that's in it,
And – which is more –
YOU WILL BE A MAN, MY SON!

*Rudyard Kipling*

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## ABSTRACT

Pysical exercise is essential for maintaining normal function of skeletal muscle. Muscle tissue also has a remarkable capacity for adaptation to changes in physical demand. In fact, without stimulation from physical activity, muscle tissue will atrophy. The mechanisms responsible for increases or decreases in muscle function are to a large extent not known. According to current opinions, one consequence of physical exercise can be muscle cell damage and inflammation. The inflammatory process is suggested to be one mechanism for muscle adaptation to exercise. Direct evidences for exercise-induced muscle inflammation in humans are weak. Nevertheless, the immune system seems to be of great importance for muscle adaptation. The capacity of the muscle tissue to adapt is largely due to the existence of satellite cells and local growth factors, but the exact molecular mechanism has not been discovered. An interaction between known and yet undiscovered factors are most likely involved in the adaptation process. Physical exercise will also change the number, activation and function of circulating leukocytes. Some of these changes are mediated via adhesion molecules, cytokines, growth factors and hormones.

The purpose of this thesis was to investigate interactions between immunological variables in human blood and skeletal muscle, in conjunction with physical exercise. The main hypothesis was that after physical exercise, circulating leukocytes will migrate to the affected muscle tissue as part of the inflammatory response. Upon completion of the damage-repair-adaptation process they will disappear from the muscle via migration or apoptosis. The magnitude of the inflammatory response should be intensity dependent. A majority of the subjects who participated in the studies were healthy males. Three different modes of eccentric exercise were used as a model to induce muscle inflammation and in one study immunological changes in the blood of soccer players during training and competition were investigated. Blood and muscle samples were taken before and at various time points after exercise. Immunohistochemical analyses of muscle sections, and blood analyses by flow cytometry, were the main tools used to assess immunological variables. The main findings were: 1) Exercise-induced muscle inflammation could not be observed in human skeletal muscle 2) The muscle biopsies induced significant skeletal muscle inflammation 3) HGF and its receptor c-Met, which are important for satellite cell activation, were expressed only in Type I skeletal muscle fibers.

It is concluded that the measured immune response to physical exercise is highly individual and depends on exercise mode and duration as well as which variables are analyzed. A significant inflammation in muscle tissue is not a likely result of physical exercise and delayed onset muscle soreness is not caused by muscle or epimysium inflammation. Furthermore, the inflammatory reaction in skeletal muscle is not depressed after strenuous eccentric physical exercise, indicating persisting normal immune function. The observed immunological events in blood and skeletal muscle in relation to physical exercise suggest a complex communication system between the two compartments. Finally, based on the observed effects of physical exercise on healthy muscle one may conclude, that physical exercise should not be precluded from the treatment of patients with inflammatory muscle diseases due to fear of increased muscle inflammation as exercise by itself does not seem to cause an inflammation in muscle tissue.

*Key words: eccentric exercise, soccer, football, adaptation, immune system, muscle, inflammation, damage, leukocytes, growth factors, cytokines, adhesion, signaling, hormones.*

# LIST OF PUBLICATIONS

I Malm, C., Lenkei, R. & Sjodin, B. (1999). Effects of eccentric exercise on the immune system in men. *J Appl Physiol* 86, 461-468.

II Malm, C., Nyberg, P., Engstrom, M., Sjodin, B., Lenkei, R., Ekblom, B. & Lundberg, I. (2000). Immunological changes in human skeletal muscle and blood after eccentric exercise and multiple biopsies. *J Physiol (Lond)* 529 Pt 1, 243-262.

III Malm, C., Sjodin, B., Sjöberg, B., Lenkei, R., Renström, P., Lundberg, I. E. & Ekblom, B. (2001). Leukocytes, cytokines, growth factors and hormones in human skeletal muscle and blood after uphill and downhill running. *J Physiol (Lond)* Submitted.

IV Malm, C., Ekblom, Ö. & Ekblom, B. (2001). Immune system alterations in response to acute and chronic soccer exercise. *Med Sci Sports Exerc* Submitted.

# CONTENTS

# GLOSSARY

(Compiled from Abbas *et al.* 2000; Adams, 1998; Balkwill *et al*. 2000; Oppenheim *et al*. 2000)

| Term | Explanation |
|---|---|
| *General* | |
| AEC | 3-amino-9-ethyl carbazole. Chromogen and substrate for the peroxidase enzyme conjugated to a secondary antibody in immunohistochemical methods. Produces a red reaction product |
| Antibody | Synonym for immunoglobulin. Glycoprotein produced by B cells and binds to antigen. Neutralizes antigens, promotes phagocytosis and activates the complement system |
| Antigen | Molecule recognized by the immune system |
| APC | Antigen presenting cells: monocytes, B cells, dendridic cells, vascular endothelial cells. Activates antigen-specific T cells |
| B cell | Expresses: CD5, CD20, CD23, CD25<br>Function: Produces antibodies when activated (plasma cells). Humoral immunity. Protection against extracellular bacteria and macromolecules |
| CD | Cluster of differentiation. System to classify cell surface molecules |
| CK | Creatine kinase. Enzyme that catalyzes the donation of phosphate from creatine phosphate to adenosine di-phosphate (ADP). Located in skeletal muscle, heart and brain. Used as a marker in blood for muscle cell membrane leakage |
| CRP | C-reactive protein. Plasma proteins that increases acutely in blood after tissue injury or inflammation. Produced in the liver and lymphocytes. Can recognize damaged cells and foreign pathogens and initiate their elimination. CRP has both a recognition and effector functions |
| Cytokine | Protein produced by many different cell types. Mediates inflammatory response and communication between cells of the immune system and other cell types |
| DAB | Diaminobenzidine tetrahydochloride. Chromogen, used as substrate for the peroxidase enzyme conjugated to a secondary antibody in immunohistochemical methods. Produces a black or brown reaction product |
| Fc | Part of the Ig molecule (heavy chain). Mediates effector function by binding to receptors (i.e. CD16, CD23) |

| | |
|---|---|
| Ig | Immunoglobulin. Synonym for antibody |
| LPS | Lipopolysaccharide (endotoxin). Substance present on the surface of gram-negative bacteria, released upon their death and stimulates innate immunity. Binds to CD14 |
| Macrophage | Expression: CD4, CD14, CD163<br>Function: Phagocytosis, release of cytokines (IL-1, IL6, IL-10, TNFα) and growth factors (HGF, LIF, VEGF). Important for muscle regeneration |
| MHC | Major Histocompatibility Complex Molecule. Display peptides for recognition by $T_C$ (MHC class I) and $T_H$ (MHC class II). MHC I is present on most nucleated cells and MHC II only on most immunocompetent cells |
| Mitogen | Any substance that induces DNA synthesis and cell division |
| Monocyte | Expresses: CD4, CD11b, CD14, CD45, CD62L, CD95<br>Function: Precursor of tissue macrophages. Secrete cytokines and growth factors |
| NK cell | Expresses: CD16, CD56, CD57, but not CD3<br>Function: Kills microbe-infected cells and cancer cells by lysis and IFN-γ secretion |
| T cell | Expression: T helper ($T_H$); CD3, CD4, T suppressor/cytotoxic ($T_{S/C}$): CD3, CD8<br>Function: $T_H$; activation of B and $T_{S/C}$ cells. $T_{H1}$ cells mediate cellular immunity. $T_{H2}$ cells mediate humoral immunity. ; $T_{S/C}$ cells lyse virus infected and cancer cells. |
| TCR | T cell receptor. Complex including CD3 and interacts with antigen on MHC |

*Cell surface molecules*

| | |
|---|---|
| CD3 | T cells<br>Associated with the T cell receptor. Involved in TCR complex signal transduction |
| CD4 | $T_H$ cells and monocytes<br>Accessory molecule for TCR antigen recognition, MHC II interaction |
| CD5 | T and B cells<br>Signaling molecule, binds to CD72. CD5+ B cells secrete IgM and may serve as protection against microbes |
| CD8 | $T_{S/C}$ cells<br>Accessory molecule for TCR antigen recognition. MHC I interaction and signal transduction |

| | |
|---|---|
| sCD8 | Soluble CD8<br>Shedded from $T_{S/C}$ upon activation |
| CD11b | (Mac-1) Granulocytes, monocytes, NK cells, T cells<br>Belongs to the integrin family. Important in firm adhesion to vascular endothelium and phagocytosis of C3bi coated particles. Binds CD54 (ICAM-1) |
| CD14 | Monocytes, macrophages, granulocytes<br>LPS binding protein, activates transcription factors (AP-1, NF-κB), gene transcription of cytokines and enzymes of the oxidative burst |
| CD15 | ($Lewis^x$) Leukocytes, endothelium<br>Adhesion to endothelium, ligand for CD62E and CD62P. Activation of monocytes |
| CD16 | NK cells, macrophages (CD16a), neutrophils (CD16b)<br>Fcγ receptor ($IgG_1$, $IgG_3$), antibody-dependent cell-mediated cytotoxicity, phagocytosis |
| CD20 | B cells<br>B cell activation or regulation |
| CD23 | Activated B cells, monocytes, macrophages<br>$F_{c\varepsilon}$ receptor, regulation of IgE synthesis. Triggers cytokine release from monocytes |
| CD25 | Activated B and T cells, activated macrophages<br>Subunit of the IL-2 receptor |
| CD28 | CD4+ (90% of all) and CD8+ T cells (50%)<br>T cell co-stimulator, activates naïve T cells, binds CD80 and CD86 on APC |
| CD38 | Activated T cells<br>Regulator of cell activation and proliferation dependant on the cellular environment, adhesion between lymphocytes and endothelial cells, intracellular signaling |
| CD45 | (Leukocyte common antigen) Bone marrow derived cells<br>Cytoplasmic phosphatase activity, signal transduction, apoptosis |
| CD45RA | Naïve T cells<br>Isoform of CD45 |
| CD45RO | Activated and memory T cells<br>Isoform of CD45 |

| | |
|---|---|
| CD56 | Regenerating and denervated muscle fibers, neuromuscular junctions, NK cells, satellite cells, dendritic cells, subsets of T and B cells, (isoform of N-CAM)<br>Mediates homotypic cell-cell adhesion |
| CD57 | NK cells, subset of T cells, monocytes, nerve cells<br>Adhesion (?) |
| CD62L | Blood B, T and NK cells, monocytes, granulocytes<br>Belongs to the selectin family. Homing receptor on leukocytes and mediates rolling on endothelium |
| CD95 | (Fas) Many different cell types<br>Fas antigen binds to Fas and mediates apoptotic signals |
| CD163 | (Mac-3) Human macrophages<br>Down-regulated by pro-inflammatory cytokines (IFNγ and TNFα) and LPS, up-regulated by IL-6 and IL-10 |
| DR | Antigen-presenting cells<br>MHC II sub-unit |
| Ki67 | All dividing cells<br>Nuclear protein and proliferation marker (not expressed in $G_0$ phase of cell cycle) |

*Growth factors and cytokines*

| | |
|---|---|
| c-met | HGF receptor<br>*Produced:* Many cells types.<br>*Function:* Signal transmission for morphogenesis (via PI3) and growth (via MAPK) |
| HGF | Hepathocyte growth factor<br>*Produced:* Liver and epithelial cells, fibroblasts, Type 1 muscle fibers<br>*Function:* Development and regeneration of tissue, tumor inhibition, morphogenesis. Only factor that can activate quiescent satellite cells |
| HIF-1 | Hypoxia-inducible factor, α and β subunits<br>*Produced:* Many cell types<br>*Function:* Oxygen sensing (α unit) transcription factor.<br>HIF-1 mRNA with hypoxia. Increses in muscle increase with exercise induces VEGF (Gustafsson *et al.* 1999) |

IFNγ — Interferon-γ. Pro-inflammatory
*Produced:* CD4+ $T_{H1}$, CD8 T cells, NK and virus exposed cells
*Function:* Blocks viral RNA translation, CD163 down-regulation, class switching of B cells, up-regulates MHC class II. Chemotactic for monocytes but not neutrophils
No change in serum IFNγ after exercise. PHA stimulated production can increase after exercise (Haahr *et al.* 1991; Baum *et al.* 1997). Serum IFNα can increase after exercise (Viti *et al.* 1985)

IGF-1 — Insulin-like growth factor-1
*Produced:* Liver, smooth muscle, satellite cells, endothelial cells
*Function:* Effector molecule of growth hormone. Cell differentiation and proliferation. Splice variant (MGF) in muscle (McKoy *et al.* 1999)
Exercise can increases serum and muscle IGF-1 (Bang *et al.* 1990; Hellsten *et al.* 1996; Singh *et al.* 1999).

IGF-1R — Insulin-like growth factor-1 receptor
*Produced:* Many cell types, including muscle
*Function:* Intracellular signaling (MAPK, PI3-kinase)

IL-1 — Interleukin-1. Pro-inflammatory, α and β form
*Produced:* Monocytes, macrophages, epithelium, fibroblasts, smooth muscles
*Function:* Increases protein breakdown in muscle. Stimulate muscle synthesis of prostaglandin E2 in endothelial cells and smooth muscle. Onset of fever in hypothalamus. Increases glycogenolysis, colagenase production. Direct effect on adrenal gland to induce steroidogenesis. Stimulate release of β-endorphin and interrupts pain transmission. Induce TNF and IL-6 production by monocytes/macrophages. Activate T cells to produce IL-2, IL-2R IL-4 and GM-CSF. Activates NK with other cytokines. Two receptors (Type 1 on T cells and Type 2 on B cells, neutrophils and bone marrow cells). Prolonged IL-1 production predisposes for in sepsis, diabetes
Increase, decrease or no change in serum after exercise (Mackinnon 1999; Pedersen 2000). Increase in muscle after exercise (Fielding *et al.* 1993) and repeated biopsies (Malm *et al.* 2000)

IL-2 — Interleukin-2. Anti-inflammatory
*Produced:* T cells (cellular immunity)
*Function:* Autocrine for T cells, drives antigen-specific expansion. B, NK neutrophil and macrophage activation. Lymphocytes expand under IL-2 influence and become target for other cytokines. IL-2 receptor α (CD25)
Increase or decrease in serum after exercise (Mackinnon 1999)

IL-4 Interleukin-4. Anti-inflammatory
*Produced:* CD4+$T_{H0}$ and $T_{H2}$ cells (humoral immunity), CD8+ T cells, mast cells, basophils.
$T_H$ differentiation to $T_{H2}$. Suppression of $T_{H1}$ cytokines.
B cell stimulation: increased viability, size, CD23, CD40, MHC class II. Regulation of IgE and $IgG_1$ production by B cells. Support or inhibit B cell proliferation depending on T cell interaction.
Macrophage anti-inflammatory effect: Increased MHC class II, LFA-1, CD23. Decreased IgG receptor expression. Inhibits IL-1, IL-6, IL-8, TNFα, $H_2O_2$. Increases V-CAM and adhesion of T cells, eosinophils, basophils, monocytes but not neutrophils on endothelial cells
Not changed in serum after exercise (Natelson *et al.* 1996; Suzuki *et al.* 2000)

IL-5 Interleukin-5. Anti-inflammatory
*Produced:* $T_{S/C}$ cells, mast cells, eosinophils
*Function:* B cell Ig secretion. Eosinophil activation, chemotaxis, survival, expansion
Not changed in serum after exercise (Malm *et al.* 2001)

IL-6 Interleukin-6. Anti-inflammatory (?)
*Produced:* Macrophages, $T_{H2}$ cells, B cells, fibroblasts, vascular endothelial cells, muscle cells
Up-regulated by mitogenic or antigenic stimuli, LPS, IL-1, IL-2, IFN, TNF, PDGF and virus. Inhibited by IL-4, IL-13.
*Function:* B cell differentiation, antibody production. T cell activation, growth and differentiation, IL-2 production. Increased platelet number. Increased synthesis of acute phase proteins, growth and inhibition of cancer cells. Involved in glycogenolysis (?)
Increases in serum after exercise of long duration (Pedersen *et al.* 2001)

IL-10 Interleukin-10. Anti-inflammatory
*Produced:* Macrophages, $T_{H2}$ cells, B cells, T memory cells
*Function:* Inhibit cytokine production by $T_{H1}$ cells and TNFα, IFNγ and IL-5 production by NK cell
Macrophages: Suppression (anti-inflammatory activation). CD163 up-regulation. Decreased antigen presenting capacity, pro-inflammatory cytokine synthesis (TNF-α, IL-1, IL-6, IL-8), prostaglandin E2, ROS, NO
B cells: Induces IgA, IgG synthesis. Enhances survival via bcl-2. Decreased secretion of TNF, IL-1, IL-8, MIP-1 from neutrophils
Increase after some (Pedersen 2000; Smith *et al.* 2000) but not all (Malm *et al.* 2001) types of exercise

LIF Leukemia inhibitory factor
*Produced:* Smooth muscle, Type 1 muscle fibers (?), LIF mRNA expressed in muscle precursor cells
Belongs to the IL-6 cytokine family
*Function:* Myoblast division, muscle regeneration. Induces synthesis of acute phase proteins
May increase in skeletal muscle after eccentric exercise (Malm *et al.* 2001)

LIF-$R_\alpha$ Leukemia inhibitory factor receptor
Belongs to the IL-6 receptor family
*Produced:* Many cell types, including muscle and endothelial cell
*Function:* Signal transduction via gp130
Not changed in skeletal muscle with exercise (Malm *et al.* 2000)

MFI Mean Fluorescence Intensity, or Median of Fluorescence Intensity
Unit for determination of antigen density on cells by flow cytometry
Represents the mean or medians of intensity expressed in channels. By changing the voltage applied to the photo multiplier tubes (the sensor detecting the fluorescence) one alters the intensity of the event (cell) which can be moved along the scale. Thus, one cannot compare the results obtained on different instruments, and even on the same instrument on different days

MESF Molecules of Soluble Fluorochrome. Absolute Units
Unit for determination of antigen density on cells by flow cytometry. Based on the use of a mixture of beads coated with the same fluorochrome as the one conjugated to the respective monoclonal antibody. The four types of beads in the mixture are coated with increasing fluorochrome amounts. A calibration plot translates the MIF into MESF units which are independent of the instrument settings and can be used across laboratories. However, MESF measures the number of fluorochrome molecules attached to a cell and so indirectly of monoclonal antibody. It is thus dependent of the number of fluorochrome molecules conjugated to a molecule of antibody. Thus in order to compare results in long time studies at the same laboratory, or in studies across laboratories, the same batch of mAb must be used

TNFα Tumor necrosis factor-α. Pro-inflammatory
Monocytes/macrophages, neutrophils, activated lymphocytes, NK cells, endothelial cells, smooth muscle cells
Anticancer effects. Induces septic chock. CD163 down-regulation. Induces cytokine production and weight loss during chronic inflammation
Serum concentration not changed by exercise, but LPS stimulated monocyte production of TNFα can increase after exercise (Mackinnon 1999; Pedersen 2000)

NOTE to the reader: Due to the chosen layout, figures do not appear in numerical order. The author apologizes for this inconvenience.

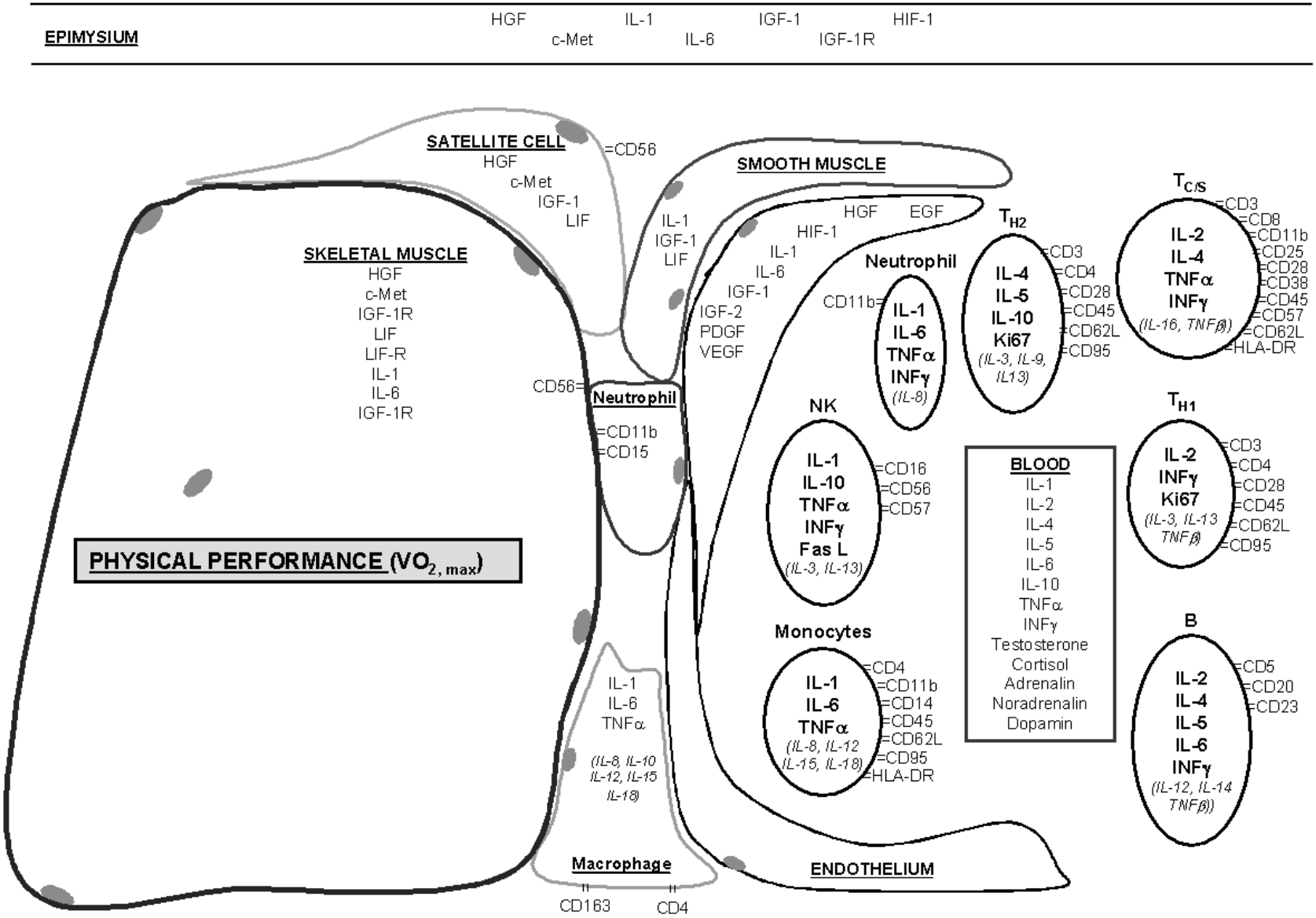



**Figure 1**

Overview of analyzed cell surface receptors, cytokines and growth factors. Each molecule is placed at the detected locations, other locations may also be possible based on other studies. Cytokines of interest but not investigated in parenthesis at site of production.

**NOTES:**

# 1 INTRODUCTION

Physical exercise affects all tissues in the human body and is essential for maintaining normal function of skeletal muscles. Despite the fact that the skeletal muscle system is the largest organ in the human body, surprisingly little is known about the mechanisms responsible for maintaining, and on demand also improving, muscle performance capacity. Research has so far demonstrated the involvement of hormones, neurosignals, growth factors, cytokines and mechanical stretch in this process but other factors not yet characterized are probably also of importance. Because of the complex interaction among these systems, the lack of complete understanding of muscle function is not surprising. Fortunately, the rapid development of new techniques and investigation methods lays the foundation for more sophisticated collection of data and more complex analyses in future research.

When exploring possible mechanism by which adaptation to physical exercise could occur, the immune system comes up as one likely candidate of importance.

The immune system consists of highly specified cells and organs, primarily developed to protect the host from invading foreign organisms and substances, but also to remove altered, infected or dead cells (Abbas 2000). It is evident that the immune system is necessary for normal growth and development and probably also for adaptation to physical exercise (Hohlfeld & Engel 1994; Chambers & McDermott 1996; Ruoslahti 1997; Lescaudron *et al.* 1999; Woods *et al.* 2000; Hawke & Garry 2001; Tatsumi *et al.* 2001).

A challenge to the immune system can elicit an immediate and a delayed type response, referred to as innate and adaptive immunity, respectively. The innate immune system includes epithelial barriers, macrophages, neutrophils, NK cells and cytokines and respond similarly to repeated infections by the same pathogen. The adaptive immune system (also called specific or acquired) includes lymphocytes (B and T cells) and can, due to its memory function, respond more vigorously to each repeated infection (Abbas 2000). An *immune response* is normally defined as the reaction of these cells and molecules to any foreign substance, but in this thesis the term *immune response* is also used to describe the immunological changes elicited by physical exercise.

Numerous investigations have described the acute immune response to physical exercise, and the research field of exercise immunology is rapidly growing. Recent findings have been summarized by Bente Klarlund Pedersen (1997) and Laurel T.

Mackinnon (1999) and related topics of interest are published annually in Exercise Immunology Review (Human Kinetics Publ., Inc., IL, USA). When reading these reviews, as well as other review articles and original investigations, it becomes apparent that observed immunological changes in response to physical exercise depend on the mode and duration of exercise, training status, genotype and feeding state of the subjects, gender, age, time of blood sampling, analytical methods and subpopulations of leukocytes analyzed (Woods *et al.* 1999). The interpretation of results obtained is further differentiated by the statistical methods used. Because of this diversity, it is not usually possible, or perhaps meaningful, to define a general immune response to physical exercise. Rather, the underlying mechanisms responsible for the observed changes in each research setting must be described in some detail.

