EXERCISE, STRESS AND IMMUNE SYSTEM FUNCTIONAL RESPONSES

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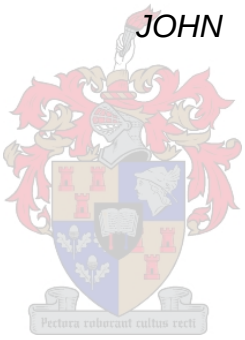
Dedicated to

JOHN



Declaration

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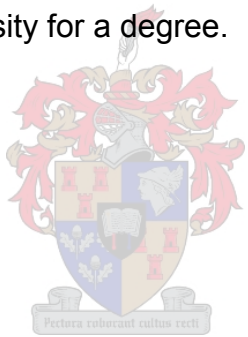

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ABSTRACT

Stress related to chronic exercise affects both the immune and endocrine systems, but there are still many issues that are poorly understood, particularly effects of stress on the functional capacity of immune cells. This thesis probed some of these issues using physiological models of physical and psychological stress. Both exercise training stress and chronic psychological stress in human subjects were shown to result in an up-regulation of spontaneous reactivity of white blood cells *in vitro*, using two different assays, namely a) a peripheral blood mononuclear cell (PBMC) culture assay measuring immune cell responsiveness and b) a relatively new flow cytometry technique for assessing activation status of cells by their expression of the surface marker CD69, in a lymphocyte subpopulation-specific manner. An up-regulation of immune cell activation in the absence of an additional stressor was associated with a decreased capacity to mount a response to a subsequent mitogen stimulus *in vitro* after chronic psychological stress and acute, extreme exercise stress. Another novel finding was that cortisol high-responders to chronic psychological stress exhibited a higher spontaneous reactivity of both $CD4^+$ and $CD8^+$ lymphocytes when compared to cortisol low-responders. This result indicates that chronic exposure to cortisol may decrease its usual inhibitory effect on spontaneous T lymphocyte responsiveness.

After optimisation of an animal model of mild, psychological stress, we demonstrated (using an IL-6 antibody) that IL-6 is necessary for a full-blown cortisol response to chronic, intermittent mild stress. Results also suggest that IL-6 plays a role in regulation of its own secretion by PBMCs in response to a stressor, by maintaining the production of IL-1β in the face of stress. Basal serum corticosterone concentration was shown to be the main determinant of the magnitude of mitogen-stimulated PBMC secretion of IL-6 *in vitro* in the stress-free controls. However, after blocking of IL-6 *in vivo*, IL-1β was identified as a major regulator of IL-6 secretion by mitogen-stimulated PBMCs *in vitro*, independently of the presence or absence of stress. The implications of these novel findings are that pro-inflammatory cytokines are sensitively regulated during mild stress.

Mean serum cortisol concentration at rest was not a useful tool to assess chronic exercise stress after training intervention. However, classification of athletes at baseline into two groups according to their resting serum cortisol concentration illustrated two distinct patterns for the responses of both cortisol and the cortisol:testosterone ratio to chronic stress.

These studies on the effects of chronic stress on parameters of the endocrine stress-axis and the immune system led to the following main conclusions: a) chronic exposure to cortisol results in a decreased inhibition of spontaneous immune cell activity at rest, b) this increased spontaneous activation of immune cells at rest in the absence of a stressor, is associated with a suppression of immune capacity to respond to a subsequent challenge, c) the latter finding is not evident under stress-free conditions where cortisol promoted immune cell IL-6 secretion, and d) IL- 1β and IL-6 are involved in the regulation of each others' secretion.