Research findings in the field of exercise immunology can be applied to affect public health, benefit patients with immunological disorders via exercise recommendations, develop pharmacological treatments for immunological and muscular diseases and influence the performance of elite athletes.

It is beyond the scope and intention of this thesis to describe and discuss all aspects of exercise immunology. For example, the topics of resistance to infections, cell functionality and mucosal immunity is not discussed because they have not been investigated in Study I-IV. Thus, after a short introduction, the focus will be on observed and hypothesized interactions between the immune system and skeletal muscle in healthy, predominantly male human subjects.

## 1.1 MUSCULAR ADAPTATION TO PHYSICAL EXERCISE

Skeletal muscle tissue has a remarkable capacity to adapt to increased, and decreased, physical demand (Saltin & Gollnick 1984). Without stimulation from physical activity, muscle tissue will undergo atrophy and decreased functional capacity, but the mechanisms responsible these are to a large extent not known. Involvement of several different systems, including the nervous, neuroendocrine, vascular and immune has been demonstrated or suggested (Grounds 1991; Felten *et al.* 1993; Ottaway & Husband 1994; Chambers & McDermott 1996).

The presence of immunocompetent cells and various growth factors appears important for optimal muscle function and adaptation (Robertson *et al.* 1992; Tidball 1995; Chambers & McDermott 1996; Hellsten *et al.* 1996; Husmann *et al.* 1996; Gustafsson *et al.* 1999). For example, in these studies macrophages have repeatedly been shown to enhance muscle regeneration and satellite cell proliferation. In future studies

other circulating or tissue resident non-muscle cells may turn out to serve similar functions.

Muscle cells can also themselves release factors with potential autocrine roles in muscle function and adaptation to different stimuli (Husmann *et al.* 1996; Goldspink 1998; Sheehan *et al.* 2000). Several cytokines have been reported to be present by muscle homogenate (Ostrowski *et al.* 1998) and at least some of them (IL-6) appears to be located within the muscle cells (Malm *et al.* 2000). A muscle specific growth factor, the mechano growth factor (MGF) has recently been reported (Goldspink 1998). It is believed that MGF is a splized variant of IGF-1, which was detected in smooth muscle cells in Study III. Investigations are needed to identify potential factors involved in muscular adaptation to physical exercise, and the potential therapeutical usefulness of these factors in treatment of muscle diseases and muscle hypotrophy. Many of these factors, if not most of them, are yet to be discovered.

## 1.2 EXERCISE-INDUCED MUSCLE DAMAGE

Exercise-induced muscle damage and inflammation in both human and animal studies are well described in the literature (Armstrong *et al.* 1991; Smith 1991; Fridén & Lieber 1992; Fielding *et al.* 1993; Sorichter *et al.* 1995; Hellsten *et al.* 1997; Child *et al.* 1999). The main conclusions made in these studies are that:

1) Exercise can induce damage and inflammation in human skeletal muscle tissue.
2) The severity of the inflammation depends on the type, duration and intensity of exercise.
3) Exercise with eccentric contractions will cause more damage and inflammation than concentric exercise of equal intensity and duration.

It has been postulated that physical exercise can cause damage to muscle cells in terms of disrupted contractile structures and cytoskeletal components ( Fridén & Lieber 1992; Fielding *et al.* 1993), loss of desmin (Lieber *et al.* 1996) and permeabilisation of the muscle cell plasma membrane (McNeil & Khakee 1992; Malm *et al.* 1996). Several previous review articles have discussed this topic (Evans & Cannon 1991; Kuipers 1994; Clarkson & Sayers 1999) and concluded that it is the eccentric component of exercise that causes the largest indications of skeletal muscle cell damage. However, skeletal muscle damage caused by voluntary physical exercise is not convincingly and repeatedly demonstrated in published scientific studies using human subjects (Malm 2001) and it was not the intention to address the issue of muscle damage in this thesis. Thus, the only

marker of damage investigated was serum CK activity (indirect method), which was analyzed in Study I-III, and the appearance of desmin (direct method), which was investigated in Study II only.

## 1.3 EXERCISE-INDUCED MUSCLE INFLAMMATION

Inflammation is a physiological event, induced by numerous stimulit and which occurs according to a well-described temporal scheme. In brief, the major events are: 1) tissue injury 2) release of vasoactive substances by the injured tissue 3) vasodilation 4) leukocyte adhesion 5) leukocyte migration from blood to the injury site and eventual 6) tissue repair (Kuby 1994). The inflammation can be of acute character, initiated by a number of different traumatic factors such as burns, chemicals, virus and bacteria (Sheldon 1992) or secondary to a specific immune reaction to muscle, as in myositis (Hohlfeld *et al.* 1997) where the initiating agent may or may not be known. Even though success is not always guaranteed, the objective of any inflammation is to repair injury and restore tissue function.

Several studies have demonstrated muscle tissue damage after physical exercise, and it has been shown that damaged muscle will induce an inflammatory response (Tidball 1995). Some investigators have also demonstrated increased leukocyte infiltration in human skeletal muscle after physical exercise (Jones *et al.* 1986; O'Reilly *et al.* 1987; Round *et al.* 1987; Fielding *et al.* 1993). Thus, if exercise-induced muscle damage, a natural consequence would be muscle inflammation. However, direct investigations of exercise-induced muscle inflammation in humans are relatively scarce (Round *et al.* 1987; Fielding *et al.* 1993; Hellsten *et al.* 1997) and in rat, exercised redistribution of leukocytes from the blood to the skeletal muscle could not be found (Espersen *et al.* 1995). Thus, it has been suggested that the evidence for exercise-induced skeletal muscle inflammation in humans may be circumstantial (Malm 2001).

## 1.4 SATELLITE CELLS

The capacity of the adult human skeletal muscle to regenerate and hypertrophy is largely due to the existence of satellite cells; a population of undifferentiated muscle precursor cells located between the basal lamina and the sarcolemma of the myotube, identified by Mauro (1961) and reviewed in the literature several times (Grounds 1991; Schultz & McCormick 1994; Chambers & McDermott 1996; Hawke & Garry 2001). They were first believed to only stem from the mesoderm of the somite, but it has recently been suggested that they might also be derived from endothelial cells (Hawke & Garry 2001).

In embryonal development satellite cells migrate, differentiate, fuse and form limb muscles. In the adult, satellite cells are readily activated when the muscle cell is damaged or stretched (Grounds & Yablonka-Reuveni 1993; Malm *et al.* 2000; Tatsumi *et al.* 2001). Upon activation they undergo mitosis, possibly only a limited number of times (Grounds & McGeachie 1987) and fuse either with existing myotubes or form new once to repair and regenerate damaged muscle tissue. Absence of muscle activity, such as during hindlimb suspension of rats or immobilization in humans, will reduce their number and proliferative activity (Darr & Schultz 1989).

Several markers of activated satellite cells have been found (c-Met, Desmin, MyoD, CD56/NCAM) while the number of markers for quiescent satellite cells are still under debate (Hawke & Garry 2001).

A myriad of factors have been implicated in satellite cell activation, but HGF is so far the only one known to activate quiescent satellite cells (Allen *et al.* 1995; Tatsumi *et al.* 1998). Chemotaxis, proliferation and differentiation on the other hand, can be ascribed to FGF, IGF-1, LIF, IL-6 and several other factors (Hawke & Garry 2001). The milieu surrounding a satellite cell will be a determining factor for its function, and macrophages appear to play an important role in muscle regeneration, likely due to their release of growth factors and contact between adhesion molecules on the cell surface.

In response to physical exercise, satellite cells are known to be activated, especially if the exercise consists of high eccentric force such as during resistance exercise (Darr & Schultz 1987; Smith *et al.* 1999). Electrical stimulation of rat muscle will also activate satellite cells (Putman *et al.* 2000). However, when using human subjects it has been shown that eccentric exercise does not activate satellite cell above what the biopsy procedure itself does (Malm *et al.* 2000). Thus, exercise-induced satellite cell activation in humans may occur when hypertrophy is induced, but it is not known if skeletal muscle adaptation to non-hypertrophic exercise also involves satellite cell activation.

## 1.5 EXERCISE AND CELLS IN CIRCULATION

The vast majority of publications in exercise immunology describe changes in number, percentage, activation and function of leukocytes in circulating blood (for some representative publications and review articles see Int J Sports Med, 1994 Vol. 15 Suppl. 3; 1997 Vol. 18 Suppl. 1; 1998 Vol. 19 Suppl. 3; 2000 Vol. 21 Suppl. 1; Exercise Immunology Review 1995-2000 Vol. 1-6 and textbooks by Laurel T. Mackinnon (1999) and Bente Klarlund Pedersen (1997)). As seen in Study IV as well as in the Result section, a crude categorization of the immune system simplifies the discussion of the

rather complex topic of exercise-induced immunological changes in the blood. The chosen categories are based on investigated variables and do not include all aspects of exercise and the immune system.

The circulating white blood cells include lymphocytes, monocytes and granulocytes. Lymphocytes can be divided into T, B and NK (natural killer) cells, monocytes differentiate into macrophages in tissue and granulocytes consist of neutrophils, basophils and eosinophils.

### 1.5.1 Neutrophils

In the blood, neutrophils constitute 50-70% of the total number of leukocytes and the normal range in adult humans is 1800-7700 cells $\mu L^{-1}$. Neutrophils are part of the innate immunity and their main function is to phagocytos and digest microbes at sites of inflammation. During the course of inflammation, neutrophils may cause additional damage to the tissue (Weiss 1989; Dallegri & Ottonello 1997).

A consistent finding in most studies is increased number of neutrophils in the blood during and after physical exercise (Kayashima *et al.* 1995; Gabriel & Kindermann 1997; Pedersen 1997; Bishop *et al.* 1999; Mackinnon 1999), and the increase appears to be dependent on the duration and intensity of exercise (Gabriel & Kindermann 1997). Thus, exercise-induced leukocytosis (increase in the number of white blood cells in the blood) can be attributed mainly to the increase in neutrophil number and to a lesser extent to changes in lymphocytes and monocytes. Immediately after secession of prolonged exercise (several hours) the number of neutrophils in the blood can be elevated for more than 6 h, but if exercise duration is increased further the number may eventually decrease below resting number (Mackinnon 1999). Acute changes in circulating numbers of leukocytes have been attributed to changes in catecholamines, while post exercise neutrophilia is under the influence of cortisol (Benschop *et al.* 1996; Pedersen & Hoffman-Goetz 2000). This is contradicted by one study which identified cortisol "responders" and "non-responders" and where post-exercise granulocytosis could not be due to increase in cortisol. During infection, neutrophilia is induced by cytokines (Abbas 2000) and cytokines could also play a role in neutrophilia after physical exercise via regulation of adhesion molecules (Meager 1999). The source of leukocytes appearing in circulation after physical exercise is the marginated pool, which can be the lung, spleen, lymphatic tissue, blood vessels or bone marrow (Benschop *et al.* 1996) but probably not skeletal muscle (Espersen *et al.* 1995). In these organs leukocyte adhere to the vessel wall and can be released by hormones such as catecholamines or shere force. A cells telomer

length is shortened by each cell division, thus older cells have short telomers compared to young cells. Because of short telomere length in the cells entering circulation, mobilization from the bone marrow and thymus (where leukocytes are produced and mature) is less likely (Pedersen & Hoffman-Goetz 2000).

Depending on mode of exercise, whethere it is acute or chronic, sampling time and method of analysis, neutrophil function during and after physical exercise has been reported to be both increased and decreased (Pyne 1994; Smith 1997; Mackinnon 1999). It is also evident that the assay used to investigate neutrophil function is of great importance because only some functions of neutrophils are affected by physical exercise (Suzuki *et al.* 1996). Suzuki *et al*. (1999; 2000) have made the conclusion that hormones and cytokines are related to changes in neutrophil function after exercise. In one study by Nieman *et al.* (1998) the intake of carbohydrates influenced neutrophil numbers and function more than the mode of exercise (running or cycling). When comparing neutrophil numbers and function at rest between trained and untrained subjects, there is no difference in cell numbers while the oxidative burst capacity may be compromised in trained subjects and athletes (Smith *et al.* 1990; Pyne 1994). Hack *et al*. (1994) demonstrated that the phagocytic capacity of neutrophils in long distance runners is compromised during intense, but not moderate training periods. There is, however, great variability in neutrophil response between subjects to similar relative exercise intensity (Smith *et al.* 1990), or possibly due to differences in mood-state (competitive or laboratory setting) of the subjects, which can affect neutrophil adhesion (Hall *et al.* 1996).

Neutrophils have the capability to migrate from the blood and infiltrate damaged or infected tissue. The recruitment of neutrophils involves endothelial activation by local factors (TNFα and IL-1 are analyzed), adhesion molecules on both the neutrophils and endothelium (CD11b, CD15 and CD62L are analyzed), activation and migration guided by locally produced chemotaxins (produced in response to TNFα and IL-1) (Dallegri & Ottonello 1997). The presence of neutrophils in human and animal skeletal muscle has been demonstrated after physical exercise, especially if the exercise involves a large eccentric component (Armstrong *et al.* 1983; Fielding *et al.* 1993; Lowe *et al.* 1995). Due to the methods and protocols used (Malm 2001), and based on the findings in Study II and III in this thesis, the neutrophil migration to skeletal muscle after physical exercise observed in several studies can probably be attributed to the muscle sampling procedure, at least in human subjects.

### 1.5.2 Monocytes/Macrophages

Monocytes are part of the innate immune system. Their functions include phagocytosis, anti-tumor activities, antigen presentation, cytokine production and tissue remodeling (Woods *et al.* 2000). In the adult human blood, the number of monocytes range between 0-800 cells $\mu L^{-1}$. The tissue numbers of macrophages (differentiated monocytes not in the blood) have not been investigated extensively. In Study II (Malm *et al.* 2000), immunohistochemical data is published as percent stained area, but the number of cells was also counted. In rested human vastus lateralis muscle 0-10 macrophages per $mm^2$ (0-8 macrophages per 100 muscle fibers) were found (unpublished data). St. Pierre & Tidball (1994) counted 12 ($ED1^+$) and 72 ($Ia^+$) macrophages per $mm^2$ in rested rat soleus muscle. The importance of both blood and tissue macrophages for muscle regeneration after injury has been demonstrated or suggested by several investigators (Grounds 1991; St. Pierre & Tidball 1994; Chambers & McDermott 1996; Massimino *et al.* 1997; Lescaudron *et al.* 1999).

Circulating monocyte number has been found to increase or not change during and after acute exercise and may be decreased during prolonged periods of intense endurance training (Gabriel *et al.* 1994; Mackinnon 1999). In Study IV, the number of monocytes was decreased immediately after two consecutive soccer games. The *in vitro* function of, as well as the cell surface receptor expression on monocytes can be altered by exercise and is dependent on mode, duration and intensity of the exercise (Tvede *et al.* 1989; Gabriel *et al.* 1994; Malm *et al.* 1999; Malm *et al.* 2000; Starkie *et al.* 2000). Monocyte function can be enhanced during periods of decreased training in endurance athletes (Gabriel *et al.* 1997).

Several macrophage functions, such as chemotaxis, adherence and phagocytosis have been shown to increase following physical exercise, but an exercise-induced reduction in MHC II expression and decreased antigen presenting capacity has also been noted (Woods *et al.* 2000). In one study (Fehr *et al.* 1989) enzyme content and phagocytic index in connective tissue macrophages were found to increase after a 15 km run to exhaustion. Most of the studies on macrophages are performed on animals, and it is not know if physical exercise can alter the function of macrophages resident in human skeletal muscle. The presence of macrophages is also important for regulation of NK cell function after physical exercise, at least in mice (Blank *et al.* 1997).

### 1.5.3 Lymphocytes

The lymphocyte population (20-40% of all leukocytes) consists of T lymphocytes (50-60% of all lymphocytes), B lymphocytes (10-15%) and NK cells (<10%). T and B cells belong to the adaptive immune system while NK cells take part in the innate immune response (Abbas 2000). T lymphocytes can be further divided into T helper ($T_H$) and T cytotoxic/suppressor ($T_{C/S}$) cells and the ratio of $T_{H1}$ to $T_{C/S}$ is normally around 2:1. Based on the cytokines they produce, $T_H$ cells can be classified as $T_{H1}$ (main cytokine produced is INF-γ) and $T_{H2}$ (main cytokines produced are IL-4, IL-5) cells. The main function of $T_H$ cells is to activate B lymphocytes (humoral response) and T lymphocytes and monocytes (cellular response). $T_{H1}$ cells stimulate phagocytosis while $T_{H2}$ cells are important in the helminthic defense and allergic reactions. $T_{C/S}$ cells mediate lysis of virus infected cells and cancer cells. When activated (then called plasma cells), B cells produce antibodies as part of the humoral immune response. The main function of NK cells is to kill virus infected and cancer cells (Abbas 2000).

The number of lymphocytes usually increases (25-100%) during acute physical exercise and returns to baseline or below within a few hours after exercise (Gabriel & Kindermann 1997; Pedersen 1997; Mackinnon 1999). This acute change is mainly due to the action oc catecholamines. If the exercise is of extreme duration, lymphocyte number may decrease below resting value even during exercise (Gabriel & Kindermann 1997). The magnitude of change has been attributed to exercise intensity and fitness level of the subjects (Mackinnon 1999).

After a period of training or when comparing athletes to non-athletes, most studies find no change in the number of lymphocytes in blood at rest (Mackinnon 2000). The number of CD3+ lymphocytes were decreased after 12 weeks of light aerobic training in one study (Shore *et al.* 1999) and after a 5 days soccer training camp in Study IV. Even in overtrained athletes normal cell counts have been observed (Gabriel *et al.* 1998).

#### *1.5.3.1 T cells*

T cells usually display a greater response to acute physical exercise than B cells, and $T_{C/S}$ (CD3+CD8+) cells are more affected than $T_H$ (CD3+CD4+) cells (Gabriel & Kindermann 1997; Mackinnon 1999). It also appears that a higher proportion of CD45RO+ (memory) than CD45RA+ (naïv) T cells are recruited to the blood during and after exercise (Malm *et al.* 1999; Pedersen & Hoffman-Goetz 2000).

During strenuous anaerobic and aerobic exercise T cells are activated (Gabriel & Kindermann 1997), while eccentric exercise can reduce CD8 expression on T cells (Malm

*et al.* 2000), or not change the number of activated T cells in circulation (Malm *et al.* 1999). With few exeptions, T lymphocyte proliferation rate decreases after physical exercise and can be related to decreased $T_H$ cell and increased NK cells numbers, but not to mode of exercise or fitness level of the subjects (Pedersen 1997; Mackinnon 1999; Pedersen & Hoffman-Goetz 2000).

Even though few studies have been performed on human subjects, it appears that both $T_H$ and $T_{C/S}$ cell numbers are fairly resistant to change with chronic physical exercise (Pedersen & Nieman 1998; Mackinnon 1999; Mackinnon 2000). When comparing the T cell populations before and after a soccer training camp (Study IV) a decrease in the number of both $T_{H1}$ and $T_{C/S}$ could be noted. However, T cell numbers increased with a period of pre-season soccer training in the same study. In one study, CD8+ cells increased after 10 weeks of 45 min, 3 time/week cycling (LaPerriere *et al.* 1994). Nieman (2000) have investigated differences in immune response between athletes and non-athletes and found that female rowers had higher PHA-induced proliferation response (T cells) compared to non-athletes but also that ConA-induced proliferation (T cells) decreased for more than 3 h after 2.5 h of running (Nieman *et al.* 1995).

*1.5.3.2 B cells*

Many exercise protocols do induce changes in the number of B cells (Mackinnon 1999), while others have concluded that B cells increase during and decrease after exercise (Pedersen 1997). When the exercise is anaerobic (Gabriel & Kindermann 1997) or consists of a large eccentric component (Malm *et al.* 2000), the number of B cells can increase after exercise. CD5+ B cell number (IgM producing) displayed a delayed decrease after eccentric exercise in one study (Malm *et al.* 1999) but not after eccentric cycling in another (Malm *et al.* 2000). In Study IV, CD5+ B cell number increased after 2 soccer games while CD5+CD23+ B cells (activated) decreased.

The capacity of B cells to produced immunoglobulins has been shown to be reduced following 1 h of cycling exercise at 80% of $VO_{2,max}$, and attributed to changes in T cell and monocyte functions (Tvede *et al.* 1989)

After periods of repetitive physical exercise, the number of B cells has been found to increase (LaPerriere *et al.* 1994) and Study IV, but after a 5 day soccer training camp in Study IV, CD5+ as well as CD23+ B cells decreased in number.

Thus, the B cell population responds in a rather heterogeneous fashion to various exercise regimes, and inconsistent findings may stem from different sub-populations analyzed.

*1.5.3.3 NK cells*

Of all lymphocytes, NK cells are considered the most respondent to physical exercise. The magnitude of change appears to be intensity, rather than duration dependent (Pedersen *et al.* 1994; Gabriel & Kindermann 1997; Pedersen 1997; Mackinnon 1999). The number of NK cells increases during exercise and returns to resting or below resting number in the hours after exercise. In some cases the NK cell number can remain decreased for several days after exercise (Figure 13). Also psychological stress can cause redistribution of the NK cell population. The control mechanism of NK cell redistribution is adrenergic (under the influence of catecholamines) (Schedlowski *et al.* 1993; Benschop *et al.* 1997).

NK cell function on a per cell basis increases during and decreases after physical exercise, at least if the exercise has been of high intensity and lasting longer than 1 h (Pedersen 1997). Exercise can decrease the expression of adhesion molecules CD44 and CD18 on NK cells in mice (Nagao *et al.* 2000), but there was no change in CD57 expression on NK cells in humans (Malm *et al.* 1999; Malm *et al.* 2000)

NK cell number at rest is usually not different between athletes and sedentary subjects, but sometimes elevated in endurance athletes (Shore *et al.* 1999; Nieman 2000). Intensified training periods can, however, result in a decreased number and function of NK cells (Mackinnon 2000), Study IV and Figure 13.

## 1.6 ADHESION AND CELL SURFACE SIGNALING MOLECULES

Adhesion molecules are expressed on circulating leukocytes and endothelium and mediate cell-cell and cell-extracellular matrix contact. They are of vital importance for cell communication, growth, migration and trafficking between different organs and compartments (Aplin *et al.* 1998; Aplin *et al.* 1999; Abbas 2000). Adhesion molecules also mediate interactions between cells of the immune system, such as T cells and APC, and guide leukocytes to sites of inflammation. In fact, interaction between cells is necessary for their survival (Giancotti & Ruoslahti 1999). According to Shephard *at al*. (Shephard *et al.* 2000) the adhesion molecules can be divided into 4 groups.

i. Selectins (CD62L is analyzed)

ii. Integrins (CD11b)

iii. The immunoglobulin superfamily (CD4, CD8, CD28, CD56)

iv. Mucins (CD45)

Each molecule, or family of molecules have their own unique functions. Aplin *et al*. (1998) also have cadherins, but not mucins, as a family of adhesion molecules. Selectins (E on endothelium, P on leukocytes and endothelium and L on leukocytes) facilitate rolling of leukocytes on the endothelium and may transmit some intracellular signals (Stamenkovic 1995). Integrins assure firm adhesion of leukocytes to the endothelium and can regulate cytoplasmic kinase activity, growth factor receptors, ion channels and the cytoskeleton (Mazzone & Ricevuti 1995; Giancotti & Ruoslahti 1999). The immunoglobulins are a large family of proteins important for development of the nervous system, antigen recognition, contact between the extreacellular milieu and the cytoskeleton (spectrin/actin), signal transduction in T and B cells and transendothelial migration (Aplin *et al.* 1998; Aplin *et al.* 1999; Abbas 2000).

Several researchers have investigated alterations in the number of cells with high or low expression of adhesion molecules, and also the cell surface expression of adhesion molecules, in response to physical exercise. It is believed that adhesion molecules are partially responsible for the redistribution of leukocytes during and after exercise (Gabriel & Kindermann 1998; Malm *et al.* 1999; Mills *et al.* 1999; Malm *et al.* 2000; Shephard *et al.* 2000) (see also 5.2). When investigating the expression of adhesion molecules, a difficult question to answer is if the changes observed, if any, are the results of changes on a per cell basis or if there is a selective redistribution of cells with different expression of these molecules to and from circulation, or a combination of both. Based on the methods used in published studies, a distinction between these two possibillities cannot be made.

On granulocytes (95-99% neutrophils), CD11b was increased during and decreased after exercise of high intensity in one study (Jordan *et al.* 1999). In general, cells in circulation appear to have higher expression of CD11b after, compared to before physical exercise (Pizza *et al.* 1996; Shephard *et al.* 2000), Study I-IV and Figure 19. An opposite change (decrease during and increase after exercise) was observed in CD62L expression after 60 min of cycling at 60% of $VO_{2,max}$ (Kurokawa *et al.* 1995) both on granulocytes and lymphocytes. The finding by Kurokawa *et al.* is rather representative for findings also in other studies (Goebel & Mills 2000; Gannon *et al.* 2001) and Studies I-IV (Figure 19) even though CD62L on CD4-CD8- lymphocytes increased 24 h after eccentric exercise in one study (Malm *et al.* 1999).

Monocytes are not as affected by physical exercise as neutrophils, but CD11b and expression on monocytes was increased after eccentric exercise in 2 studies (Pizza *et al.* 1996; Malm *et al.* 1999), as was CD62L expression on monocytes (Malm *et al.* 1999).

Even though NK cells usually change in response to physical exercise (see above) the expression of adhesion molecules on the NK cells in circulation did not changed after 60 min of cycling at 60% of $VO_{2,max}$ (CD11b, CD11c) (Kurokawa *et al.* 1995) or eccentric exercise (CD57) (Malm *et al.* 1999; Malm *et al.* 2000). In a recent study by Gannon *et al.* (Gannon *et al.* 2001) it was demonstrated that CD49d and $CD11a^{hi}$ NK cells were recruited to the blood during exercise, and that the MFI of CD11a on each cell population correlated positively to the change in number of that cell population (CD4+, CD8+ and CD56+ lymphocytes). Down-modulation of adhesion molecules CD44 and CD18 on NK cells after noradrenalin infusion in mice has also been noted (Nagao *et al.* 2000), supporting the idea that NK cell redistribution is controlled by catecholamines via adhesion.

Expression of adhesion molecules on leukocytes can also change with periods of training in track athletes (Baum *et al.* 1994).

The contradictive findings among studies are summarized by Shephard *at al.* (2000) and exemplified by Gannon *et al*. (2001) where it is apparent that the change in each subpopulation of lymphocytes to any given exercise protocol, is unique with regards to their expression of adhesion molecules. Some of the changes in adhesion molecules observed in response to physical exercise can be attributed to changes in catecholamines (Rehman *et al.* 1997) and possibly also to changes in cytokines (Meager 1999). It is of interest to note that neutrophil adhesion capacity can be influenced by active imagery (Hall *et al.* 1996). Thus, results regarding the influence of physical exercise on some immunological variables may partially depend on if the studies are carried out in a competitive or laboratory setting. The methods for determination of antigen density is also of great importance (see Glossary on MFI and MESF).

An interpretation of these findings can only be speculative, but it could be suggested that firm adhesion (CD11b) is decreased and rolling (CD62L) is increased indiucating that cell signaling rather that cell migration is prioritized.

An array of signaling molecules, not specifically involved in the adhesion process is present on the surface of leukocytes. These molecules, such as CD14 an d CD95 on monocytes and CD5, CD20, CD23 and CD95 on lymphocytes all transmit communication signals between the extracellular and intracellular environment. Any change in expression or affinity of these molecules may change cell function. CD14 is the bacterial lipopolysaccharide (LPS) binding protein on monocytes and macrophages (Schultz & McCormick 1994). When LPS binds to CD14, the Toll-like receptor transmits

a signal to induce cytokine production and the respiratory burst (Abbas 2000). Changes in CD14 expression or affinity will thus influence the capacity to fight bacterial infections, and can also influence cytokine production by T cells, and monocyte expression of CD54 (ICAM-1) (Hirohata & Oka 1993). CD5+ B cells secrete IgM antibodies and changed number of CD5+ B cells in circulation may affect the first line of defense against microbes. The function of CD20 and CD23 is still not fully understood, but may involve B cell activation (Abbas 2000; Riley & Sliwkowski 2000). CD95 (Fas) is the receptor for Fas ligand and transmits apoptotic signals by activation of caspases (Abbas 2000). CD95 can also inhibit glucose transport in T cells (Jurkat cells) (Berridge *et al.* 1996). Changed expression of CD95 can consequently determine life or death of the cell.

Changes in cell surface expression of signaling molecules have,to the knowledge of the author, not been extensively studied in response to physical exercise but some alterations have been observed (Malm *et al.* 1999; Malm *et al.* 2000; Mooren *et al.* 2001). Due to the small number of investigations, no general conclusion regarding exercise-induced changes in leukocyte signaling capacity can be made. The observed delayed changes in signaling molecules in Study IV are thus novel findings.

The significanse of these findings is that communication between circulating cells and endothelium could be altered with physical exercise via changed expression of cell surface molecules.

## 1.7 CYTOKINES

Cytokines are proteins produced and released by monocytes, macrophages, epithelium, fibroblasts, smooth muscles cells (Belec *et al.* 1997; Abbas 2000; Balkwill 2000) and skeletal muscle undergoing regenerative changes (Authier *et al.* 1997; Belec *et al.* 1997) (see Glossary). In their classic function they are effector molecules of leukocytes, and mediators of cell communication between lymphocytes, monocytes and other target cells (thus sometimes named lymphokines and monokines). Many cytokines are called interleukins (IL) because they were originally discovered to be produced by, and acting among, leukocytes. However, all cytokines affecting leukocytes are not called interleukins, and interleukins can affect other cells than leukocytes (Abbas 2000; Balkwill 2000). The complexity of "The Cytokine Network" as it is known today, has been summarized in books edited by Balkwill (2000), Abbas, Lichtman & Pober (2000) and Oppenheim & Feldmann (2000). The effect of any given cytokine on a target cell depends on numerous confounding factors, and is exemplified by Abbas *et al*. (2000); *pleiotropism* (different effect on different cells), *redundancy* (overlapping effect), *synergy* (several

cytokines having additive effect) and *antagonism* (inhibition by one cytokine on the effect of another). Thus, in order to fully elucidate the role of cytokines in any given situation, the simultaneously analysis of several cytokines is preferential.

Cytokines have a role in both the innate and the humoral *in vivo* immune response (Abbas 2000), and are often classified as "pro" and "anti" inflammatory (Pedersen *et al.* 1998; Ostrowski *et al.* 1999). The pro-inflammatory cytokines include IL-1, IL-6, TNF-α and INF-γ while some of the anti-inflammatory cytokines are IL2-, IL-4, IL-10 and IL-1ra (receptor antagonist) (Abbas 2000; Balkwill 2000). This classification is, however, not absolute due to the overlapping functions of many cytokines. IL-6, for example was recently suggested to be more anti- than pro-inflammatory (Pedersen 2000).

Many cytokines are constitutionally expressed, but production and secretion increase in response to microbes and inflammatory signals. Some of the functions mediated by cytokines include regulation of Ig production, cell differentiation and cell proliferation (Abbas 2000; Balkwill 2000), expression of adhesion molecules (Blue *et al.* 1993; Song *et al.* 1997; Meager 1999) and induction or inhibition of apoptosis (Monney *et al.* 1998; Flad *et al.* 1999). Cytokines and hormones share many similar characteristics, and it has been suggested that the difference between them is only a matter of definition (Smith 1997).

Most of the data on cytokines and physical exercise has been obtained on IL-1, IL-6 and TNFα. Thus, a short description of these 3 cytokines is presented below. (See Glossary for other cytokines.)

*Interleukin-1* (IL-1) exists in an α and β form, which both bind to the same receptor and exert similar biological functions (mediation of inflammatory response) (Dinarello 1994; Abbas 2000). In general, IL-1α acts locally and IL-1β systemically. IL-1 is produced by many cell types, including activated monocytes, macrophages, epithelium, fibroblasts and smooth muscle (Oppenheim & Feldmann 2000). TNFα is one of the primary inducers of IL-1. In low doses, IL-1 acts locally (IL-1α) to stimulate leukocyte adhesion and inflammatory events. In higher concentrations IL-1 acts systemically (IL-1β) (Smith 1997) and has been linked to protein synthesis (Cooney *et al.* 1999), production of acute phase reactants (i.e. CRP) by the liver (Abbas 2000), fever and the release of growth hormone (Smith 1997). IL-1 also stimulates the release of β-endorphin and interrupts pain transmission and the release of other cytokines such as TNFα IL-2, IL-4 and IL-6 (Dinarello 1994). An involvement of IL-1 in inflammatory myopathies is

documented (Authier *et al.* 1997; Lundberg *et al.* 2000) and prolonged IL-1 production can predisopse individuals for sepsis and diabetes (Dinarello 1994; Balkwill 2000). A connection between IL-1, the cytoskeleton and adhesion molecules was recently discovered by Zhu *et al*. (1998), where IL-1 signaling was affected by whether or not the cells were bound to integrin. In conjunction with the localization of the pro-IL-1β converting enzyme (ICE) in mitochondria, the accumulation of mitochondria at neuromuscular junctions and localization of IL-1β also at neuromuscular junctions (Authier *et al.* 1997), the possibility for a connection between IL-1, energy metabolism, adhesion molecules, intracellular signaling and physical activity appears to exist. Also, a specific receptor for IL-1 has been detected in vascular smooth muscle (French *et al.* ).

Serum IL-1 concentration can increase in response to physical exercise, even though of less magnitude than IL-6 and IL-1ra (Table 1).

IL-1 is present in healthy human skeletal muscle, and increases when the muscle is damaged or regenerating (Cannon *et al.* 1989; Fielding *et al.* 1993; Malm *et al.* 2000). IL-1 can decrease skeletal muscle protein synthesis (Cooney *et al.* 1999), promote glucose uptake by skeletal muscle (Jarrous & Lang 1995) and prostaglandin E2 synthesis in endothelial and smooth muscle cells (Baracos *et al.* 1983). IL-1 can also act as a vasodilator (Minghini *et al.* 1998). In the study by Fielding *et al*. (1993) the increased IL-1β accumulation in human skeletal muscle after exercise was related to neutrophil infiltration, but this was not the case in two other investigations (Authier *et al.* 1997; Malm *et al.* 2000). Authier *at al*. (1997) suggested that IL-1 serves a function in muscle fiber regeneration rather than inflammation.

*Interleukin-6* (IL-6) is produced by a large number of cells, but the main sources are macrophages, fibroblasts and endothelial cells (Oppenheim & Feldmann 2000). Smooth muscle cells (Helle *et al.* 1991), $T_{H2}$ cells, B cells, fibroblasts, myoblasts (Bartoccioni *et al.* 1994) and muscle cells (Nagaraju *et al.* 1998; Malm *et al.* 2000) can also produce IL-6. Production of IL-6 is up-regulated by mitogenic or antigenic stimuli, LPS, IL-1, IL-2, IFN, TNF, PDGF and virus. Inhibition of production is mediated by IL-4 and IL-13 (Helle *et al.* 1991; Abbas 2000; Balkwill 2000).

IL-6 has been considered a pro-inflammatory cytokine, but is perhaps better described as an anti-inflammatory cytokines because it responds to, rather than is the causes of, inflammation (Pedersen 2000). IL-6 acts via gp130, a receptor subunit shared by LIF (see below) (Taga & Kishimoto 1997). The many functions of IL-6 include

stimulation of B cell differentiation and antibody production, T cell activation, growth and differentiation, IL-2 production, increased platelet number, synthesis of acute phase proteins, release of growth hormone and inhibition of cancer cells (Taga & Kishimoto 1997; Abbas 2000; Balkwill 2000). In rat skeletal muscle, IL-6 can induce protein breakdown *in vitro* (Goodman 1994).

Because IL-6 increases in serum after prolonged, strenuous physical exercise of eccentric character (Bruunsgaard *et al.* 1997) it was initially believed that IL-6 was a sign of muscle damage. However, the same research group recently suggested that the increase in serum IL-6 may serve a function in the control of metabolism and be involved in glycogenolysis (Ostrowski *et al.* 1998; Pedersen *et al.* 2001). In a study by Crosier *et al*. (1999) they could find no difference in eccentric exercise-induced IL-6 before and after a period of eccentric training, even though the increase in soluble myoglobin diminished after training, supporting the notion that IL-6 is not increased in the blood due to skeletal muscle damage. In this context, the finding by Venkatraman & Pendergast (1998), that IL-6 decreased after endurance exercise when the intake of dietary fat was low is puzzling. With a low fat intake, the exercise-induced IL-6 increase should be enhanced because glycogen deposits are more rapidly utilized. *In vitro*, IL-6 has been shown both to stimulate (Mattacks & Pond 1999) and not to stimulate (Feingold et al. 1992) lipolysis. It is possible that co-stimulators like IL-4 and TNFα are needed for optimal effect of IL-6 (Mattacks & Pond 1999).

IL-6 is present within the skeletal muscle cell (Malm *et al.* 2000) and appears to be the source of IL-6 appearing in blood after exercise (Ostrowski *et al.* 1998).

*Tumor necrosis factor-α* (TNFα), as well as IL-1, mediates innate immunity and TNFα is the primary mediator of acute inflammation (Abbas 2000). TNFα is released in large amounts from monocytes and macrophages when gram-negative bacteria are encountered (LPS stimulation), but also viruses, IL-1, IL-2 and hypothermia can induce TNFα. Besides monocytes, neutrophils, activated lymphocytes, NK cells, endothelial cells and smooth muscle cells are sources of TNFα production (Oppenheim & Feldmann 2000). TNFα acts in a concentration dependent manner, where low quantities stimulate local inflammation with neutrophil and macrophage infiltration and moderate quantities cause systemic effects such as fever and production of acute phase proteins (i.e. CRP). If the plasma concentration of TNFα becomes very high, the result will be septic shock

**Table 1.** *Change in blood concentration(plasma or serum) of IL-1, IL-6 and TNFα with exercise. (Selected studies)*

| Exercise | Cytokine | Effect | Reference |
|---|---|---|---|
| Marathon | IL-1 | ↑ plasma | (Ostrowski *et al.* 1999) |
| | IL-6 | ↑ plasma | |
| | TNFα | ↑ plasma | |
| Marathon | IL-1 | ±0 plasma | (Suzuki *et al.* 2000) |
| | IL-6 | ↑ plasma | |
| | TNFα | Not detectable | |
| Marathon | IL-1 | ±0 plasma , ↓ number of IL-1α, IL-6 producing cells | (Starkie *et al.* 2001) |
| | IL-6 | ↑ plasma | |
| | TNFα | ↑ plasma | |
| Cycling 1 h at 75% | IL-1 | ±0 plasma IL-1β, α | (Ullum *et al.* 1994) |
| | IL-6 | ↑ plasma | |
| | TNFα | ±0 plasma | |
| Running at 80% to exhaustion | IL-1 | ±0 plasma | (Venkatraman & Pendergast 1998) |
| | IL-6 | ↓ plasma (diet dependent) | |
| | TNFα | ±0 plasma | |
| Swimming, cycling, running to max (mean 68 min) | IL-1 | ±0 serum | (Weinstock *et al.* 1997) |
| | | ↓ LPS stimulated IL-1β production | |
| | IL-6 | ↑ serum | |
| | TNFα | ↑ serum | |
| Eccentric cycling | IL-1 | Plasma IL-1β not detectable | (Bruunsgaard *et al.* 1997) |
| | IL-6 | ↑ plasma | |
| | TNFα | Plasma TNFα not detectable | |
| Running 6 h | IL-1 | ±0 plasma | (Drenth *et al.* 1995) |
| | | ↓ LPS stimulated IL-1β | |
| | IL-6 | ↑ plasma | |
| | TNFα | ±0 plasma | |
| | | ±0 LPS stimulated TNFα | |
| Eccentric weight lifting | IL-1 | ↓ serum | (Smith *et al.* 2000) |
| | IL-6 | ↑ serum | |
| | TNFα | ±0 serum | |
| 3 h of cycling and walking | IL-1 | ↑ plasma | (Moldoveanu *et al.* 2000) |
| | | IL-1β mRNA in monocytes did not change | |
| | IL-6 | ↑ plasma | |
| | | mRNA not changed | |
| | TNFα | ↑ plasma | |
| | | mRNA not changed | |

(Abbas 2000). Some of the *in vitro* effects of TNFα are increased adhesion molecule expression on endothelium (Blue *et al.* 1993), release of chemotactic substances and apoptosis (Monney *et al.* 1998; Flad *et al.* 1999). Also, the macrophage receptor CD163 (which mediates anti-inflammatory activites) can be downregulated by TNFα (Buechler *et al.* 2000). The *in vivo* effects include decreased blood glucose, necrosis of tumors and cachexia (loss of muscle mass) in rats (Abbas 2000; Balkwill 2000; Oppenheim & Feldmann 2000).

In response to physical exercise, the TNFα concentration in blood may increase or not change (Pedersen *et al.* 1998; Pedersen 2000) and Table 1. In some studies no detectable levels were found in serum, indicating that the methods used may not have been sensitive enough (TNFα serum concentration is in the range of 1-2 pg $mL^{-1}$, Study III). In some studies LPS stimulated monocyte production of TNFα has been shown to increase after exercise (Mackinnon 1999), but can not account for the post exercise increase in serum concentration (Starkie *et al.* 2001).

In one study (Greiwe *et al.* 2001) TNFα decreased in skeletal muscle of elderly human subjects after a period of resistance training. The decrease was positively related to increased protein synthesis. This fits nicely with the findings in another study (Li *et al.* 1998) where differentiated mice myotubes treated with TNFα lost myosin heavy chain protein in a time and dose related manner. However, infusion of TNFα in human subjects had no short term effect on muscle metabolism in a study by De Blaauw et al. (De Blaauw *et al.* 1997). TNFα can induce an acute decrease in cotractile function (Hopkins 1996). Thus, TNFα (as well as IL-1 and IL-6) may influence skeletal muscle metabolism and function, possibly only in a longer perspective (Reid & Li 2001), but Pedersen (Pedersen *et al.* 1998) concluds that *in vitro* data is not supported by *in vivo* experiments. As with IL-6, TNFα may work in synergy with other factors (such as increase in VCAM-1 with IL-4 (Barks *et al.* 1997)), and infusion of TNFα (or any other factor) alone does not necessarily mimic the physiological reality.

## 1.8 GROWTH FACTORS

Adult human skeletal muscle has the capacity to regenerate after injury, and the adaptability to physical exercise is well known. The exact molecular mechanism for this function has not been discovered, but a group of molecules collectively called growth factors (GF) have received some attention and reviewed recently by Husmann *et al.* (1996), Goldspink (1999), Grounds (1991) and Chambers & McDermott (1996). Growth

factors are locally produced peptides, which act in an autocrine or paracrine way in the muscle, but can also have systemic effects. Contraction or stretching of skeletal muscle cells are necessary for maintenance of normal muscle cell function, but the link between the mechanical stretching of the muscle cell and gene activation has not been discovered. Ruoslathi simply states, "stretching is good for the cell" (Ruoslahti 1997). Based on the intricate and complex constellation of events governing life and death in other cells types, a simple explanation to the issues of skeletal muscle development, regeneration and adaptation is unlikely. A combination of known and yet undiscovered factors are most likely involved.

Numerous GFs are know to affect skeletal muscle both *in vivo* and *in vitro*, and if muscle hypertrophy or regeneration is desired, the final outcome of their actions are activation, proliferation and differentiation of satellite cells, discussed in Section 1.5. The role of growth factors in non-hypertrophy adaptation, such as in response to endurance exercise, is not known. Much of the work on growth factors is performed on cell cultures, and Grounds (1991) cautions against the extrapolation to *in vivo* functions. Nevertheless, based on the results from published studies, a few growth factors were selected as possibly influencing muscle function after physical exercise. Described below are some of the functions of these factors summarized (from Husmann *et al.* 1996; Goldspink 1999; Grounds 1991; Chambers & McDermott 1996).

Fibroblast growth factor (FGF) is stored in the extracellular matrix (Yamada *et al.* 1989), released from damaged muscle cells, infiltrating macrophages and endothelial cells and stimulates satellite cell proliferation while inhibiting differentiation. FGF also mediates chemotaxis and angiogenesis. Transforming growth factor-β (TGF-β) inhibits satellite cell proliferation, differentiation and fusion. TGF-β first initiates, then downregulates inflammatory events, but if accumulated in excess, TGF-β predispose the host for infections. Thus, TGF-β has both local and systemic functions (Wahl 1994). Both FGF and TGF-β are stored locally and can be released immediately after injury and are therefore important in acute inflammation. Platelet-derived growth factor (PDGF) can be released by platelets, blood vessels and macrophages. PDGF increases cell proliferation and decreases cell differentiation and is also a mitogen and chemotactic agent for satellite cells. Hypoxia-inducible factor-1α (HIF-1α) is an oxygen-sensing transcription factor (Wenger 2000), which can influence erythropoietin gene induction and was increased in human skeletal muscle after physical exercise (Gustafsson *et al.* 1999). In this study HIF-

1α was related to changes in vascular endothelial growth factor (VEGF). Thus HIF-1α and VEGF could play a role in skeletal muscle adaptation to exercise.

Three growth factors and their receptors that were selected and investigated in this thesis are briefly discussed:

*Leukemia inhibitory factor* (LIF) belongs to the IL-6 cytokine family, and exerts its function via the shared gp130 receptor protein (Taga & Kishimoto 1997; Paradis & Gendron 2000).

LIF can be detected in smooth muscle, normal and regenerating skeletal muscle and LIF mRNA is expressed in muscle precursor cells (Kami & Senba 1998; Schoser *et al.* 1998; Kami *et al.* 2000). Several growth factors and cytokines (IL-1β, TNFα, TGF-β, G-CSF, PDGF, bFGF, and IGF) can trigger LIF production (Sun *et al.* 1996).

LIF and IL-6 have many similar functions such as induction of acute phase proteins and macrophage differentiation. LIF is important both for normal embryonic development of skeletal muscle and for muscle regeneration, because it stimulates myoblast proliferation and nerve innervations and survival. Because the LIF receptor (LIF-R) has been localized to type 1 muscle cells, LIF may affect type I muscle fibers more than type II, and myotubes are irresponsive to LIF stimulation because they lack LIF-R (Schoser *et al.* 1998; Kami *et al.* 2000).

When administrated after induced muscle injury, muscle regeneration was enhanced in mice subjected to crush injury to the muscle (Barnard *et al.* 1994) but in rats no effect of LIF injection on muscle regeneration after chemical muscle injury (bivacaine hydrochloride) was found (Gregorevic *et al.* 2000).

The influence of physical exercise on LIF has not been investigated previously.

*Hepathocyte growth factor* (HGF) is expressed in the liver, skeletal muscle, vascular smooth muscle cells, satellite cells, fibroblasts, lung and spleen (Matsumoto *et al.* 1995)

HGF is a pluripotent growth factor which functions via its receptor, the proto-oncogene c-Met. The structural organization of several different tissues, besides liver where it was first identified, is strongly influenced by HGF. Cell growth, motility, chemoattractant for motoneurons, proliferation of endothelial and epithelial cells and organ regeneration are some of the actions transmitted by HGF (Birchmeier & Gherardi 1998; Gal-Levi *et al.* 1998). Without HGF and c-Met, skeletal muscle and other organs

are not properly formed in the developing embryo (Birchmeier & Gherardi 1998). Most importantly in the perspective of this thesis, is the exclusive role of HGF as the only factor capable of activating quiescent satellite cells (Gal-Levi *et al.* 1998; Tatsumi *et al.* 1998). The role of HGF/c-Met in adult humans has not been investigated *in vivo*, but in cell cultures both autocrine and paracrine functions of HGF on satellite cells were recently described (Sheehan *et al.* 2000; Warn *et al.* 2001). Among several growth factors investigated in one study, HGF had the largest effect on myoblast migration and also that the effect was dose-dependent (Corti *et al.* 2001).

Induction of HGF can occur when a tissue is injured. Released prostaglandins (PGs) from the site of injury are potent inducers of HGF gene transcription (Matsumoto *et al.* 1995), while INFγ can upregulate c-Met in alveolar epithelial cells (Nagahori *et al.* 1999). HGF is present in uninjured muscle but may be in an inactive form (bound to heparan sulphate proteoglycan) (Birchmeier & Gherardi 1998; Tatsumi *et al.* 1998). As with many other cytokines, hormones and growth factors, it has been shown that HGF can act in synergy with other factors, and enhanced proliferation was observed when HGF and FGF (fibroblast growth factor) were added together (Sheehan & Allen 1999). *In vitro*, INFγ enhanced the migratory activity of HFG on cultured alveolar epithelial cells (Nagahori *et al.* 1999).

The influence of physical exercise on HGF has not been investigated previously.

*Insulin-like growth factor-1* (IGF-1) can be found in the blood, liver, smooth muscle. satellite cells and endothelial cells (Adams 1998).

IGF-1 is the effector molecule of growth hormone (GH) and stimulates somatic growth, cell differentiation and proliferation. Systemic IGF-1 is produced mainly in the liver (Haydar *et al.* 2000), but IGF-1 can also act in a autocrine/paracrine fashion when locally produced and has been suggested to be a key factor in skeletal muscle regeneration and adaptation (Adams 1998). Protection of stress-induced apoptosis in cardiac myoblasts by IGF-1 has also been reported (Mockridge *et al.* 2000)

Local and systemic IGF-1s are independant of each other, and a splice variant of IGF-1, named mechano-growth factor (MGF) exists in skeletal muscle (Yang *et al.* 1996). MGF is suggested to be a link between mechanical stretch of the muscle and gene response (Goldspink 1999; McKoy *et al.* 1999).

The IGF-1 receptor is localized both in the cell membrane and in the nucleus. When IGF-1 binds to its receptor cell differentiation, anabolism and proliferation can occur. The

actions of IGF-1 can also be regulated by the presence of IGF-1 binding protein in the blood and extravascular space. If the binding protein increases with GH injection this may explain why administration of GH (which increases IGF-1production) does not affect protein synthesis rate and muscle function in human subjects (Adams 1998). Locally produced IGF-1/MGF appears to have an important function in muscle hypertrophy but the function in non-hypertrophy adaptation to exercise is unknown. Myonuclear number in rats subject to hindlimb suspension was maintained with either exercise or exercise and GH/IGF-1 injections, but not with GH/IGF-1 only (Allen *et al.* 1997) indicating that systemic GH/IGF-1 is not the key to the muscle adaptation puzzle.