OPSOMMING

Chroniese oefening-verwante stres beïnvloed beide the immuun- en endokriene sisteme, maar daar is nog baie aspekte wat swak begryp word, veral m.b.t. die effekte van stres op die funksionele kapasiteit van immuunselle. Hierdie tesis het sommige van dié vraagpunte ondersoek deur gebruik te maak van fisiologiese en psigologiese stres. Beide oefening program-verwante stres en chroniese psigologiese stres in proefpersone het 'n op-regulering van spontane witbloedselreaktiwiteit *in vitro* tot gevolg gehad, wat d.m.v twee verskillende metodes aangetoon is, naamlik a) 'n perifere bloed mononukluêre selkultuur (PBMS-kultuur) bepaling van immuunsel reaktiwiteit en b) 'n relatief nuwe vloeisitometriese tegniek vir die assessering van aktiveringsstatus van selle, deur hul uitdrukking van die oppervlakmerker CD69, op 'n limfosiet subpopulasie-spesifieke wyse. 'n Opregulering van immuunselaktiwiteit in die afwesigheid van 'n addisionele stressor is geassosieer met 'n verlaagde kapsiteit om te reageer op 'n latere mitogeniese prikkel *in vitro*, na chroniese psigologiese stres en akute, erge oefeningstres. Nog 'n nuwe bevinding was dat kortisol hoog-respondeerders, in reaksie op chroniese psigologiese stres, 'n hoër spontane reaktiwiteit van beide $CD4^+$- and $CD8^+$-limfosiete toon in vergelyking met kortisol laag-resopndeerders. Hierdie bevinding toon aan dat chroniese blootstelling aan kortisol die inhiberende effek daarvan op spontane reaktiwiteit van T-limfosiete verminder.

Na optimalisering van 'n rotmodel van gematigde, psigologiese stres, het ons gedemonstreer (deur gebruik te maak van 'n IL-6 teenliggaam) dat IL-6 nodig is vir 'n volledige kortisolreaksie op chroniese, onderbroke, gematigde stres. Die resultate dui daarop dat IL-6 'n rol in die regulering van sy eie sekresie deur PBMSe in reaksie tot 'n stressor speel, deur die handhawing van produksie van IL-1β in die teenwoordigheid van stres. Basale serum kortisolkonsentrasie is as die belangrikste beslissende faktor in die omvang van mitogeen-gestimuleerde PBMS sekresie van IL-6 *in vitro* in die stresvrye kontroles aangedui. Na blokkering van IL-6 *in vivo*, is IL-1β egter as 'n belangrike reguleerder van IL-6 sekresie deur mitogeen-gestimuleerde PBMSe *in vitro* geïdentifiseer, onafhanklik van die teenwoordigheid of afwesigheid van stres. Die implikasie van hierdie nuwe bevindinge is dat pro-inflammatoriese sitokiene tydens gematigde stres sensitief gereguleer word.

Die gemiddelde serum kortisolkonsentrasie in ‘n rustende toestand was nie ‘n gepaste instrument om chroniese oefeningstres na ‘n oefenprogram-ingreep te assesseer nie. Na basislyn klassifikasie van atlete in twee groepe volgens hul rustende serum kortisolkonsentrasie, is twee afsonderlike patrone vir die reaksie van beide kortisol en die kortisol:testosteroon verhouding egter aangetoon.

Hierdie studies rakende die effekte van chroniese stres op parameters van die endokriene stres-as en die immuunsisteem het tot die volgende vernaamste gevolgtrekkings gelei: a) chroniese blootstelling aan kortisol het ‘n verlaagde inhibisie van spontane immuunselaktiwiteit tydens rustende toestande tot gevolg, b) hierdie verhoogde spontane aktivering van immuunselle tydens ‘n rustende toestand word geassosieer met ‘n onderdrukking van immuunkapasiteit om te reageer op ‘n daaropvolgende prikkel, c) laasgenoemde bevinding is nie sigbaar tydens stresvrye toestande, wanneer kortisol IL-6 sekresie bevorder, nie en d) IL- 1β en IL-6 is betrokke by die regulering van mekaar se sekresie.