In response to physical exercise, serum IGF-1 increases acutely (Bang *et al.* 1990) and after 10 weeks of resistance training in elderly, muscle IGF-1 increased significantly (Singh *et al.* 1999) as did IGF-1/MGF mRNA in rabbit skeletal muscle after 4 days of stretch (McKoy *et al.* 1999) and IGF-1 expression in both capillaries and satellite cells in humans after 7 days of military training (Hellsten *et al.* 1996).

# 2 HYPOTHESIS

The purpose of this thesis was to investigate interactions between immunological variables in human blood and skeletal muscle in conjunction with physical exercise.

Interactions between immunological events in blood and skeletal muscle at rest and after physical exercise are proposed as a means of adaptation and increased physical performance. These interactions may involve, but not be limited to:

i. Leukocyte migration from blood to muscle tissue

ii. Cell surface expression of signaling and adhesion molecules

iii. Changes in serum hormone concentration

iv. Release of muscle specific proteins due to permeabilized cell membranes

v. Serum and tissue concentration of cytokines and growth factors.

The main hypothesis during the completion of this thesis has been that physical exercise will induce inflammation in human skeletal muscle. After strenuous physical exercise, leukocytes from the blood will migrate to the affected muscle tissue as part of the inflammatory response, and disappear upon completion of the damage-repair-adaptation process. The magnitude of the inflammation / infiltration should be dependent on the intensity of, and eccentric component in the exercise.

Exercise-induced skeletal muscle damage and inflammation should be detectable in the blood as increased presence of commonly used markers for inflammation.

# 3 METHODS

## 3.1 SUBJECTS

The work included in this thesis is exclusively based on human subjects. The majority of the subjects were males. In Study III where females were not excluded only 3 volunteered. A majority of the 56 subjects who participated in the studies were healthy males, and many of them students at Stockholm University College of Physical Education and Sports or Stockholm University. As seen in Table 1, most subjects were trained or well trained but not elite athletes. A few subjects had a maximal oxygen consumption ($VO_{2,max}$) of below 35 ml $min^{-1}$ $kg^{-1}$ and considered untrained. The ethics committee at Karolinska Institutet approved all experimental protocols and all subjects signed an informed consent before participating in any testing or exercise.

## 3.2 EXERCISE PROTOCOLS

Previous investigations have determined that, compared to concentric exercise, eccentric exercise induces more ultra-structural alterations, inflammation and delayed onset muscle soreness (DOMS) (Armstrong *et al.* 1983; Fridén *et al.* 1983; Clarkson *et al.* 1986; Evans & Cannon 1991; Appell *et al.* 1992; Pyne 1994; MacIntyre *et al.* 1995; Lieber *et al.* 1996; MacIntyre *et al.* 1996; Brown *et al.* 1997; Clarkson & Sayers 1999). Thus, different modes of eccentric exercise were used as a model to induced muscle and epimysium inflammation in Studies I-III.

### 3.2.1 Walking "Borsov steps"

In the morning of the exercise day, the subjects performed a 10-min warm-up on a stationary cycle ergometer followed by 5 x 30 eccentric-type walking steps (15 steps per leg) with 1 min rest between each set. The type of eccentric exercise used (Borsov-steps) will induce severe muscle soreness in the gluteus and quadriceps muscles. The steps are executed by kneeling down with the leading leg to a 90° angle, touching the ground with the knee of the lagging leg and returning to a standing, upright position.

### 3.2.2 Eccentric cycling

The electrically powered bicycle, a Krogh cycle, used in this study has previously been used for eccentric cycling exercise (Fridén *et al.* 1983). It consists of an electrical motor, an electrical induction clutch and a modified cycle ergometer (Figure 2). Subjects were instructed to maintain 60 rpm for 30 min at a work rate equal to the highest

concentric cycling work rate maintained for 2 min during the concentric cycling $VO_{2,max}$ test. $VO_2$ was also measured during the last 5 min of exercise.

### 3.2.3 Downhill and uphill running

Running speed during the 45 min exercise was chosen based on $VO_2$ measurements from each subject when running at 2 different speeds (60-90 sec at each speed) at the assigned incline or decline. These measurements were performed as part of the warm-up for the $VO_{2,max}$ test. The Downhill 4° group was aimed to run at 50% of $VO_{2,max}$ and the Uphill 4° group to run at 75% of $VO_{2,max}$. In order to maximize the eccentric component of the exercise while minimizing the risk of injury, subjects in the Downhill 8° group were asked to run at the maximum speed maintainable for 45 min (Figure 3). All subjects managed to run at the chosen speed for the 45 min. Between 20-23 min and 42-45 min of exercise $VO_2$ and heart rate were recorded.

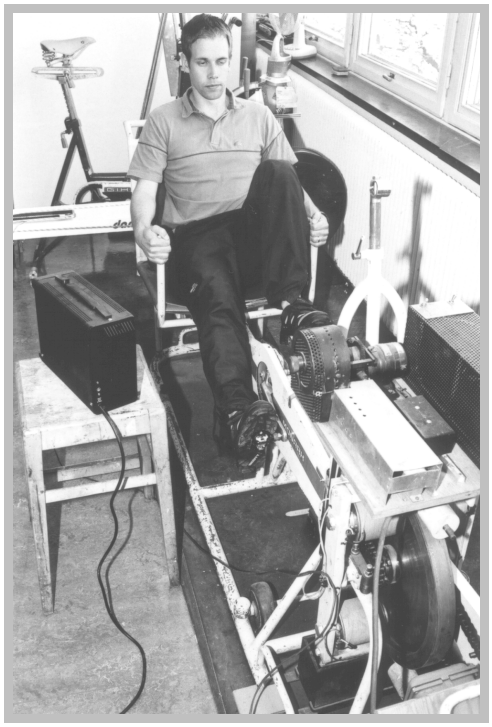


**Figure 2**
Eccentric cycling

**Figure 3**
Downhill running at 8°

### 3.2.4 Soccer exercise

Two consecutive competitive (2 x 45 min) soccer games were played (afternoon February 27 and morning February 28) and a five days training camp completed April 5-9. During the time between the two games and the training camp (6 weeks) the training consisted of 25 practice session and 7 games (2 x 45 min). The average practice session lasted approximately 2 h and contained 65% playing time, 25% mixed exercises and 10%

strength training. Exercise intensity during competitive match-play has been estimated to 70-75% of $VO_{2,max}$. Exercise during the training camp consisted of a daily morning run of about 8 km and two 90 min soccer-specific training sessions per day. Two of the 90 min practice sessions were exchanged for a 2 x 45 min practice game.

### 3.3 PERFORMANCE TESTS

Standard indicators of physical performance were measured ($VO_{2,max}$, 30 sec Wingate, blood lactate, muscle strength) and used to characterize subjects and investigate changes in physical performance and metabolism with different exercise protocols. The tests performed in each study were chosen based both physiological considerations and practical manageability.

*Study I:* Before and 48 h after the eccentric exercise an anaerobic cycling test was performed which consisted of a 10 min warm-up, 30 s all-out cycling, 5 min recovery followed by 10 x 10 sec all-out cycling interspersed with 50 sec rest periods, all at a work load of 7% of total body weight.

*Study II:* A standard incremental concentric cycling test was performed, starting at a work rate of 100 W at 60 revolutions per minute with a 50 W increase in work rate every two minutes until exhaustion and maximal oxygen uptake was determined.

*Study III:* Not later than two days before the 45 min running exercise each subject's $V_{O2,max}$ was determined. After a 10 to 15 min warm-up at a speed chosen by the subject and a brief resting period (approximately 5 min) the test started at an incline of 1° and an increase of 1° every minute until exhaustion. Running speed was constant and set at each subjects estimated 10 km racing pace.

*Study IV:* $VO_{2,max}$ was measured during a maximal treadmill test, starting at 12 km $h^{-1}$ and elevation to 2°. Speed and elevation was then increased alternatively by 1 km $h^{-1}$ and 1°, respectively, until exhaustion. A standardized sub-maximal cycling test was also performed, and heart rate (HR) recorded before and 24 h, 48 h and 72 h after the second game. During the sub-maximal test the subject cycled at 75 W and 150 W until steady state HR was achieved (<5 beats change per minute), but at least 4 min on each work rate.

**Table 2.** *Subject characteristics and exercise protocols from Studies I-IV; mean (range)*

| Study | N | Age (year) | Body weight (kg) | $VO_{2,max}$ (ml $min^{-1}$ $kg^{-1}$) | Wingate (W $kg^{-1}$) | Exercise |
|---|---|---|---|---|---|---|
| I | 12 | 26 (22-35) | 77 (65-92) | | 1.63 (1.58-1.86)[a] | Borsov steps (5 x 30 steps) |
| II | 13 | 24 (19-32) | 75 (63-95) | 49 (34-68) | | Eccentric cycling (30 min at 37% of $VO_{2,max}$) |
| III | 21 | 27 (18-58) | 75 (62-94) | 51 (28-67) | | Dowhill and uphill running (45 min at 48-81% of $VO_{2,max}$) |
| IV | 10 | | | 59 (49-67) | | Soccer exercise (Games and practice) |

[a] 95% confidence interval

### 3.3.1 Main equipment used

Expired air collected into Douglas bags were analyzed using a Beckman LB-2 $CO_2$ analyser (Stockholm, Sweden), an Applied Electrochemistry Inc. $O_2$ analyser (Sunnyvale, CA, USA) and a Warren E. Collins Inc. spirometer (Braintree, MA, USA).

Concentric cycling exercise was performed on a Monarch 818E ergometer (Varberg, Sweden).

Anaerobic cycling tests were performed on an electrically controlled, modified Cardionics Wingate ergometer (Cardionics Inc., Bandhagen, Sweden).

All running was performed on a treadmill from Rodby Electronics, Enhörna Sweden.

In Study I, on line oxygen consumption was measured with the Medical Graphics Corp. CPX system (St Paul, MN, USA), and in Studies II and III, an AMIS 2001 from Inovision A/S, Denmark was used. The changes in on-line equipment was made because calibration procedures and comparisons between the Medical Graphics unit and Douglas bags revealed a deviation of 5-8% and considered unsatisfactory (data not shown).

A computerized dynamometer (the SPARK System) (Seger *et al.* 1988) was used for a functional test of the knee extension muscles, including the vastus lateralis.

Blood lactate was analyzed with an YSI 2300 STAT (Yellow Spring, OH, USA).

Heart rate was recorded with heart rate monitors from Polar Electro, Finland.

## 3.4 SAMPLING

### 3.4.1 Muscle and epimysium

Muscle biopsies were taken from the vastus lateralis using the forceps biopsy technique. After local epidermal anesthesia (Carbocain 20 mg mL-1, ASTRA, Södertälje, Sweden) a 1.5 cm incision was made through the dermis and epimysium and one 50-100 mg muscle tissue sample removed. In Study III, an approximately 3x5 mm piece of the epimysium was cut by a surgical scalpel after which a 50-100 mg muscle tissue sample was removed by the forceps biopsy technique (conchotome). The removed sample was

placed in Tissue-Tek medium (Miles Laboratories, IN, USA), frozen in isopropanol in liquid nitrogen within 1 minute and stored at -70° C before sectioning (6 μm). Sections were placed on Superfrost glass slides (Novakemi AB, Enskede, Sweden), air dried over night (leukocyte antigens) or fixed in 2% formaldehyde and air dried for 30 min (cytokines) and stored at -70°C until staining.

### 3.4.2 Blood

Blood samples were drawn from a fore arm vein before and at various times (up to 7 days) after exercise and always before the use of any anesthetics in the muscle biopsy procedure. For flow cytometry analyses, blood was collected into Vacutainer tubes containing ethylendiaminetetraacetic acid (EDTA; Becton Dickinson, France), kept at room temperature and analyzed within 24 h. For C-reactive protein (CRP), cortisol, albumin, sex hormone-binding globulin (SHBG), testosterone and soluble CD8 (sCD8) analyses blood was collected into untreated Vacutainer tubes, centrifuged at +4°C and serum stored at -70°C. Blood for analysis of catecholamines was collected into heparinized Vacutainer tubes submerged in ice cold water (Becton Dickinson, France), centrifuged at +4°C and serum stored at -70°C. Creatine kinase (CK) analyses were performed on either untreated or heparinized blood.

For whole blood lactate measurements, 25 μL blood was drawn from a fingertip capillary diluted with 50 μL YSI buffer with Triton X and analyzed with the YSI 2300 STAT lactate analyzer (YSI Corp., Yellow spring, OH, USA).

## 3.5 ANALYSES

### 3.5.1 Immunohistochemistry

The Vectastain ABC Elite chemicals and rapid staining protocol (Vector Laboratories, Burlingame, CA, USA) and the Histostain Plus™ kit (Zymed Laboratories Inc., South San Francisco, USA) were used when investigating expression of leukocyte phenotypic antigens with monoclonal antibodies in muscle and fascia sections. For cytokine staining, a modified Vectastain protocol was used (Ulfgren *et al.* 1995). The main feature in the modified protocol is the use of saponin for cell membrane permeabilization (Sigma-Aldrich, Stockholm, Sweden) and hepes (Life Technologies Ltd., Paisley, Scotland) in the Earle's balanced salt solution (EBSS; Life Technologies Ltd., Paisley, Scotland) buffer.

Optimal concentration of each antibody was determined before analysis. In short, the immunhistochemical staining procedure includes the following main steps: 1)

Blocking of endogenous peroxidase with $H_2O_2$ 2) Blocking of unspecific antigens with normal serum 3) Incubation with a primary antibody (mouse anti-human) for the antigen investigated 4) Incubation with biotinylated secondary antibody (goat anti-mouse) 5) Incubation with an avidin-biotin-peroxidase complex 6) developing with a peroxidase substrate (AEC or DAB) 7) Counterstaining with Mayer's Hematoxylin (Apoteksbolaget, Malmö, Sweden).

### 3.5.2 Image analysis

For quantification of positively stained tissue sections, a semi-automated image analysis system was used (Leica Microsystems, Sweden). The software was customized to perform optimal detection and data processing for the different staining protocols used. The system consists of a high-resolution 3CCD camera (DXC-950P, Sony Corporation, Tokyo, Japan) mounted on a Leica Fluovert FS microscope and connected to the QWin 500IW v.2.2 image analysis system (Leica Microsystems, Kista, Sweden) via a stabilized power supply (Inmac Power Line Conditioner 8831-7, Inmac, Santa Clara, CA, USA).

Analyses were performed on three separate areas of each muscle section for a total area of approximately 1.5 $mm^2$ per antigen and biopsy. The analyses included detection of positively stained tissue, manual counting of the total number of muscle fibers, measurement of detected area and counting of positively stained leukocytes by the QWin system. Detection threshold for positively stained tissue was set for each antigen on the pre-exercise biopsy and kept constant for all biopsies from the same subject. Optimal threshold for each individual and antigens was determined.

### 3.5.3 Flow cytometry

Blood leukocytes phenotypes and cytokines were analyzed by three or four color flow cytometry at Nova Medical Research, St. Görans Hospital, Stockholm, Sweden under the management of Dr Rodica Lenkei. The method for three-color flow cytometry has been described in detail (Lenkei & Andersson 1995) and is based on a high degree of standardization. Briefly, the staining was performed on whole blood for 30 min, on ice, followed by erythrocyte lysing with Ortho-mune Lysing Reagent (Johnson & Johnson, Raritan, NJ, USA). The staining panel comprised combinations on monoclonal antibodies (MAbs), conjugated with fluorescien isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophill protein (PerCP) all from Becton Dickinson, Mountain View, Ca, USA, (BDIS) with exception of CD45RO-PE MAb (Dakopatts, Denmark). The samples were analyzed with a daily calibrated FACScan Flow Cytometer (BDIS) using different microbeads: QC Windows and Quantum 1000 from Flow Cytometry Standards

Corporation and CaliBRITE (BDIS), as usually done for quantification of cellular antigens with Flow Cytometry. As the fluorescence intensity of the calibration beads was constant, and no adjustments were done to compensation circuits, the Mean Fluorescence Intensity (MFI) can be considered a relevant indicator for the expression of surface CD antigen molecules. Cell surface molecule density was also expressed as Molecules of Equivalent Soluble Fluorochrome (MESF). The difference is explained below:

*Mean Fluorescence Intensity, or Median of Fluorescence Intensity* (MIF) is a relative unit for determination of antigen density on cells by flow cytometry. MIF represents the mean or medians of intensity expressed in each channel. By changing the voltage applied to the photo multiplier tubes (the sensor detecting the fluorescence) one alters the intensity of the event (cell) which can be moved along the scale. Thus, one cannot compare the results obtained on different instruments, and even on the same instrument on different days

*Molecules of Soluble Fluorochrome* (MESF) is an absolute unit for determination of antigen density on cells by flow cytometry. Based on the use of a mixture of beads coated with the same fluorochrome as the one conjugated to the respective monoclonal antibody. The four types of beads in the mixture are coated with increasing fluorochrome amounts. A calibration plot translates the MIF into MESF units that are independent of the instrument settings and can be used across laboratories. However, MESF measures the number of fluorochrome molecules attached to a cell and so indirectly of monoclonal antibody. It is thus dependent of the number of fluorochrome molecules conjugated to a molecule of antibody. Thus, in order to compare results in long time studies at the same laboratory, or in studies across laboratories, the same batch of mAb must be used

White blood cell count and differentials were estimated with a Coulter® STKS hemocytometer (Coulter Electronics, USA). Because cell numbers were determined in whole blood, corrections for changes in plasma volume were not made.

Soluble CD8 (sCD8) was estimated by enzyme-linked immunosorbent assay (ELISA; Endogen, Inc. Woburn, MA, USA) according to the manufacturer's instructions.

Serum cytokines were measured by Cytometric Bead Array method for Flow Cytometry, with the Human $T_{H1}/T_{H2}$ Cytokine kit from Becton Dickinson (BD Pharmingen, San Diego, CA., USA) according to producer's methodology. The flow cytometric results were translated into amount of cytokines by using the software included in the kit.

The Ki-67 antigen (a marker for cell proliferation rate) was detected after lymphocyte permeabilization by using a whole blood method. Briefly, the cells were stained with the mAbs directed to antigens expressed on lymphocyte surface washed and treated with paraformaldehyde followed by a permeabilization step with saponin buffer, and thereafter stained with Ki-67.

### 3.5.4 Hormones, CK and CRP

Cortisol, testosterone, CRP and some of the CK analyses were performed by the Department of Clinical Chemistry, Karolinska Hospital under the management of Regina Solborg.

Serum cortisol was determined by a standard immunofluorescent method (Department of Clinical Chemistry, Karolinska Hospital, Solna, Sweden).

Serum testosterone and sex hormone binding globulin (SHGB) concentration were determined by a standard time-resolved fluoroimmunoassay (Kit B050-101 and B070-101 respectively, AutoDelphiaTM, Wallac Oy, Finland). The biological available (free) testosterone concentration (not bound to SHGB or albumin) was calculated as T*(1+0.601*C) where T = unbound testosterone and C = plasma albumin concentration

C-reactive protein (CRP) and albumin were analyzed by means of particle-enhanced immunonephelometry (Dade Behring Marburg, Germany).

CK activity was measured using a standard laboratory kit (CK MPR2, Boehringer-Mannheim, Germany) and a DU-70 spectrophotometer (Beckman Instruments AB, Bromma, Sweden).

Adrenaline, noradrenaline and dopamine were analyzed in heparinized plasma, using high-pressure liquid chromatography (HPLC) with electrochemical detection (CMA Microdialysis AB, Solna, Sweden) and a Nucleosil-100 SA 5 μm column (NC100-5SA-250D, Hichrome, Berkshire, England). The coefficient of variance (n=8) was for; noradrenaline 4.6%, adrenaline 6.0% and dopamine 7.4%.

## 3.6 METHODOLOGICAL CONSIDERATIONS

(Immunohistochemistry and image analysis)

To determine the reliability of the image analysis system, 2 separate calibration procedures were performed.

*Control of the system's reliability in repeated measurements of any given image*: One image from one single tissue section was stained with CD11b, developed in AEC and then captured and analyzed 39 separate times. The coefficient of variance for repeated measurement of the same tissue section was 0.08% (Figure 4). The system set up

(microscope, power supply, CCD camera etc) can therefore be considered very stable and highly standardized.

*Variation in antigen detection within one muscle sample*: The size of a muscle biopsy taken with the forceps technique is in the range of 50-100 mg, only representing a small fraction of the muscles (vastus lateralis). Because of the small sample size, a critical question to ask is if a randomly chosen section from a muscle biopsy can be used to correctly estimate events in an entire muscle. Also, the immunohistochemical staining procedure involves several critical steps where section thickness, antibody concentrations and developing times can alter the staining intensity. Thus, several factors can influence the detectable amount of marked and visualized molecules within a tissue section (normally referred to as "positive area"). To investigate the within tissue variation of a given molecule, 29 serial sections were cut, stained with CD163, developed with AEC and analyzed in the above describe image analysis system. The coefficient of variance for analysis of the 29 serial sections from the same muscle sample was 42%, indicating a variation in antigen expression (in this case CD163) within the tissue sample (Figure 5).

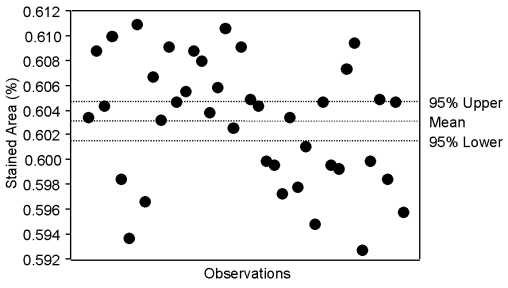



**Figure 4**
Repeated analysis (39 times) of the same micrograph

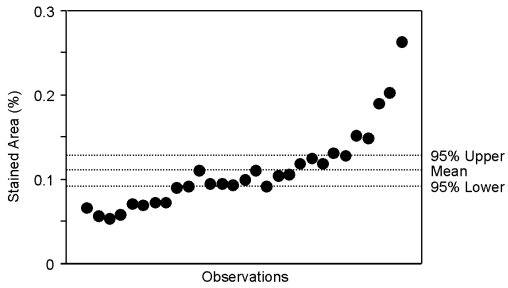



**Figure 5**
Analysis of 29 serial sections (6 μm) from one muscle sample

The implication of this calibration procedure is that the system setup has a detection error of ± 0.08% (CD11b) and that there can be a variation in antigen expression (CD163) of at least ± 42% within a muscle sample. A Power analysis using the above data from CD163 demonstrates that with $\alpha$=0.05, $\beta$=0.20, mean (SD)=544 (236) $\mu m^2$ and N=5, a difference in stained area of 419 $\mu m^2$, or 0.08% of the total area in the detection field (5.21 x $10^5$ $\mu m^2$) can be measured.

The variation in staining intensity due to differences in composition of the chemicals used, room temperature variations, developing times and other influencing factors were kept at a minimum, but the significance of each factor is difficult to measure.

In Study II, all muscle tissue sections from one individual was always stained simultaneously to minimize the risk that any difference over time (biopsy 1 to 7) would be due to the staining procedure. In Study III, muscle sections from 7 individuals were stained simultaneously, all sections thus stained on 3 separate occasions. When comparing the results in Studies II and III, it is apparent that the detected amount of CD antigens in human skeletal muscle at rest can be reproduced within clinically significant limits between studies, and the results from CD11b (the C3bi receptor) are also identical to the numbers previously published in our laboratory (using different equipment) by Ylva Hellsten (Hellsten *et al.* 1996)

## 3.7 STATISTICAL ANALYSES

Due to the skewed distribution of many blood variables (phenotypes, hormones, cytokines), non-parametric methods or log-transformation is needed (Lenkei & Andersson 1995) and have been used in Studies I-IV. However, when combining data from Studies I-IV, normal distribution (skeweness < 1) was observed in all phenotypes at rest (N=51) and the analysis of cross sectional data uses parametric statistics on normal numbers (not log-transformed). This indicates that when taking random samples from the population, larger variation is obtained compared to when subjects are selected for research (i.e. healthy, non-smoking men, aged 20-35). This should be kept in mind when extrapolating or generalizing data from any study to a larger population.

# 4 RESULTS

This thesis is based on the analysis of 22 cell surface receptors, 1 nuclear protein, 7 cytokines and 5 hormones in human blood and 7 cell surface receptors and 10 cytokines and growth factors in human skeletal muscle and epimysium. Six different modes of physical exercise and one control group have been used and data are compiled in 4 different manuscripts. Some of the combined results from the blood and muscle analyses are displayed in Figures 6-28 and emphasizes the diversity and complexity of the human immune system, in these cases when responding to various modes of physical exercise or multiple muscle biopsies. When presented as below, the effects of different exercise protocols on the main immunological variables investigated in the blood can be visualized based on the question "*how does a specific immunological variable change in response to a given mode of exercise*?" One alternative would have been to present the immunological response to each mode of exercise. Such a presentation rapidly becomes incomprehensible because it attempts to answer the question "*how does the immune system change in response to a given mode of exercise*?" Only the number of variables investigated limits the answer to the second question.

Displayed below are some selected variables which have been investigated in all or most of the studies. Changes are shown in percent in order to compare changed numbers between different variables.

In brief, the 4 studies included:

Study I: Borsov-steps, no Control group. No biopsy.

Study II: Eccentric cycling and Control group. Seven biopsies from each subject.

Study III: Downhill running at 4° and 8°, Uphill running at 4° and Control group. One biopsy from each subject at 48 h.

Study IV: Soccer exercise: Two games and a 5 day training camp. No biopsy.

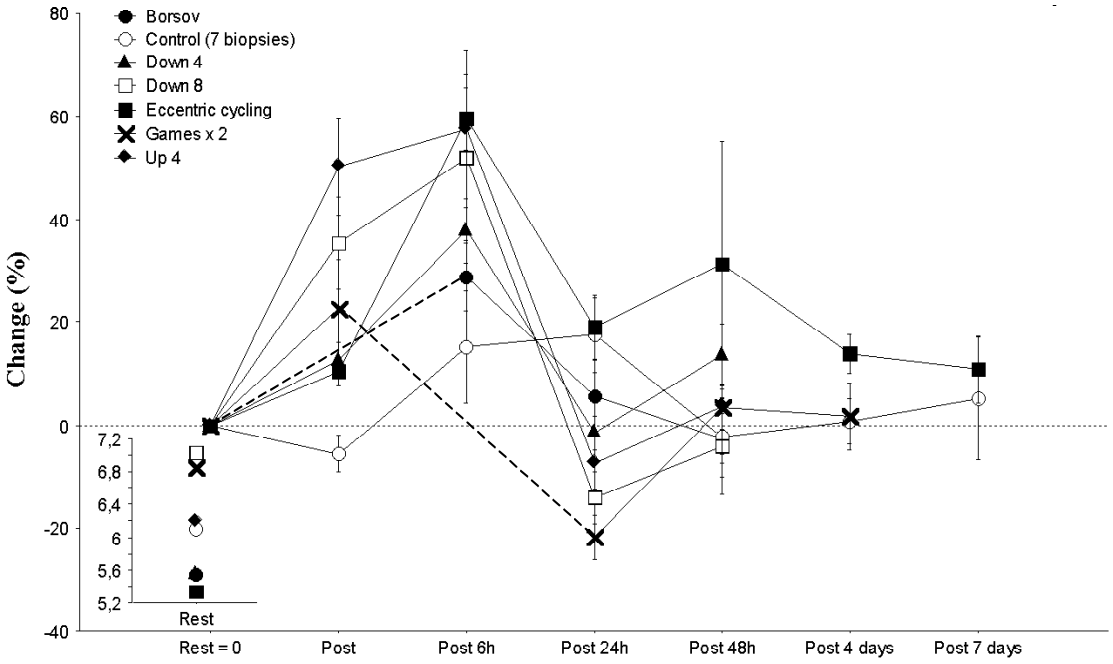



**Figure 6**
Changes in blood leukocyte numbers in response to different exercise protocols. Data from Studies I-IV. Resting numbers inserted (cells mL x $10^6$)

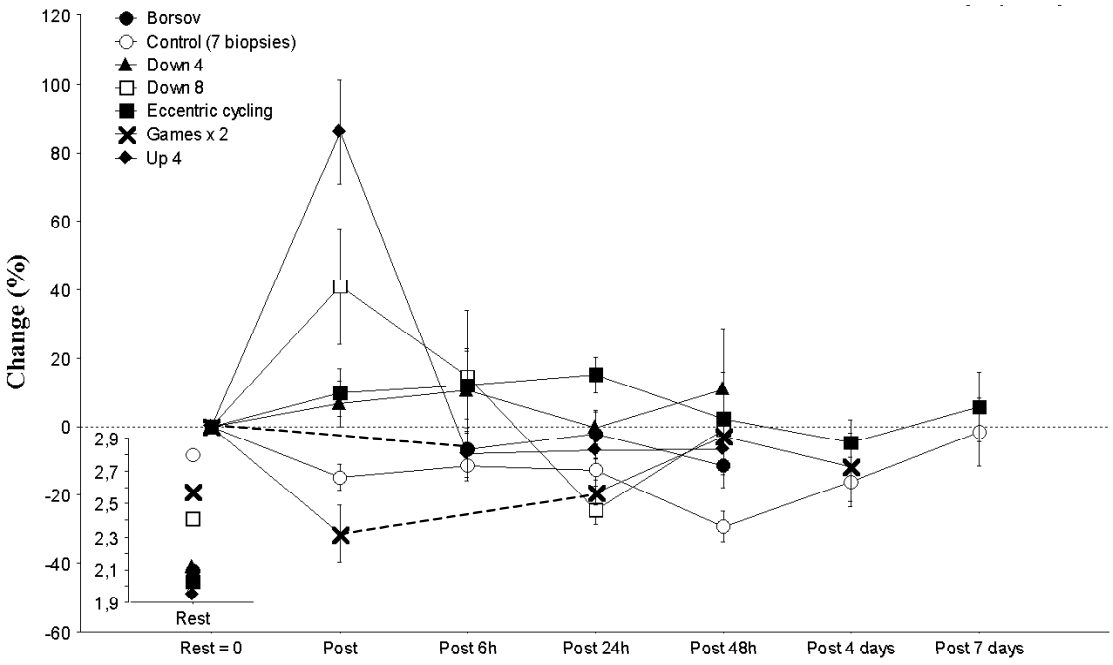



**Figure 7**
Changes in blood lymphocyte numbers in response to different exercise protocols. Data from Studies I-IV. Resting numbers inserted (cells mL x $10^6$)

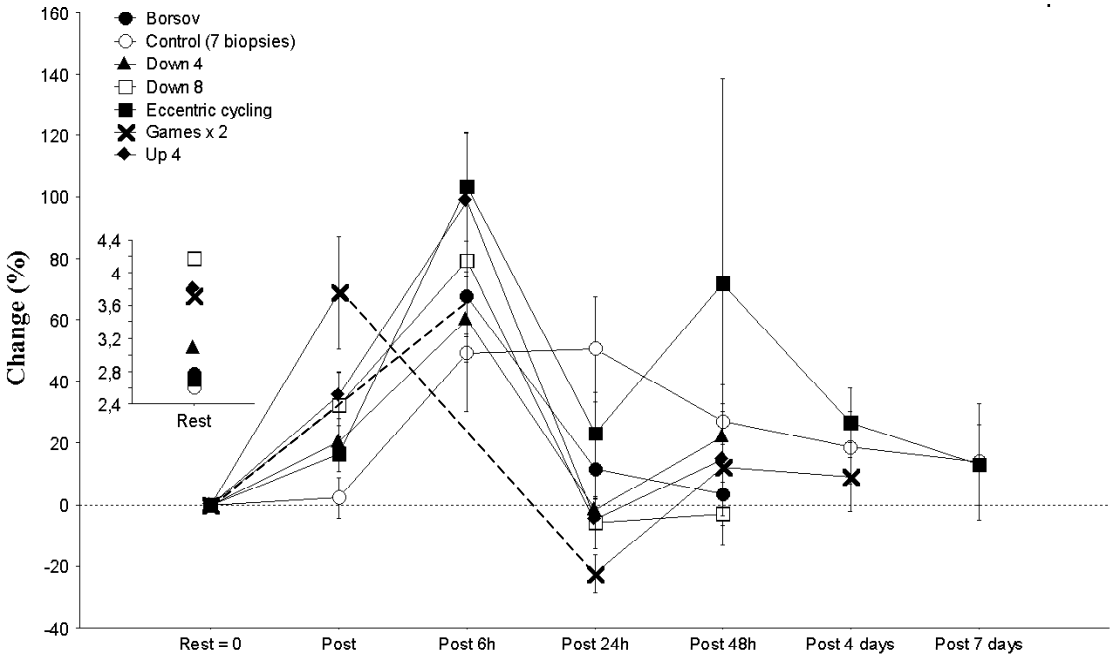



**Figure 8**
Changes in blood neutrophil numbers in response to different exercise protocols. Data from Studies I-IV. Resting numbers inserted (cells mL x $10^6$)

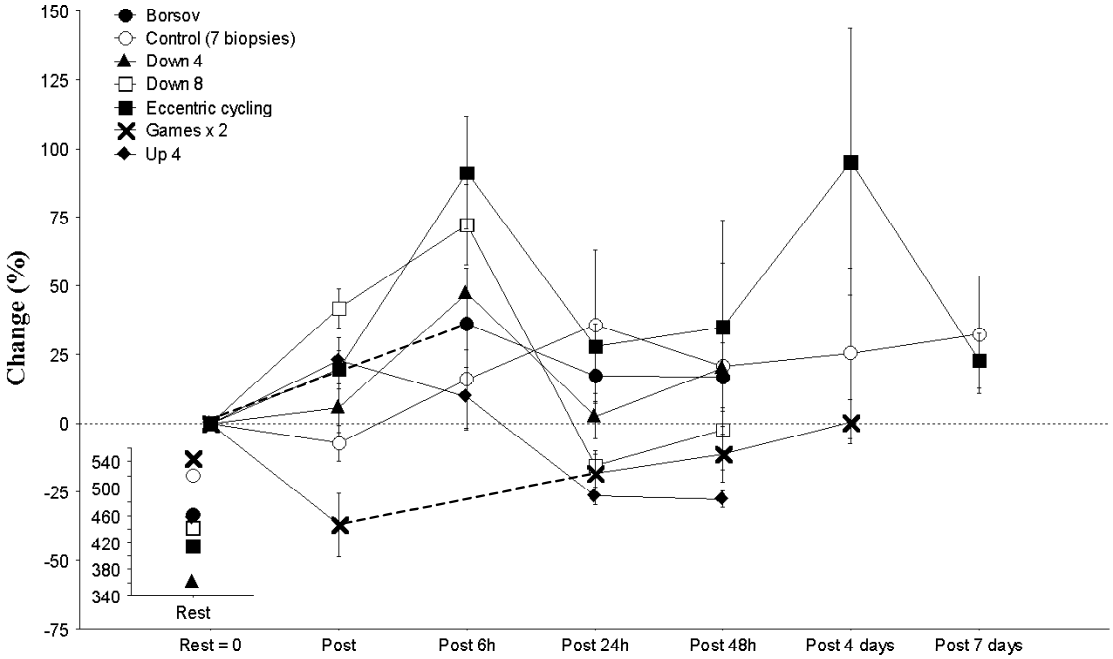



**Figure 9**
Changes in blood monocyte numbers in response to different exercise protocols. Data from Studies I-IV. Resting numbers inserted (cells mL x $10^3$)

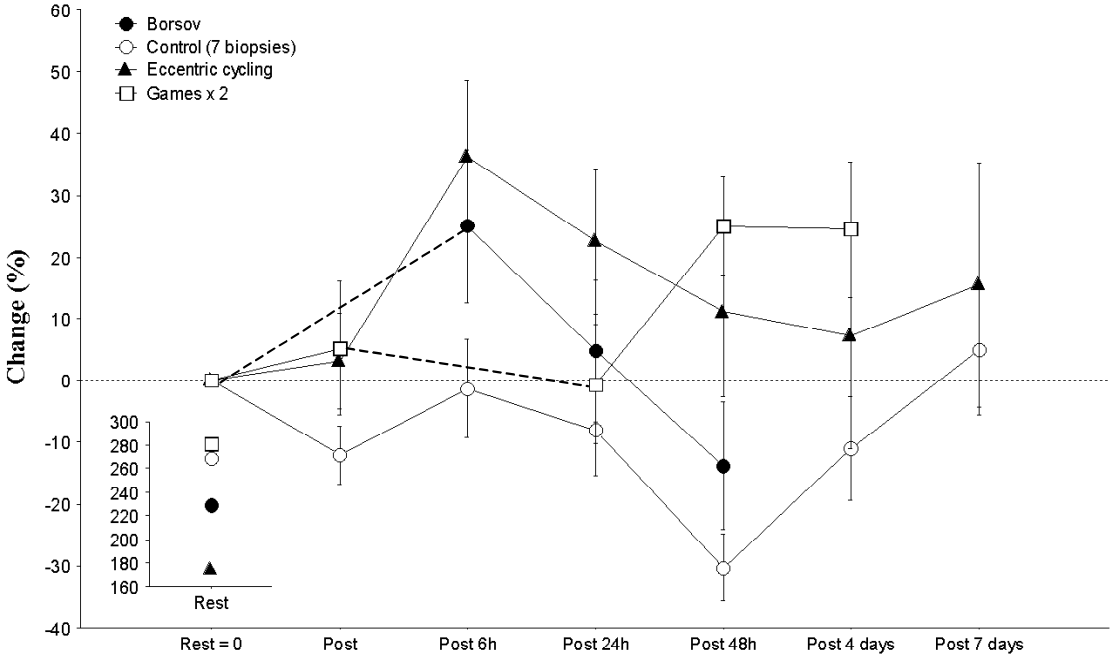



**Figure 10**
Changes in numbers of B cell numbers in the blood in response to different exercise protocols. Data from Studies I, II and IV. Resting numbers inserted (cells mL x $10^3$)

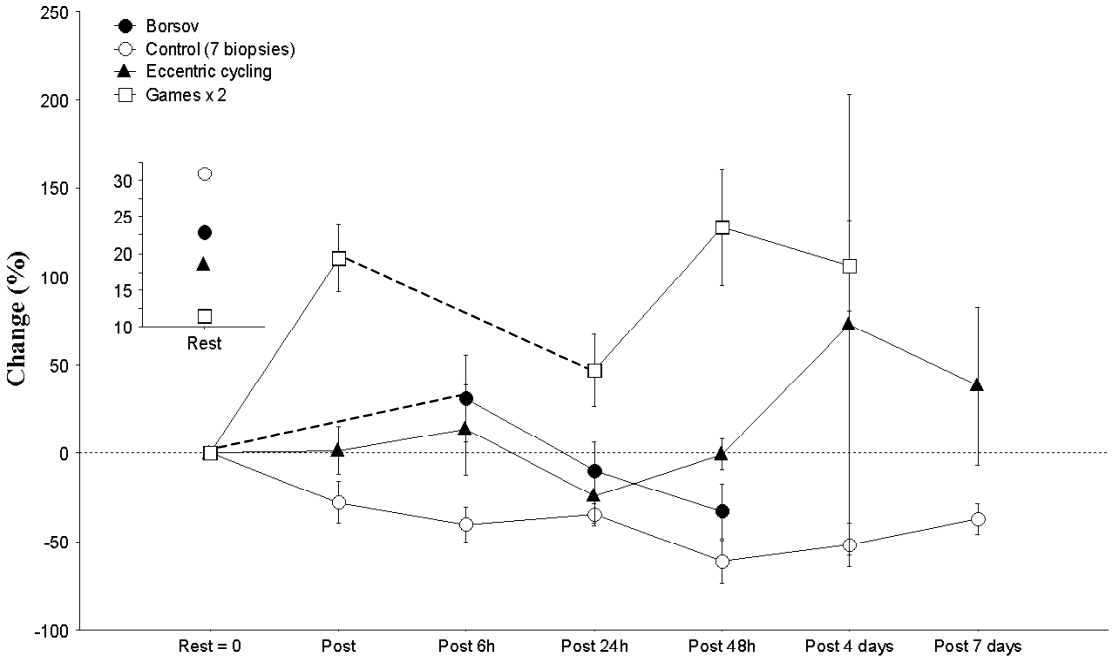



**Figure 11**
Changes in numbers of circulating activated B cell numbers in the blood in response to different exercise protocols. Data from Studies I, II and IV. Resting numbers inserted (cells mL x $10^3$)

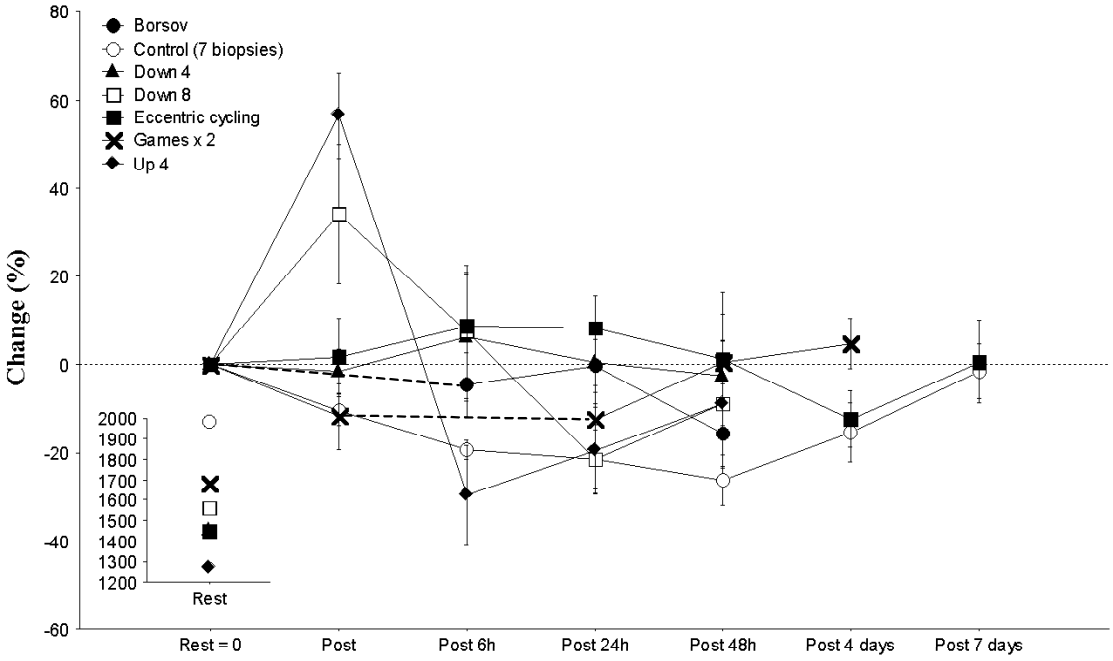



**Figure 12**
Changes in circulating T cell (CD3+ lymphocyte) numbers in the blood in response to different exercise protocols. Data from Studies I-IV. Resting numbers inserted (cells mL x $10^3$)

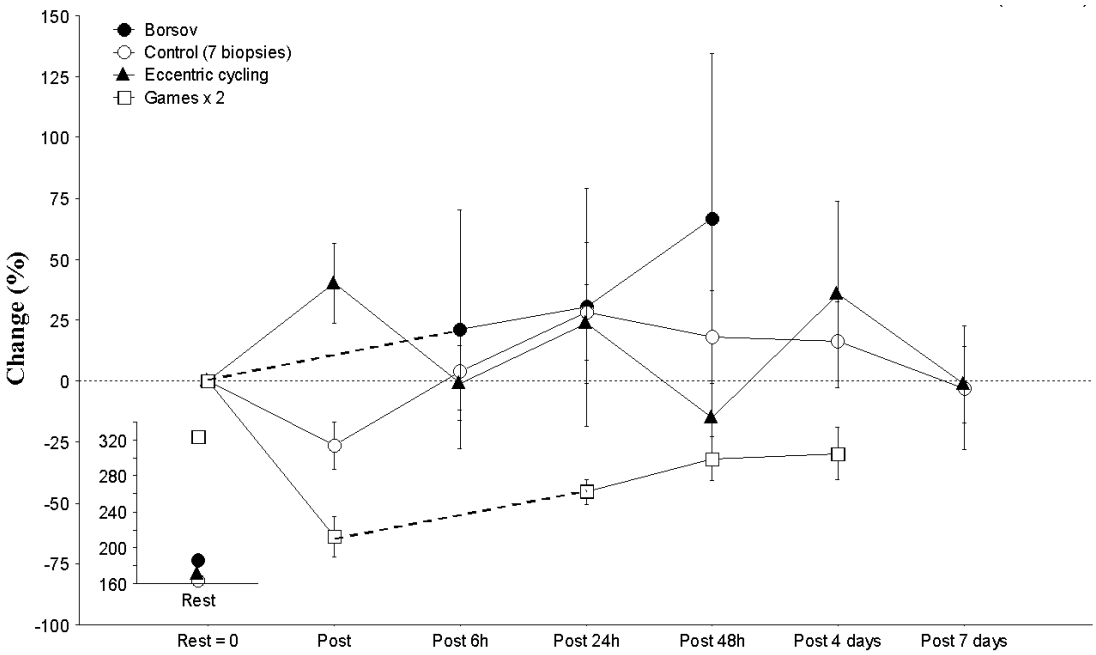



**Figure 13**
Changes in NK cell (CD56+CD16+CD57-CD3-) numbers in the blood in response to different exercise protocols. Data from Studies I, II and IV. Resting numbers inserted (cells mL x $10^3$)

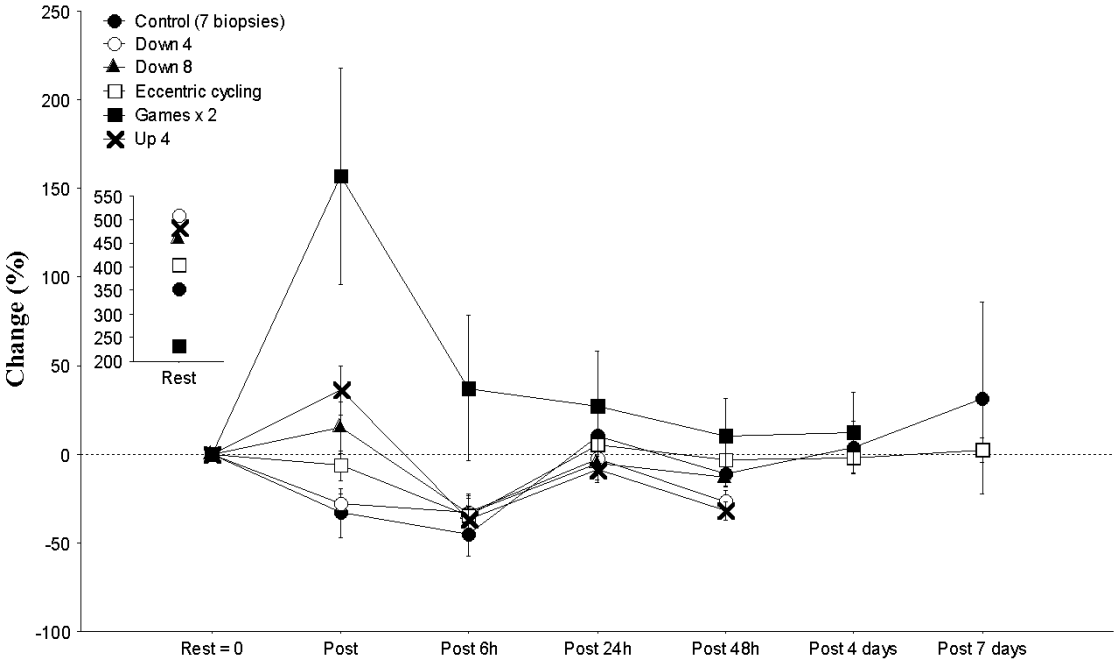



**Figure 14**
Changes in serum cortisol concentration in response to different exercise protocols. Data from Studies II-IV. Resting concentration inserted (nmol $L^{-1}$)

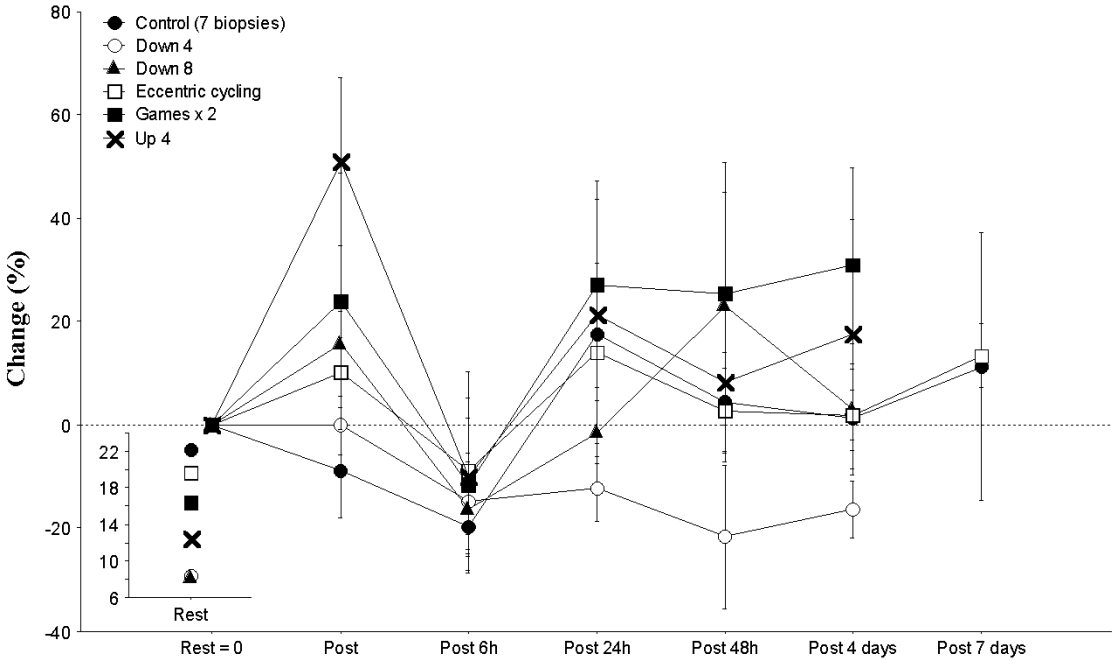



**Figure 15**
Changes in free serum testosterone (not bound to SHBG or albumin) concentration in response to different exercise protocols. Data from Studies II-IV. Resting concentration inserted (nmol $L^{-1}$)

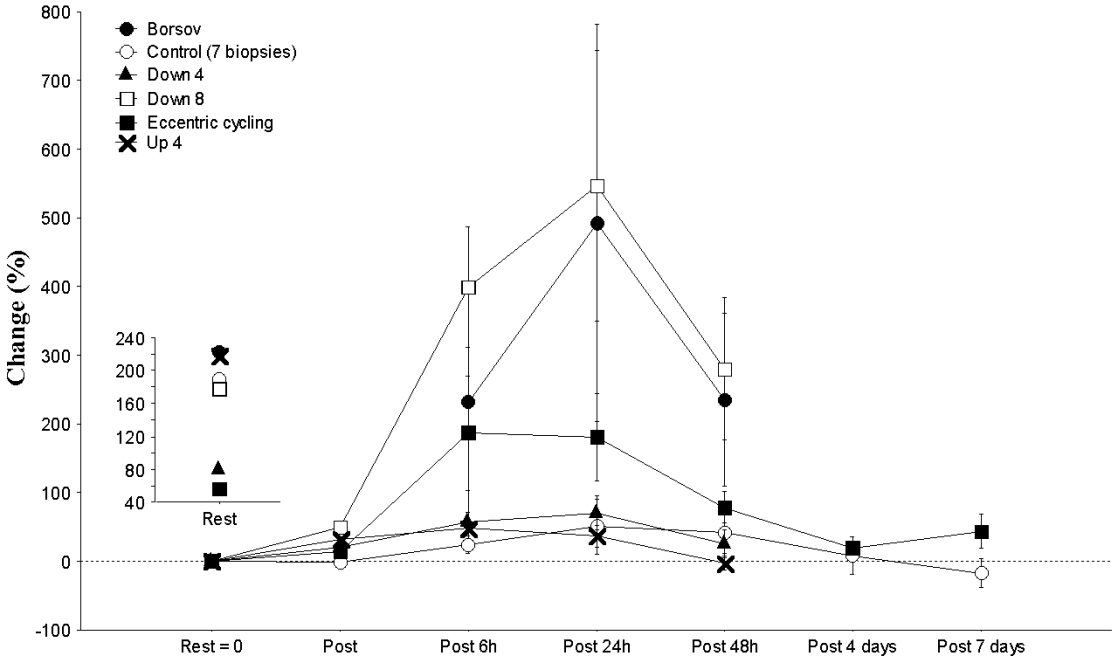



**Figure 16**
Changes in serum CK activity in response to different exercise protocols. Data from Studies I-III. Resting concentration inserted (Units $L^{-1}$)

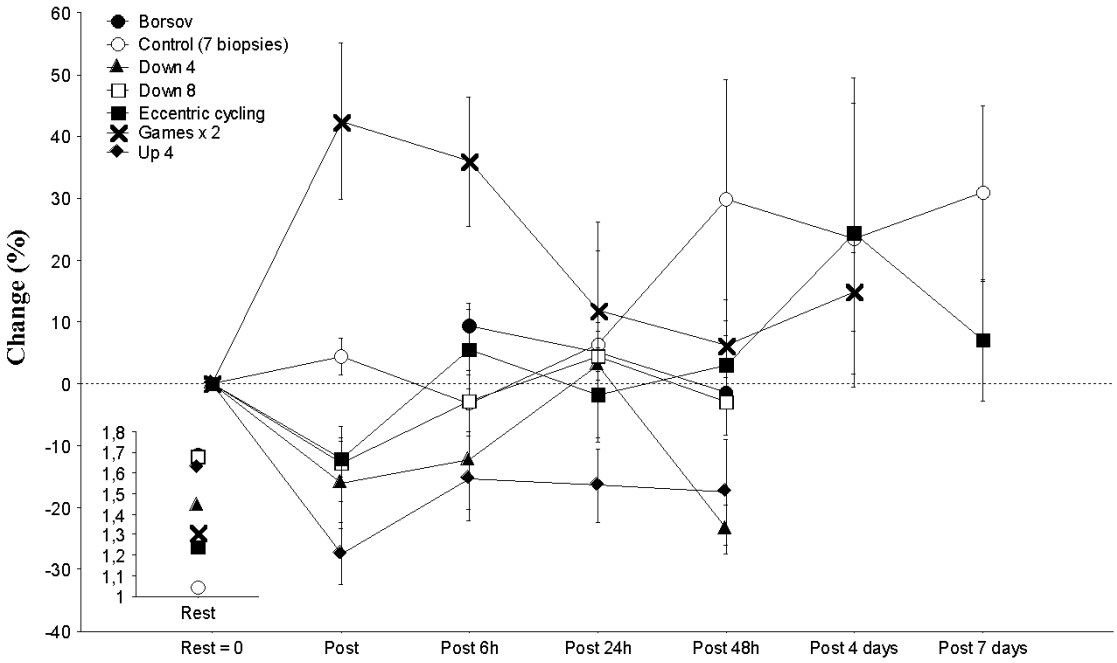



**Figure 17**
Changes in CD4/CD8 ratio in response to different exercise protocols. Data from Studies I-III. Resting ratio inserted

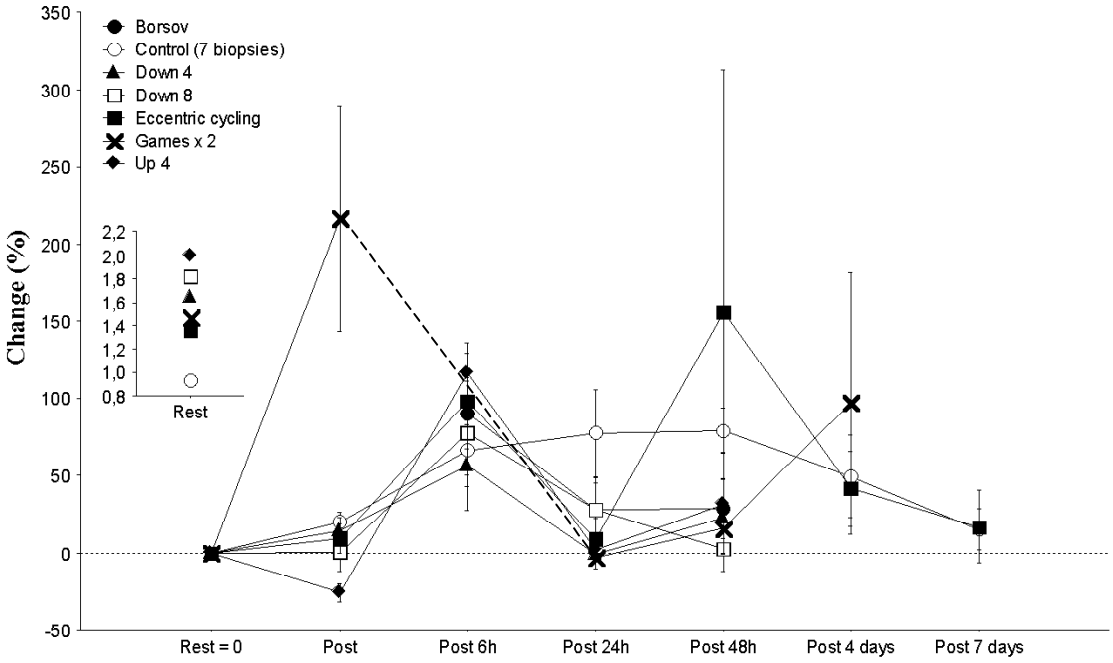



**Figure 18**
Changes in Neutrophil/Lymphocyte ratio in response to different exercise protocols. Data from Studies I-IV. Resting ratio inserted

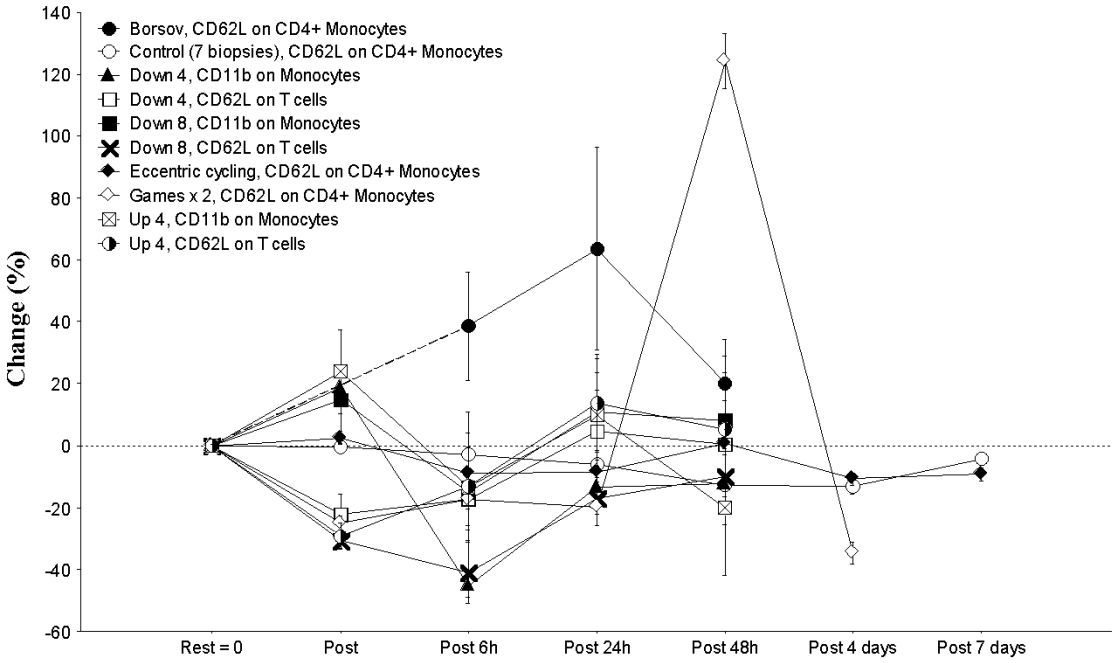



**Figure 19**
Changes in cell surface expression (MFI or MESF) of adhesion molecules in response to different exercise protocols. Note that all increased are CD11b and all decreased are CD62L. Data from Studies I-IV

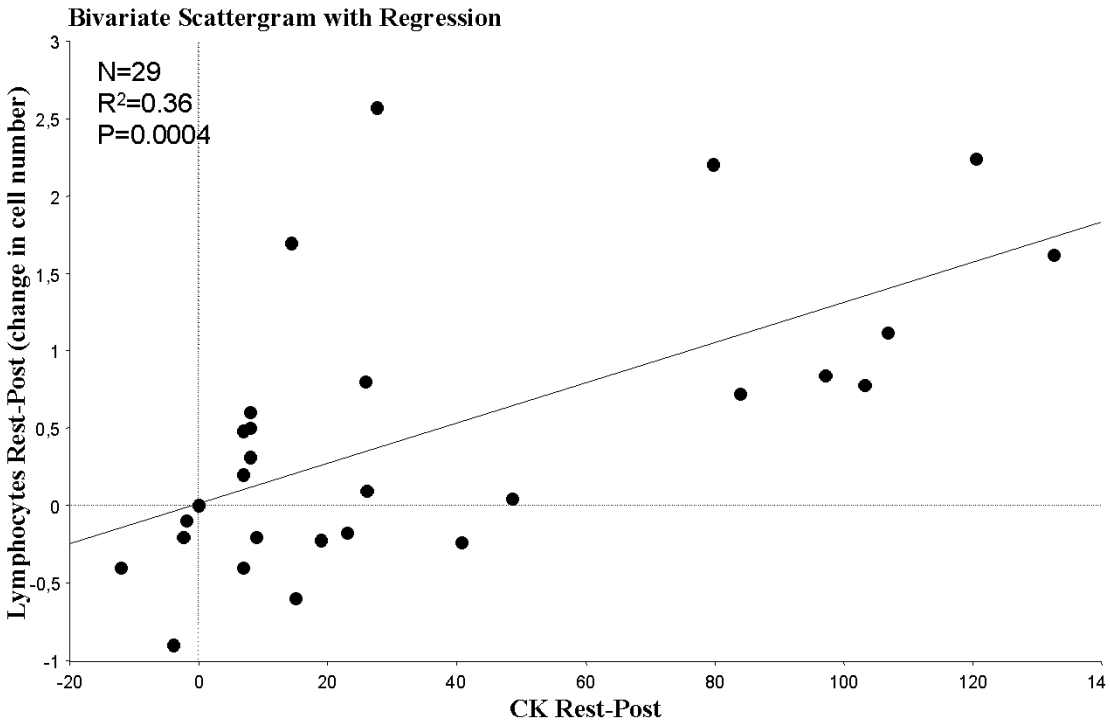



**Figure 20**
Correlation between change in lymphocyte numbers and serum CK activity. Data from Studies I-III

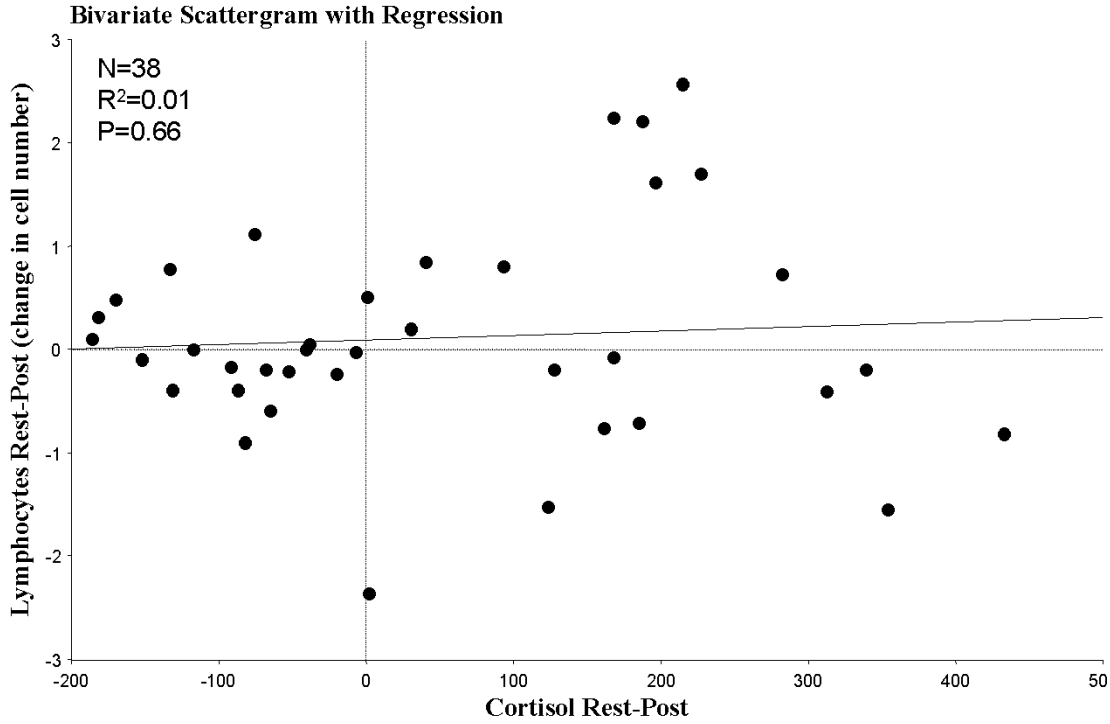



**Figure 21**
Correlation between change in lymphocyte numbers and serum cortisol concentration. Data from Studies II-IV

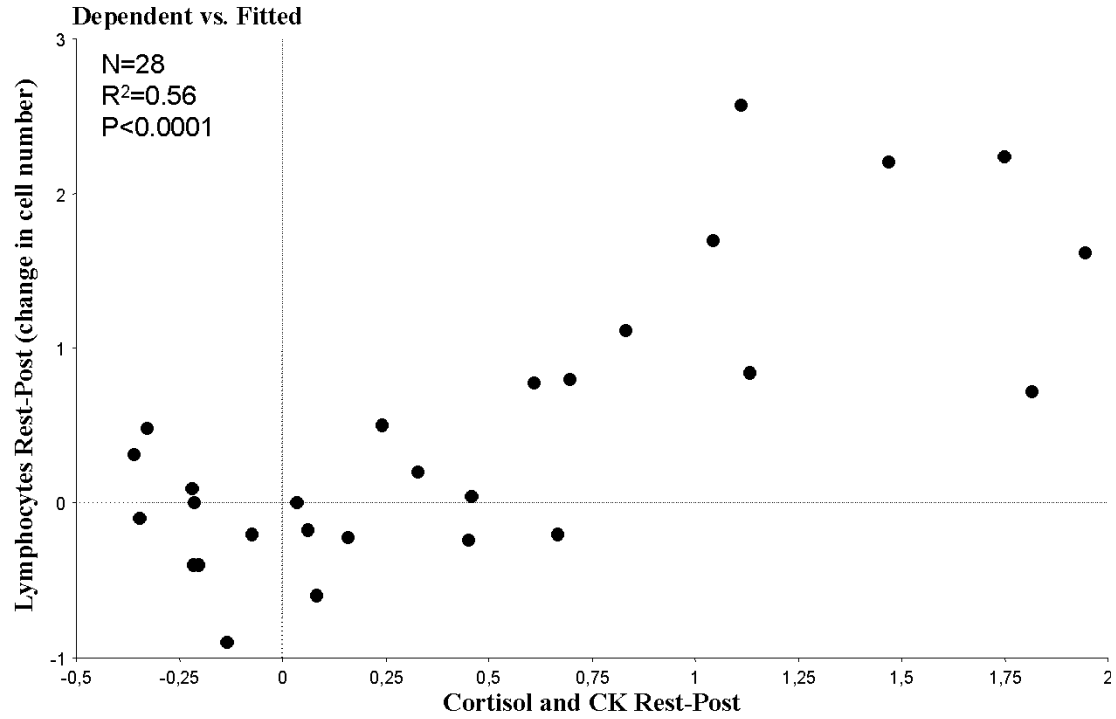



**Figure 22**
Multiple regression between change in lymphocyte numbers and serum CK activity (t = 3) and cortisol (t = 4). Data from Studies II-III

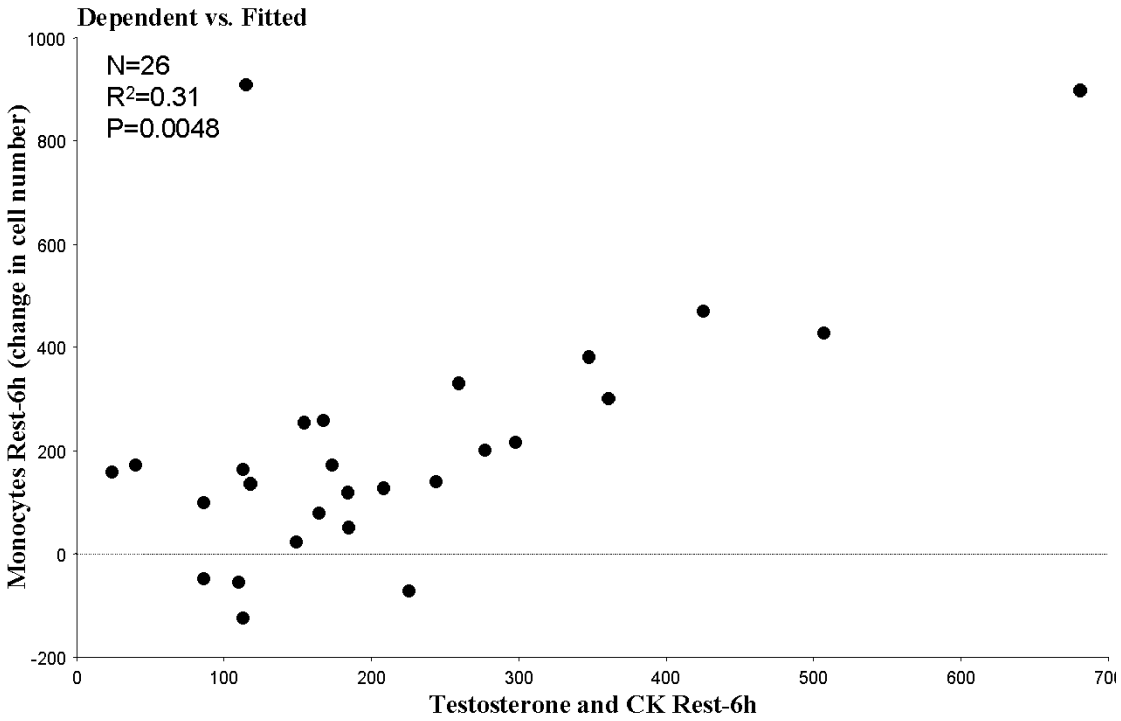



**Figure 23**
Multiple regression between change in monocyte numbers and serum CK activity (t = 2) and serum testosterone concentration (t = 2). Data from Studies II-III

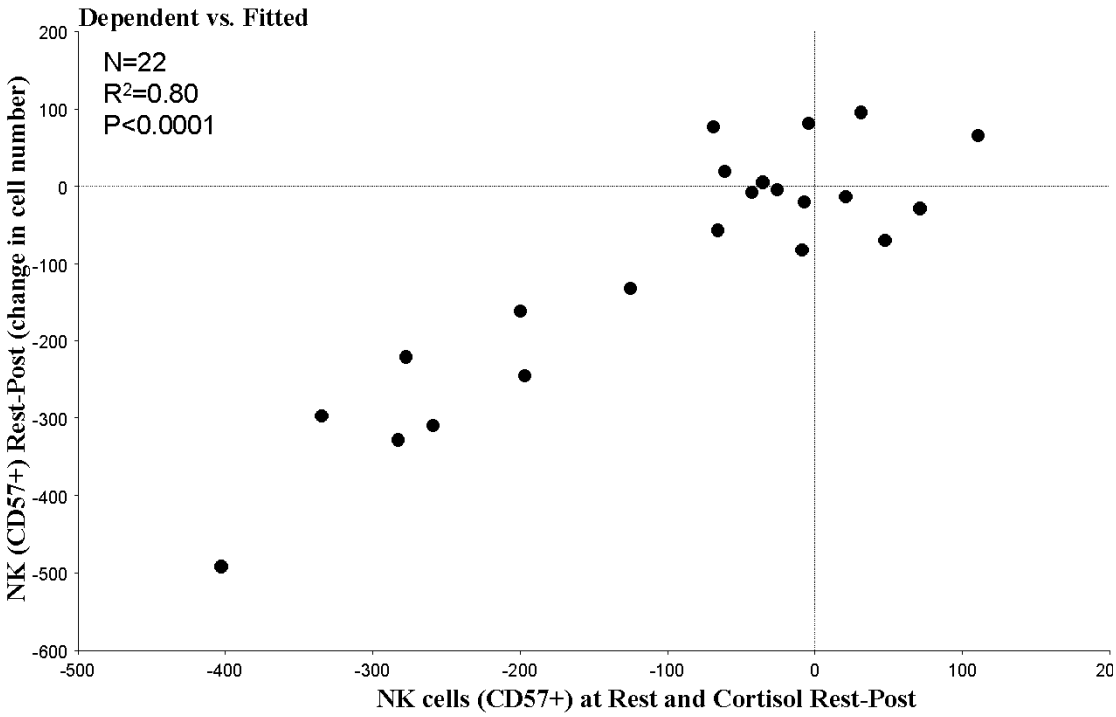



**Figure 24**
Multiple regression between change in NK cell numbers and NK cell numbers at rest (t = -8) and serum cortisol concentration (t = -2). Data from Studies II and IV

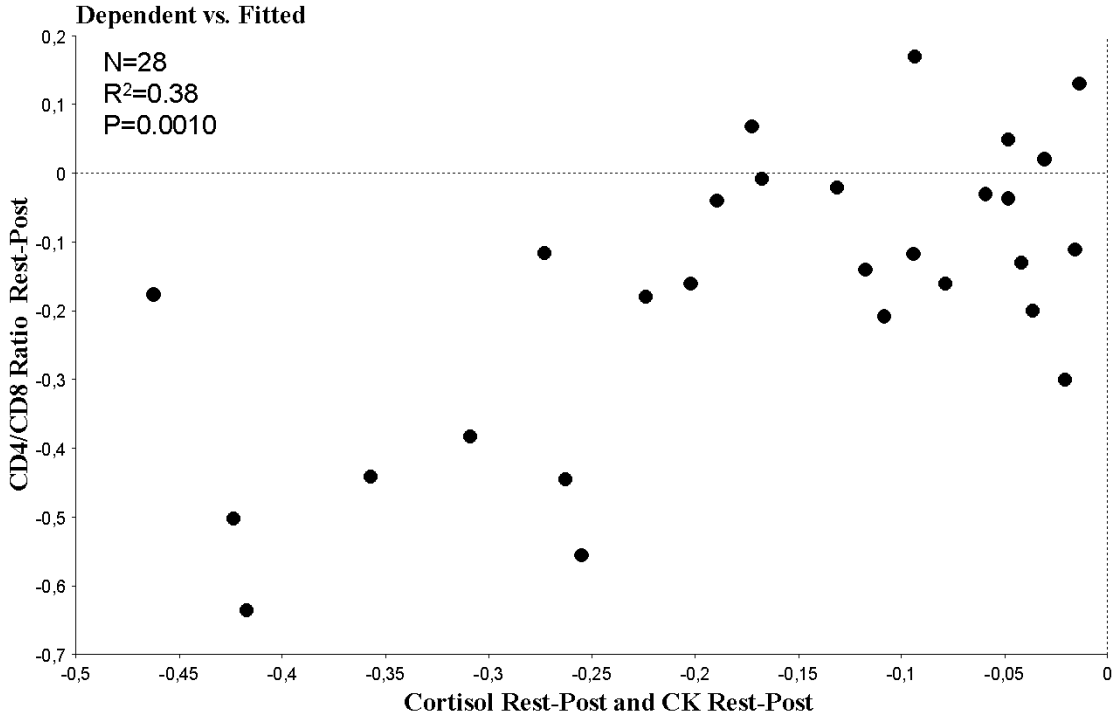



**Figure 25**
Multiple regression between change in CD4/CD8 Ratio and change in serum cortisol concentration (t = -2) and serum CK activity (t = -2). Data from Studies II-IV

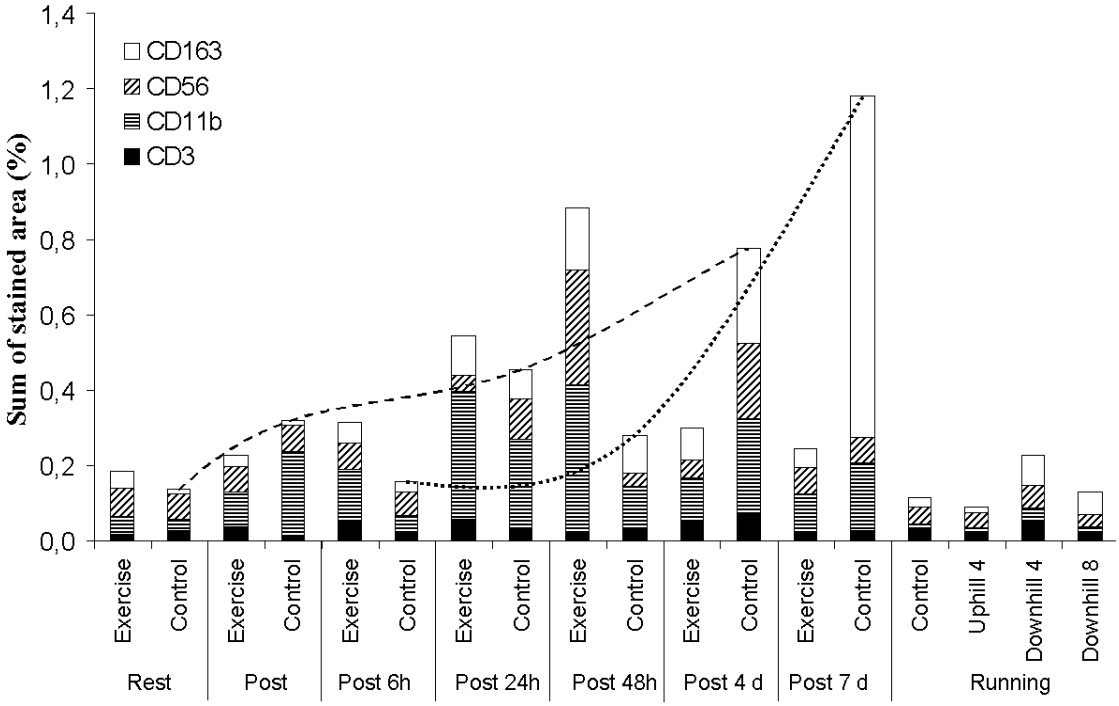



**Figure 26**
The sum of stained area for CD3, CD11b, CD56 and CD163, indicating the total amount of leukocytes and activated satellite cells in skeletal muscle tissue at rest and at various time points after 30 min of eccentric cycling and muscle biopsies (from Rest to Post 7 d) and 48 h after downhill or uphill running.

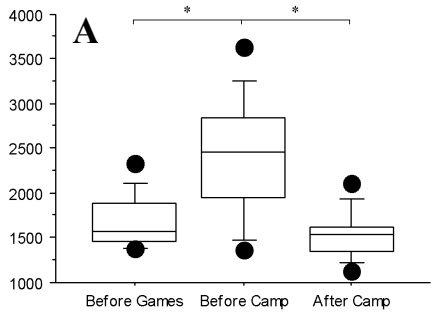
– – – Control subject's left leg (Rest, Post, 24h, 4 d)

····· Control subject's right leg (Post 6h, 48h, 7 d). Data from Studies II and III

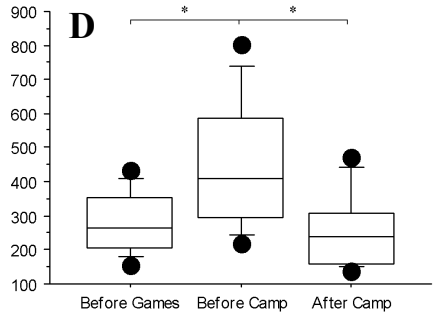
**Table 2.** *Fisher's r to z correlation between changes in variables investigated in blood. Cross section analysis with data from Studies I-IV*

| | N | Correlation | P-Value |
|---|---|---|---|
| **Leukocytes Rest-6h, Lymphocytes Rest-Post** | **29** | **0,63** | **<0,01** |
| **Leukocytes Rest-6h, Neutrophils Rest-6h** | **41** | **0,90** | **<0,01** |
| **Leukocytes Rest-6h, CK Rest-Post** | **29** | **0,52** | **<0,01** |
| Leukocytes Rest-6h, CK Rest-24h | 38 | 0,33 | 0,04 |
| Leukocytes Rest-6h, Cortisol Rest-Post | 28 | 0,41 | 0,03 |
| **Leukocytes Rest-6h, Monocytes Rest-6h** | **41** | **0,44** | **<0,01** |
| Leukocytes Rest-6h, Testosterone Rest-Post | 27 | 0,34 | 0,08 |
| Leukocytes Rest-6h, Testosterone Rest-6h | 27 | 0,23 | 0,26 |
| Leukocytes Rest-6h, NK (CD57+) Rest | 24 | 0,25 | 0,25 |
| **Leukocytes Rest-6h, NK (CD57+) Rest-Post** | **12** | **0,75** | **<0,01** |
| **Lymphocytes Rest-Post, Neutrophils Rest-6h** | **29** | **0,60** | **<0,01** |
| **Lymphocytes Rest-Post, CK Rest-Post** | **29** | **0,62** | **<0,01** |
| Lymphocytes Rest-Post, CK Rest-24h | 29 | 0,39 | 0,03 |
| Lymphocytes Rest-Post, Cortisol Rest-Post | 38 | 0,07 | 0,67 |
| Lymphocytes Rest-Post, Monocytes Rest-6h | 29 | 0,04 | 0,83 |
| Lymphocytes Rest-Post, Testosterone Rest-Post | 37 | 0,15 | 0,38 |
| Lymphocytes Rest-Post, Testosterone Rest-6h | 37 | -0,05 | 0,77 |
| **Lymphocytes Rest-Post, NK (CD57+) Rest** | **22** | **-0,68** | **<0,01** |
| **Lymphocytes Rest-Post, NK (CD57+) Rest-Post** | **22** | **0,78** | **<0,01** |
| **Neutrophils Rest-6h, CK Rest-Post** | **29** | **0,46** | **<0,01** |
| Neutrophils Rest-6h, CK Rest-24h | 38 | 0,30 | 0,07 |
| Neutrophils Rest-6h, Cortisol Rest-Post | 28 | 0,39 | 0,04 |
| Neutrophils Rest-6h, Monocytes Rest-6h | 41 | 0,17 | 0,28 |
| Neutrophils Rest-6h, Testosterone Rest-Post | 27 | 0,27 | 0,17 |
| Neutrophils Rest-6h, Testosterone Rest-6h | 27 | 0,03 | 0,87 |
| Neutrophils Rest-6h, NK (CD57+) Rest | 24 | 0,20 | 0,36 |
| Neutrophils Rest-6h, NK (CD57+) Rest-Post | 12 | 0,55 | 0,06 |
| **CK Rest-Post, CK Rest-24h** | **38** | **0,45** | **<0,01** |
| CK Rest-Post, Cortisol Rest-Post | 28 | 0,44 | 0,02 |
| CK Rest-Post, Monocytes Rest-6h | 38 | -0,01 | 0,96 |
| CK Rest-Post, Testosterone Rest-Post | 27 | 0,08 | 0,69 |
| CK Rest-Post, Testosterone Rest-6h | 27 | 0,11 | 0,59 |
| CK Rest-Post, NK (CD57+) Rest | 21 | -0,06 | 0,86 |
| CK Rest-Post, NK (CD57+) Rest-Post | 12 | 0,40 | 0,20 |
| CK Rest-24h, Cortisol Rest-Post | 38 | 0,34 | 0,07 |
| CK Rest-24h, Monocytes Rest-6h | 28 | 0,19 | 0,25 |
| CK Rest-24h, Testosterone Rest-Post | 37 | 0,08 | 0,70 |
| CK Rest-24h, Testosterone Rest-6h | 37 | 0,16 | 0,44 |
| CK Rest-24h, NK (CD57+) Rest | 22 | 0,17 | 0,47 |
| CK Rest-24h, NK (CD57+) Rest-Post | 22 | 0,52 | 0,08 |
| Cortisol Rest-Post, Monocytes Rest-6h | 41 | 0,16 | 0,43 |
| Cortisol Rest-Post, Testosterone Rest-Post | 27 | 0,35 | 0,03 |
| Cortisol Rest-Post, Testosterone Rest-6h | 27 | 0,06 | 0,71 |
| Cortisol Rest-Post, NK (CD57+) Rest | 24 | 0,33 | 0,14 |
| Cortisol Rest-Post, NK (CD57+) Rest-Post | 12 | -0,49 | 0,02 |
| Monocytes Rest-6h, Testosterone Rest-Post | 37 | 0,39 | 0,04 |
| **Monocytes Rest-6h, Testosterone Rest-6h** | **37** | **0,51** | **<0,01** |
| Monocytes Rest-6h, NK (CD57+) Rest | 22 | 0,05 | 0,81 |
| Monocytes Rest-6h, NK (CD57+) Rest-Post | 22 | 0,50 | 0,10 |
| **Testosterone Rest-Post, Testosterone Rest-6h** | **37** | **0,42** | **<0,01** |
| Testosterone Rest-Post, NK (CD57+) Rest | 22 | -0,10 | 0,66 |
| Testosterone Rest-Post, NK (CD57+) Rest-Post | 22 | 0,10 | 0,65 |
| Testosterone Rest-6h, NK (CD57+) Rest | 34 | -0,01 | 0,96 |
| Testosterone Rest-6h, NK (CD57+) Rest-Post | 22 | -0,08 | 0,72 |
| **NK (CD57+) Rest, NK (CD57+) Rest-Post** | **22** | **-0,88** | **<0,01** |

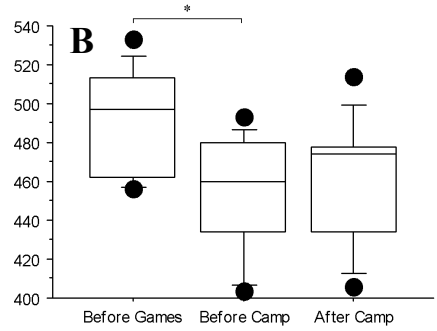
Cross sectional data was not log-transformed because numbers at rest were normally distributed



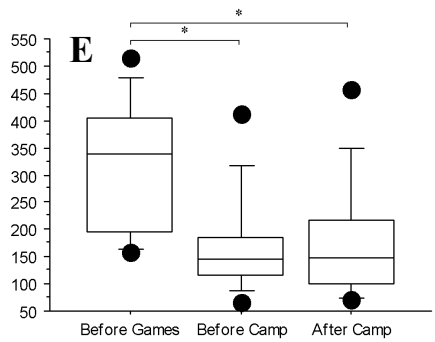

**CD3+ Lymphocytes (T cells)**



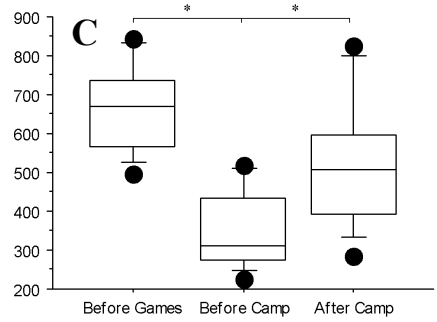

**CD20 Lymphocytes (B cells)**




**CD3 on CD8+ Lymphocytes**




**CD56+CD16+CD3-CD57+ (NK cells)**




**CD11b on Leukocytes**

**Figure 27**
Changes in cell surface antigen expression (MESF) (A-C) and cell numbers (cells $\mu L^{-1}$) (D-E) in blood from soccer players at rest. Data from Study IV

# 5 DISCUSSION

The complexity of the immune system is beyond the knowledge of today.

Changes in immune function determine health, even life and death, because every single cell in an organism is in one way or another in communication with circulating and tissue resident effector cells of the immune system.

Because physical exercise can alter many immunological variables, exercise is a convenient model for exploration of the *in vivo* response by the immune system to a given stressor. Unfortunately, standardization of protocols and elimination of external and internal confounding factors are hard to get by problems when using human subjects.

Consequently, we decided to take the approach that "more is better" when collecting immunological data in an exercise physiology perspective. The rather extensive amount of data rendered from Studies I-IV is discussed in detail in each publication. Therefore, this section will focus on some common and some discreet entities discovered when performing meta-analysis comparing different modes of physical exercise.

The damaged and inflammation inflicted by a muscle biopsy is an overlooked methodological artifact. The consequences of this observation will be addressed.

When extracting the results and conclusions from Studies I-IV, the following were highly ranked by the author:

i. Exercise-induced muscle inflammation, investigated by the detection of CD11b, CD163, IL-6, IL-1β and TNFα antigens could not be observed in human skeletal muscle tissue or epimysium. The leukocyte infiltration and increased detection of IL-1 observed in both eccentrically exercised and control muscle samples in Study II could not be repeated in Study III (where only 1, as compared to 7 in Study II muscle sample was taken from each subject).

ii. The muscle biopsy procedure (forceps technique) induced significant skeletal muscle inflammation in Study II. It appears as if the detected amount of all infiltration markers combined was larger in the exercised group compared to controls (Figure 26). This indicates that the immune function of humans skeletal muscle is not depressed, but enhanced 24 to 48 h after strenuous eccentric physical exercise.

iii. Because of the lack of significant inflammation in healthy humans skeletal muscle after strenuous eccentric and concentric physical exercise, individuals suffering from inflammatory muscle diseases may benefit from physical exercise without the negative effects of increased muscle inflammation.

iv. Delayed onset muscle soreness is not caused by muscle or epimysium inflammation.

v. Increased number of circulating neutrophils is the only investigated variable with a homogenous response independently of exercise mode.

vi. Hormonal changes in serum can only partially explain changes in circulating leukocyte and monocyte subpopulations after exercise.

vii. The numerous correlations between immunological events in blood and skeletal muscle before and after physical exercise and/or multiple biopsies suggests a complex communication system between the two compartments (Figure 26).

viii. The lack of correlation between CK and markers of muscle inflammation in Study II and III, and observed correlation between CK and changes in number of circulating lymphocytes, monocytes as well as CD4/CD8 ratio suggest that either a) CK has a direct effect on these variables and/or b) the correlations are only secondary, i.e. some other molecule(s) with immunoregulatory potential escape(s) the muscle cell together with CK.

ix. Hepathocyte growth factor (HGF) and its receptor c-Met are abundant in Type I skeletal muscle cells (Study III, Figure 3) making HGF one candidate for the described molecule(s) in vii.

Based on these eight paragraphs, and a few additional remarks, some critical topics related to exercise immunology and muscle adaptation can be discussed:

a) Immunological interactions between blood and skeletal muscle (5.1)
b) Exercise-induced leukocyte infiltration and inflammation in skeletal muscle (5.2)
c) The stress-immune paradox (5.3)
d) Importance of local factors in skeletal muscle adaptation to physical exercise (5.4)
e) The clinical, every-day-life and athletic point of view (5.5)

In the following discussion I will emphasize some of the main findings in described in the Result section. I shall attempt to address each issues in a short and to the point manner. The discussion will make no effort to be encyclopedic. Because of the

exponential expansion of medical research, this would be beyond the authors capability, incomprehensive for most readers and outdated within a few years.

## 5.1 EVIDENCE OF COMMUNICATION

Prior to conducting any of my experiments, the literature was thoroughly reviewed. Previous findings indicated that a link between skeletal muscle and leukocytes in the blood existed. For example, glutamine is released to the blood primarily from skeletal muscle (Parry-Billings *et al.* 1990), is an important substrate for leukocytes (Griffiths & Keast 1990), decreases in blood after strenuous physical exercise (Newsholme 1994; Keast *et al.* 1995; Walsh *et al.* 1998) and can consequently affect immune function (Shephard & Shek 1995; Pedersen & Toft 2000). Other denominators, such as changed blood glucose and muscle glycogen concentration can influence substrate availability for blood and tissue leukocytes and consequently affect their function. Recently is was demonstrated that neutrophil adhesion can be altered by proton pump inhibition (Yoshida *et al.* 2000). Changed proton concentration in a tissue (blood, endothelium, muscle) with exercise could therefore influence cell surface receptor affinity. Some studies had observed changed adhesion capacity on circulating leukocytes (Warlow & Ogston 1974; Baum *et al.* 1994; Gabriel *et al.* 1994). Because leukocyte adhesion is closely linked to intra- and inter-cellular signaling (Rosales *et al.* 1995; Stamenkovic 1995; Celi *et al.* 1997; Giancotti & Ruoslahti 1999) the literature provides a theoretical link between skeletal muscle metabolism and leukocytes adhesion.

Correlation between immunological events in human skeletal muscle and blood was subsequently demonstrated both in Studies II and III. In a recent review (Malm 2001), a hypothesis based primarily on the findings in Study II (Malm *et al.* 2000) was presented (Figure 28) based on observed correlations (always $R^2>0.8$, $P<0.01$). These findings fitted rather nicely with previous observations that physical exercise can cause muscle damage and leukocyte infiltration (Hikida *et al.* 1983; Round *et al.* 1987; Fielding *et al.* 1993).

There was, however, a discrepancy in the puzzle: the biopsy procedure appeared to have caused disturbances in the measurements. Thus, the cause and effect of these relationships could not be defined. Results from Study III indicate that the proposed model (Figure 28) is not valid for exercised induced changes in skeletal muscle, but summarizes the immunological damage-repair response to muscle biopsies.

Changes in cell surface expression of adhesion molecule on leukocytes and a redistribution of the number of leukocytes expressing adhesion molecules could be noted both after Borsov-steps and eccentric cycling, but not in the control group subjected also

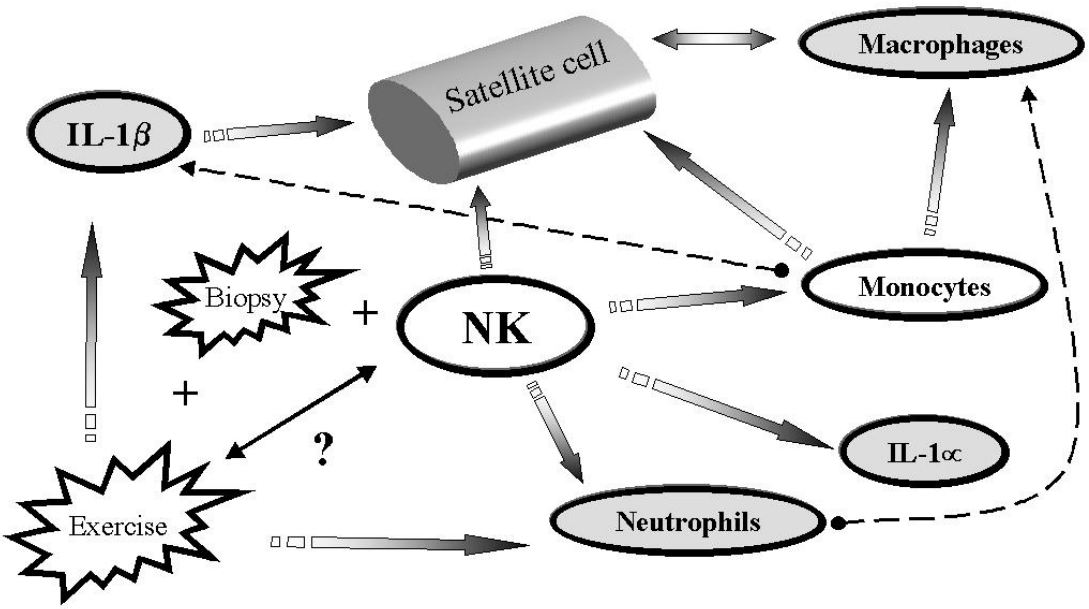



**Figure 28**
Interactions between immunological variables analyzed in blood and skeletal muscle after eccentric exercise and 7 biopsies, or 7 biopsies without prior exercise (Malm 2001). Based on results from Study III, it is concluded that this illustration represents immunological events triggered by the surgical trauma of the muscle biopsy procedure. Shaded figures represent muscle tissue and open figures represent blood.

to 7 consecutive muscle biopsies. Changed leukocyte adhesion capacity was a consistent finding in all studies (Figure 19). With few exceptions, CD11b increases and CD62L decreases acutely after physical exercise, independently of exercise mode (Pizza *et al.* 1996; Malm *et al.* 1999; Malm *et al.* 2000; Shephard *et al.* 2000). One interpretation is that cells with high capacity for firm adhesion (CD11b) is released into the blood and that cells with high capacity for endothelial rolling (CD62L) adhere to the endothelium. This could trigger signaling by adhesion but without migration.

Creatine kinase (CK) is a commonly used marker in blood to assess tissue damage, particularly skeletal muscle damage after exercise, but its value as such has been strongly questioned (Warren *et al.* 1999; Malm 2001). In Figures 20-22, the correlation between changed CK and lymphocyte number in Studies I-III is plotted. In Table 2 additional correlation between CK and leukocytes are calculated. The correlations were rather weak (i.e. Figure 20: $R^2 = 0.36$, $P<0.01$), but strengthened when the change in cortisol was added to the regression model (Figure 22: $R^2 = 0.56$, $P<0.01$). A similar correlation was observed to the change in monocyte number (Figure 23) where CK and testosterone changes could account for an estimated 30% of the changes in monocyte number (notice the outlier at x = 100, y = 900. Taking out this subject gives $R^2 = 0.65$, $P<0.0001$). There is little evidence that CK would be released from some other tissue than skeletal muscle after physical exercise, and increased serum CK must therefore be an indication that the

plasma membrane of the muscle cells has been permeabilized, and that the release is larger that the break-down/uptake by the liver. The observed correlation between CK, hormones and circulating leukocytes, together with the known effect of testosterone and cortisol on cell membrane function (Lawrence *et al.* 1984) and observed difference in serum CK changes and leukocyte redistribution between men and women in response to the same relative workrate (MacIntyre *et al.* 2000; Stupka *et al.* 2000) support the idea that CK, like IL-6 may *not be a sign of damage but of communication*. If CK escapes the muscle cells, so must other molecules.

I would like to propose that the measured changes in serum CK activity after physical exercise either has a direct effect on circulating leukocytes, or that some other molecules with immunoregulatory potential escape the muscle cell together with CK. The serum concentration of this/these molecule(s) should change dynamically with serum CK activity, at least for the hours immediately after secession of exercise.

Ostrowski *et al*. (1998) have shown that IL-6 is released from muscle (measured by A-V difference). In Studies II and III we detected IL-6 both in muscle cells and in epimysium. Pedersen *et al*. (2001) suggested that IL-6 from the muscle transmits signals regarding the metabolic status of the muscle. Other cytokines and growth factors such as HGF, LIF and IGF-1 seem to be much more abundant in human skeletal muscle than IL-6 (Studies II and III) and have the potential to be released from the muscle tissue, pending the correct signal is given. Particularly, large amounts (stained HGF area in muscle is in the order of 1000 times IL-6, while the stained area is equal in epimysium) of HGF are present in type I muscle fibers (Study III). HGF is also the only known factor that can activate quiescent satellite cells (Allen *et al.* 1995; Tatsumi *et al.* 1998; Sheehan *et al.* 2000). CK has a molecular weight of approximately 50 kD and the active form of HGF has a 69 kD α-subunit and a 34 kD β-subunit (Nakamura *et al.* 1993) (both subunits are necessary for c-Met signaling). Though somewhat larger, it is not impossible that HGF, or some other factor, may escape the muscle cell simultaneously as CK. In fact, Jackson *et al*. (1991) concluded that protein leakage from rat skeletal muscle after trauma was not size dependent. The author is not aware of any study where HGF in blood has been measured in response to physical exercise in man or mouse. The released molecule (CK or some other factor) can then exert autocrine, exocrine or endocrine functions in muscle tissue and/or on circulating leukocytes. No strong correlations were found between leukocytes and IL-4, IL-5, IL-6, IL-10, TNFα or INFγ in Study IV. The author is not aware of any other attempts directly correlate these changes to physical exercise. Some

growth factors such as VEGF that is present in human skeletal muscle (Gustafsson *et al.* 1999) are also released to the blood after physical exercise (Schobersberger *et al.* 2000). It may, however, not be possible to correctly describe events in skeletal muscle (or any other organ) by measuring blood variables (Warren *et al.* 1999), and immunological synergism can not easily be predicted (Felten *et al.* 1993).

An immunological-skeletal muscle "internet" as in Figure 29 can be imagined, but the details are yet to be worked out. Like in the electronic version, all events occurring at any one time on "the net" can probably not be measured simultaneously.

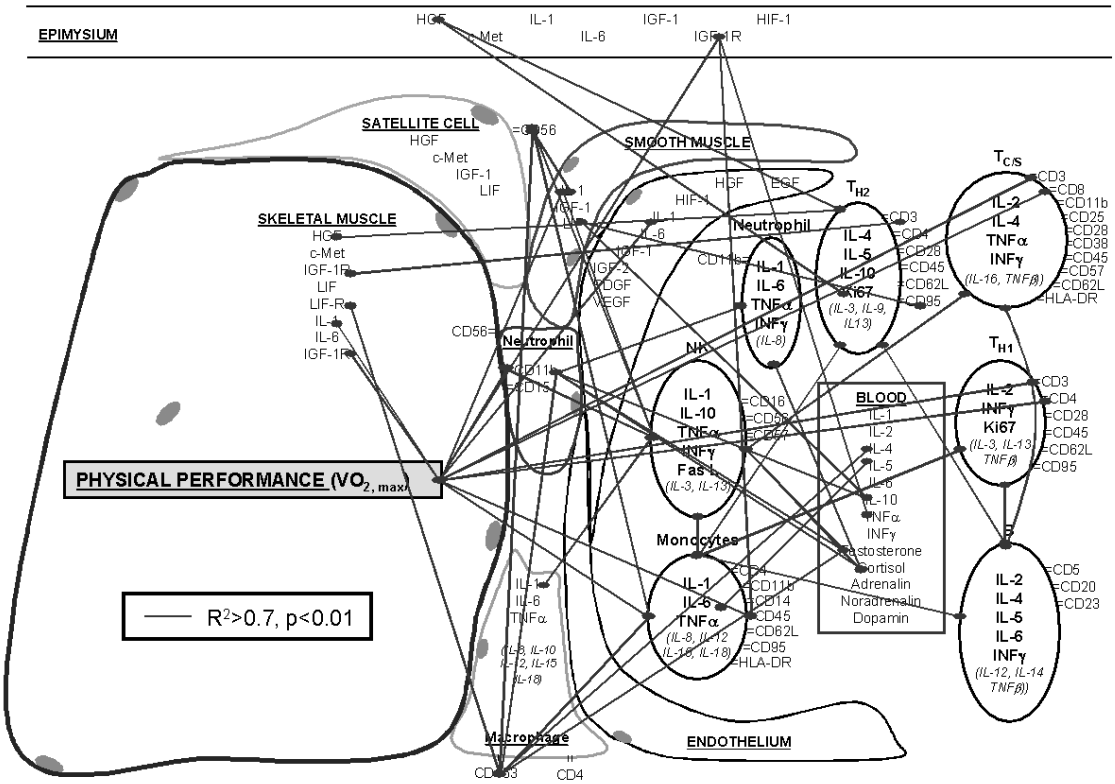



**Figure 29**
The immunological internet. A limited view of the complex response by the immune system to physical exercise (compare to Figure 1). All immunological interactions occurring during and after a bout of physical exercise are without doubt many times more. (Correlations with $R^2$>0.7 and P<0.05 in Studies I-IV are shown as solid dot-end lines)

## 5.2 WHERE DO ALL THE CELLS GO?

For optimal immune function, the location of leukocytes is of importance. At rest, only about 1 % of all leukocytes can be found in circulating blood, the rest residing in the lymph system, spleen, liver, skin, gut and other organs (Abbas 2000). When a potential pathogen is encountered, a rapid migration of leukocytes to the site of invasion occurs and

the intruder is attacked. Thus, any physiological event changing leukocyte trafficking can potentially also the change immunological capacity to battle an infection or repair damage.

A redistribution of leukocytes during and after physical exercise among tissues in the human body is manifested by changes in circulating cell numbers and proportions (Nieman *et al.* 1989; Tvede *et al.* 1989; Ferry *et al.* 1990; Benschop *et al.* 1996; Shinkai *et al.* 1996; Pedersen *et al.* 1997; Suzuki *et al.* 1999). In general, leukocytes increase in numbers during and immediately after exercise and decrease to normal, or below normal, at some times (a few hours) after exercise has ended. It is believed that they enter circulation from the marginated pool, mainly consisting of leukocyte adherent to the walls of blood vessels. During exercise catecholamines and shear force release the cells into circulation (Pedersen 1997; Mackinnon 1999). Most of the neutrophils entering circulation in response to physical exercise come from the lung (Peters *et al.* 1992). Others have suggested that after physical exercise some of the leukocytes migrate from circulation to the exercised skeletal muscle (Hikida *et al.* 1983; Round *et al.* 1987; MacIntyre *et al.* 1996; Pedersen & Hoffman-Goetz 2000). However, the latter suggestion was not supported by a study in rats, where leukocyte numbers decreased in the lung, liver and kidney 1 h after exercise to exhaustion but did not increase in skeletal muscle tissue (Espersen *et al.* 1995).

Thus, the question remains; where *do* all the cells go?

In agreement with the literature, a general leukocytosis mainly due to an increase in neutrophil numbers, was also observed in Studies I-IV, and summarized in Figure 6 and 8. As stated by several previous investigators, the immune response to physical exercise is depending on mode, duration, intensity, training status, feeding state and several other factors (Nieman *et al.* 1994; Gabriel & Kindermann 1997; Nieman *et al.* 1997; Pedersen 1997; Bishop *et al.* 1999; Mackinnon 1999). Some of them are difficult to control and account for in a laboratory setting.

From Figures 6-19 it is obvious that different exercise protocols induced different immune responses. The intensity-dependent response described by others (Gabriel & Kindermann 1997) is probably a simplification. For example, the largest change (+100%) in neutrophil number in blood was measured 6 h after 30 min eccentric cycling at 30-40% of $VO_{2,max}$ and 45 min of 4° uphill running at 55-65% of $VO_{2,max}$. After 45 min of 8° downhill running at 75-85% of $VO_{2,max}$ neutrophil number increased only by 80% (Figure 8). Even though the intensity-dependent response may be valid when the mode of exercise

is the same, it is clearly not so when comparing uphill running, downhill running and eccentric cycling. The importance of factors such as the eccentric and concentric component of the exercise mentioned above, different hormonal variations (Figure 14-16) and initial immune function are of such importance that relating changes to relative exercise intensity does not explain observed variations in leukocyte numbers during and after exercise. As can be seen in Table 2, when combining data from Studies I, II and IV, the strongest determining factor for changed number of NK cells from before to after exercise was resting NK cell numbers (catecholamines not included). The more cells available, the larger response. Perhaps the initial approach for data collection "more is better", also is applied in some cases by the immune system.

Skeletal muscle is probably not the organ to which leukocytes migrate after physical exercise. The small amount of leukocytes resident in skeletal muscle cannot account for the exercise-induced leukocytosis. Based on the number of leukocytes in the vastus lateralis muscle, assuming equal distribution in all muscle tissue, it was calculated that the number of leukocytes in the human musculature is about 10% of the number in blood.

Based on the results from Studies II and III, and influenced by observations made by Dhabhar *et al*. (2000), I suggest that the skin could be one site of leukocyte re-localization after physical exercise. The motivation for this suggestion is that in a fight or flight situation, an immunological shield is needed in case of injury (Nagatomi *et al.* 2000).

Many attempts have been made to explain the cause of delayed onset muscle soreness (DOMS) (Armstrong 1984; Smith *et al.* 1989; Appell *et al.* 1992; Crenshaw *et al.* 1994; MacIntyre *et al.* 1995). Suggestions have been made that the phenomenon of DOMS is caused by muscle inflammation (Hikida *et al.* 1983; Smith 1991), or the swelling of the muscle fibres ( Fridén *et al.* 1988; Smith 1991; Nosaka & Clarkson 1996) that may or may not be caused by muscle inflammation. According to the findings in Studies II and III, exercise induced muscle or epimysium inflammation cannot be the cause of DOMS. Muscle pain could also be inflicted by the release of substances from the muscle cells (Stebbins *et al.* 1990) or from non-muscle cells such as endothelial cells, tissue mast cells or macrophages. A number of such substances (e.g. bradykinin, substance P, PGE2) are known to cause pain (Dray & Perkins 1993; Babenko *et al.* 1999) and nociceptors have been found in the interstitial space in human skeletal muscle (Marchettini *et al.* 1996). At the writing moment, the author is not aware of any published study which has investigated any of these substances in muscle tissue after DOMS-

inflicting exercise in human subjects. A second possible explanation of DOMS could be muscle swelling caused by other factors than inflammation, such as increased protein metabolism in the muscle cells and subsequent increase in osmotic pressure. Because DOMS decreases the likeliness that the affected person will undertake any strenuous physical exercise, DOMS could serve a physiological purpose in allowing adequate time for muscle recovery and adaptation.

## 5.3 THE STRESS-IMMUNE PARADOX

Decreased immune function, increased risk for infections and skeletal muscle inflammation are three possible outcomes of strenuous physical exercise according to the current literature (Armstrong 1984; Fielding *et al.* 1993; Brenner *et al.* 1994; Nieman 1994; Pyne 1994; Pedersen & Bruunsgaard 1995; Gleeson 2000). Psychological stress is known to increase the risk for viral infections in humans (Cohen *et al.* 1991) but Ilbäck *et al*. (Ilback *et al.* 1991) concluded, from studies in rats, that "Strenuous exercise immediately before infection drastically reduced susceptibility to either of the bacteria (Streptococcus pneumoniae and Francisella tularensis)". In a recent review by Friman & Wesslen (2000) takes the standpoint that acute exercise will enhance resistance to infections. They also conclude that the adaptability to exercise may decrease during some infections but not other. In a study in our laboratory of marathon runners, an increased risk of infection was observed only in the group of runners who reported an infection during the 3 weeks prior to the race. Runners with no infection prior to the race had no increased risk after the race (Ekblom *et al*. submitted).

This could be referred to as a "stress-immune paradox" (Dhabhar & McEwen 1997). In a time of acute physical and/or psychological stress (chased by a lion way back then, or by a shark even longer ago on the scale of evolution) the immune system should be enhanced, not depressed, to protect the host. Stress is also considered detrimental for people with inflammatory diseases, but if the immune system was depressed by stress, so should the inflammatory response (Dhabhar & McEwen 1997).

In a series of experiment, Dhabhar and colleagues (Dhabhar & McEwen 1996; Dhabhar & McEwen 1997; Dhabhar 1998; Dhabhar 2000) have shown that in the rat (to the knowledge of the author, no human data is available), at least one immune function is consistently enhanced in response both to physical and psychological stress of acute character, namely the delayed type hypersensitivity (DTH). After a period of chronic stress DTH was depressed, and this finding was explained by the attenuated cortisol and lymphocyte increase in response to acute stress after a period of chronic stress. DTH is a

T cell dependent activation of macrophages and inflammation in response to a previously encountered antigen (Kuby 1994). Because DTH involves both adaptive ($T_H$ cells) and innate (macrophages and inflammation) immunity, some function(s) in either or both systems must be enhanced in response to acute stress.

Bringing this paradox into the field of exercise physiology, one can ponder upon the following: 1) Stress is often considered negative, and the term should perhaps not be used when referring to physical exercise (unless you really do not want to exercise), 2) Circumstantial evidence has led to the conclusion that voluntary physical exercise induces skeletal muscle inflammation in healthy humans.

If some leukocytes migrate to the skeletal muscle after physical exercise, the migration is not of significant magnitude and no where near the amount observed in, for example, myositis. Whether or not physical exercise can exacerbate some inflammatory events in diseased human skeletal muscle is not extensively investigated. One study suggests that this is not the case (Alexanderson *et al.* 2000). In studies by Ekblom *et al*. (1975) and Nordemar *et al*. (1976a; 1976b; 1981) physical exercise was well tolerated by individuals suffering from rheumatoid arthritis. Inflammation is an immunological event, and if the immune system is depressed after strenuous physical exercise, then the inflammatory capacity should also be depressed. Because no signs of exercise-induced skeletal muscle inflammation was found in Studies I-IV, at a first glance the notion of depressed immune function appears to be supported. However, inflammation is also the reaction to tissue damage or infection. The latter is probably not relevant in the context of these studies, and the former has not been convincingly demonstrated in human skeletal muscle after voluntary physical exercise (unless, of course the muscle is ruptured by a trauma).

It could also be suggested that the immune system is depressed in such way that an inflammation, initiated by exercise-induced muscle damage, is prevented by the very same exercise. Judging from Figure 26 this is not the case. In exercised, as well as in non-exercised human skeletal muscle, inflammation was induced by the needle biopsy inflicted damage (Malm *et al.* 2000). *The inflammatory response to injury is consequently not depressed in human skeletal muscle after strenuous physical exercise.*

On the contrary, a sign of enhanced immune function immediately after physical exercise is the increased number of neutrophils in circulation. This increase appears to be independent of exercise mode and duration (Figure 8). In fact, neutrophils were also increased in response to the muscle biopsies (an intruder or injury) in the control group in

Study II. Nagatomi *et al*. (2000) suggested that the observed acute changes in circulating blood in response to strenuous physical exercise is a "preparatory phase for the efficient defense at the surface of the body". Increased number of neutrophils in circulation will do exactly that.

Up until now, the literature has been in agreement that strenuous chronic physical exercise and/or psychological stress can depress immune function. If the physical exercise or psychological stress is above the adaptive capacity of the organism, over a longer period of time (weeks to months) the effect will be deleterious (Cohen *et al.* 1991; Fry *et al.* 1991; Pedersen *et al.* 1996; Dhabhar & McEwen 1997; Mackinnon 2000; Nieman 2000). However, the mechanisms responsible for decreased immune function has not yet been identified. Furthermore, all functions of the immune system are not decreased during periods of increased exercise intensity and volume. For example, in figure 27 C the adhesion capacity (CD11b MESF) of leukocytes in circulation increased significantly after 5 days of intensified soccer training. In a study by Gabriel et al. (1998) the number of activated T cells (CD3+HLA/DR+) in circulation was increased, as was the cell surface expression of CD45RO on T cells.

It would be an evolutionary paradox that the immune system should abandon us when we need it the most.

Darwin once stated "survival of the fittest", not "survival of the depressed".

## 5.4 LOCALS OF IMPORTANCE

So far in the discussion it has been argued that skeletal muscle adaptation to physical exercise is not a does not invlove muscle cell damage, inflammation and infiltration of circulating leukocyte. For everyone who has performed physical exercise it is, however, apparent that events perceived by our central nervous system take place in the muscle tissue after physical exercise. If not inflammation, then what is it?

Substantial evidence from research performed during the past 10 years demonstrates the importance of local factors within the skeletal muscle tissue for development, regeneration and possibly also adaptation to changes in physical demand.

Based on findings in the literature 9 factors were selected for investigation (all data not yet published). Results from Study III visualize the physical localization of at least three systems of growth factors (HGF, IGF-1 and LIF) and their respective receptors (c-Met, IGF-R, LIF-R) within the skeletal muscle, smooth muscle and endothelial cells. The findings are summarized in Figure 30.

The functionality of these systems has not been investigated in this thesis, but with results primarily from *in vitro* and animal studies the findings may be discussed. The functions of these growth factors are also discussed in section 1.8.

Leukocytes, growth factors and cytokines present in muscle tissue are necessary both for normal muscle function and tissue repair following injury (Hohlfeld & Engel 1994; Mitchell *et al.* 1995; Tidball 1995; Chambers & McDermott 1996; Husmann *et al.* 1996; Lescaudron *et al.* 1999). The role of leukocytes in muscle tissue has been discussed elsewhere (sections 1, 5.1 and 5.2) and the following discussion will focus on the schematic presentation in Figure 30.

HGF/c-Met systems have been discovered in several tissues, including liver, endothelium and skeletal muscle (Birchmeier & Gherardi 1998) and are involved in muscle development, growth, morphogenesis and tumour protection (Birchmeier & Gherardi 1998). Both HGF and c-Met were detected in slow twitch (Type 1) muscle fibers and changed in response to physical exercise. Recently it has been shown that HGF is released from stretched satellite cells in culture and that the released HGF can activate adjacent satellite cells (Tatsumi *et al.* 2001). In light of the discussion of serum CK release (see 5.1) there is a possibility that HGF can be released from permeabilized Type 1 muscle fibers. If this is the case, and HGF is the only factor capable of activating satellite cells, events related to Type 1 muscle fibers may determine satellite cell activation.

IGF-1 is another molecule, which can induce skeletal muscle hypertrophy, both via a systemic, growth hormone dependent pathway and via local autocrine/paracrine action (Adams 1998). IGF-1 can also protect cardiomyocytes and fibroblasts from apoptosis (Mockridge *et al.* 2000). Result from Study III support previous findings of a localized IGF-1 system involved in muscle adaptation. A possible connection between endothelium and muscle cells was also demonstrated by the finding that IGF-1 was detected in the walls of blood vessels, and IGF-1R located within the muscle cells and close to blood vessels.

An isoform of IGF-1 has been isolated from skeletal muscle. Because it was observed to increase with muscle contractions, the discoverer named it mechano growth factor (MGF) and this molecule could be a link between muscle contraction and gene expression (McKoy *et al.* 1999).

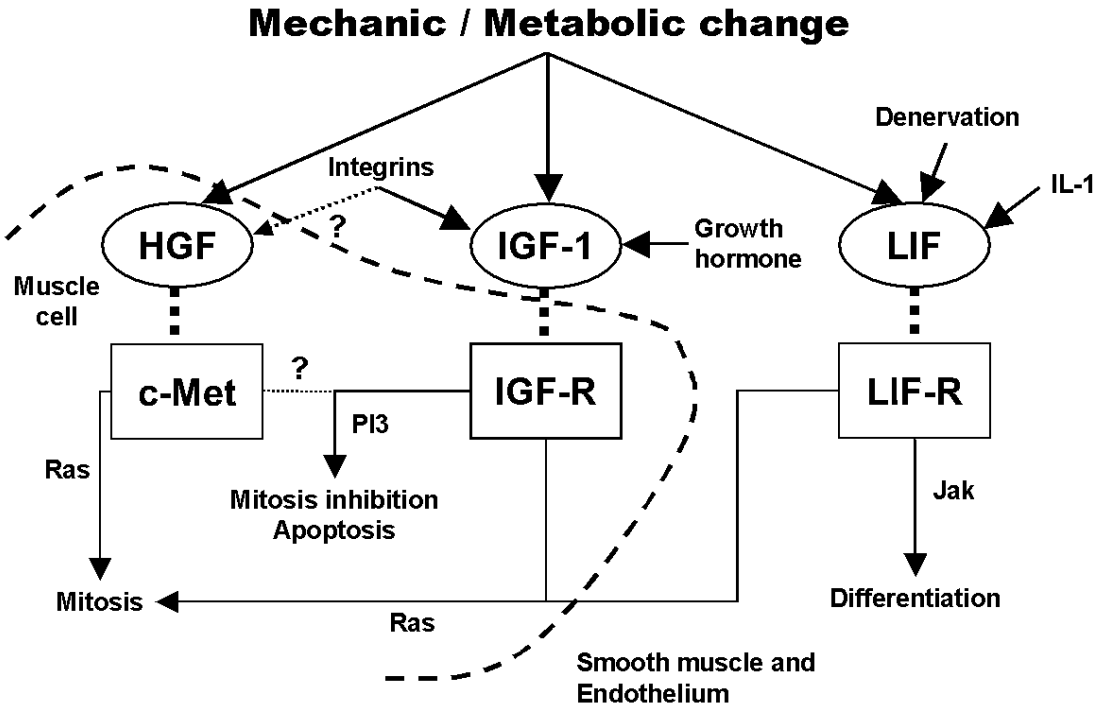



**Figure 30**
Schematic presentation of growth factors and corresponding receptors detected in Study III

LIF has previously been detected in human skeletal muscle (Schoser *et al.* 1998).. In response to some, but not all, types of injuries LIF stimulates muscle regeneration (Barnard *et al.* 1994; Gregorevic *et al.* 2000). In Study III, LIF expression in human skeletal muscle was restricted to endothelial cells and epimysium. Schoser *et al*. also detected LIF in denervated myofibers (Schoser *et al.* 1998). Therefore epimysium could be a potential source of LIF production and release. The LIF-R was not detected in most muscle sections while epimysium sections displayed some staining. Neither LIF nor LIF-R was affected by 45 min of downhill or uphill running. LIF-R may be involved in muscle regeneration (Barnard *et al.* 1994; Gregorevic *et al.* 2000) and has been shown to induce an alternative activation of CD163+ macrophages (Goerdt *et al.* 1999). The low detection of LIF-R in human skeletal muscle suggests that circulating or locally produced LIF may not affect human skeletal muscle. Alternatively, the LIF-R could be unregulated during inflammation, something which did not occur 48 h after downhill or uphill running. It is also possible that different species (man versus rat) and a difference between systemic and local function of LIF and other growth factors result in inconsistent observations by researchers. The detected LIF and LIF-R in human muscle cells in a previous study (Schoser *et al.* 1998) was not confirmed in the Study III. Different polyclonal antibodies and the lack of saponin permeabilization the previous study may result in different staining compared to Study III. Detected amount of LIF was related to

IL-10, an anti-inflammatory cytokine which has been suggested to modulate the reaction to injury (Frangogiannis *et al.* 2000) by affecting macrophages function and to down regulate inflammatory cytokines such as IL-6 and IL-1. An interesting observation is that IL-6, IL-10 and LIF share a common receptor subunit (gp130) (Lai *et al.* 1996).

Interleukin 1β (IL-1β) was detected in both muscle and epimysium sections. One previous study has reported increased staining for IL-1β after exercise (Fielding *et al.* 1993) but these findings were most likely due to a previous muscle biopsy, as earlier indicated (Malm *et al.* 2000) and presently confirmed by the lack of IL-1β increase 48 h after exercise. This is in agreement with an earlier study (Authier *et al.* 1997) where IL-1β was associated with muscle regeneration and not inflammation.

Data from Study III demonstrates the presence (whether it is the result of accumulation or production is not known) of cytokines and growth factors which in different *in vivo* and *in vitro* experiments have been shown to posses immunoregulatory and muscle regenerating properties. Their exact functions in relation to human skeletal muscle adaptation to physical exercise are currently not known.

In summary, these findings suggest that local factors are of significant importance in human skeletal muscle adaptation to physical exercise.

## 5.5 THEN WHAT'S THE USE?

When all is said and done, what is the relevance of the findings and conclusions in this thesis? Based on the initial hypothesis, and with a few new ideas gathered along the way, I believe that the somewhat controversial conclusions in this thesis can be applied in a few different areas of research, everyday life, physical activity and sports. (Roman numbers i-v refer to Hypothesis.)

i. Leukocytes do not migrate from blood to skeletal muscle in response to physical exercise. Exercise-induced skeletal muscle inflammation could not be confirmed.

ii. Cell surface expression of signaling and adhesion molecules is related to immunological events in humans skeletal muscle, suggesting a signal-by-adhesion communication.

iii. Serum hormones affect circulating leukocytes, but appear to have less significance in skeletal muscle immunology.

iv. There is a release of some intramuscular proteins (CK) to the blood, not related to skeletal muscle inflammation but to circulating leukocytes.

v. Serum cytokines could only be related to a limited number of variables in skeletal muscle. Local growth factors are suggested to play a more pronounced role in skeletal muscle adaptation to physical exercise.

*Research*

Because physical exercise does not induce significant skeletal muscle inflammation, especially not involving cytotoxic T cells, which are the effector cells of several inflammatory muscle diseases, people suffering from these diseases should not restrain from physical exercise based on the notion that exercise induces inflammation in healthy human skeletal muscle. Studies are of course needed to investigate the effect of physical exercise on diseased human skeletal muscle. Also, the inflammatory response in humans skeletal muscle is not suppressed even after strenuous, eccentric exercise. Other means of skeletal muscle adaptation than the classical damage-inflammation-repair-adaptation pathway should be investigated in future studies.

*Everyday life*

For the recreational athlete, and everyone who just tries to stay in physical shape, muscle soreness is an often experienced but unwanted effect of physical exercise. It is intriguing that such a common occurrence can not be explained by today's science, for inflammation it is not.

*Exercise physiology and sports*

One common observation in these studies is the large individual differences in the immune response to the same relative or absolute workrate. This could be one factor determining the difference in results among athletes exposed to similar training programs, and explain why some stay healthy while others get sick when the major competition is just days ahead.

When designing training programs for athletes it is of importance that optimal adaptation is achieved. If we can understand the mechanism behind muscle adaptation, we can also optimize training and athletic performance.

We have not yet reached that goal, just taken the first few steps…

As of today, this is my contribution to the field of exercise physiology

September 3, 2001

# 6 ACKNOWLEDGEMENTS

We all owe our successes in life to the sacrifices and efforts of other people.

I plead guilty to the following:

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