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LIST OF PUBLICATIONS AND CONGRESS CONTRIBUTIONS

NATIONAL

Posters

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- **Smith C,** Myburgh KH. Selected immune and endocrine responses to increased training intensity. 31st annual congress of Physiological Society of Southern Africa, Potchefstroom, South Africa, 2003

- **Smith C**, Myburgh KH. IL-1β, IL-6 and peripheral blood mononuclear cells' responses to intermittent immobilisation: a rat model of chronic inflammatory stress. 32nd annual congress of Physiological Society of Southern Africa, Coffee Bay, South Africa, 2004

Oral Presentation

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Peer-Reviewed Paper

- **Smith C,** Myburgh KH. Treatment with *Sutherlandia frutescens subs. microphylla* alters the corticosterone response to chronic intermittent immobilisation stress in rats. South African Journal of Science 100: 229–232, 2004

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- Myburgh KH, du Toit J, **Smith C**. Changes in performance and resting cortisol in response to 8 weeks of high intensity training. Med Sci Sports Exerc 34(5S):S276, 2002. Saint Louis, Saint Louis, USA, Lippinco H Williams and Wilkins.

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LIST OF ABBREVIATIONS

ACSM	American College of Sports Medicine
ACTH	adrenocorticotrophic hormone
AIDS	acquired immune deficiency syndrome
ANOVA	analysis of variance
APC	antigen presenting cell
BMI	body mass index
CBG	corticosteroid binding globulin
CD	cluster designation
CD45RO	marker for helper T memory cells
CHO	carbohydrate
ConA	concanavalin A
CRH	corticotrophin releasing hormone
CRP	C reactive protein
DHEA	dehydroxyepiandrosterone
DHEAs	dehydroxyepiandrosterone-sulphate
DOMS	delayed onset muscle soreness
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GABA	gamma-aminobutyric acid
GR	glucocorticoid receptor
HDL	high-density lipoprotein
HPA-axis	hypothalamo-pituitary-adrenal axis
IFN	interferon
IgG	immunoglobulin class G
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MR	mineralocorticoid receptor

mRNA	messenger ribonucleic acid
NIDDM	non-insulin dependent diabetes mellitus
NK	natural killer lymphocyte
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PHA	phytohaemagglutinin
PPO	peak power output
PWM	pokeweed mitogen
1-RM	one-repetition maximum
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SEM	standard error of the mean
SHBG	sex hormone binding globulin
SST	serum separation tubes
TAT	tyrosine aminotransferase
TGF-β	transforming growth factor-beta
T_H1	helper T lymphocyte type I
T_H2	helper T lymphocyte type II
T_H3	helper T lymphocyte type III
TNF	tumour necrosis factor
5TT	5 km time trial
40TT	40 km time trial
VO_{2max}	maximum oxygen consumption
WBC	white blood cell

Chapter 1

Introduction

1.1 General Introduction

Ever since participation in sport changed from an amateur game to a professional career, athletes have found themselves under ever-increasing demand to improve their performance. In their quest for gold however, the ever-increasing volume and intensity of training, and therefore simultaneously decreasing recovery time, may become counterproductive.

Theoretically, an “ideal” training regimen would allow for enough recovery time between exercise bouts, for the athlete to start each training session without negative influences of residual strain from the previous session. However, this is neither the reality, nor the “ideal” in the modern competitive environment of sport. Rather, the athletes intentionally overload to adapt to a higher level of performance. In the process, they cope with the increased levels of stress (both physiological and psychological), rebound from episodes of overreaching, or fail in one way or another. It is the latter that is of particular concern to exercise physiologists, since the inability to rebound from training overload may result in the full-blown overtraining syndrome, which is characterised by among others, long-term decreased performance, chronic inflammation, immunosuppression and abnormal resting hormonal profiles (Barron *et al.*, 1985; Fry *et al.*, 1991b; Gabriel *et al.*, 1998; Hartmann & Mester, 2000; Ketner & Mellion, 1995; Kuipers & Keizer, 1988; Urhausen & Kindermann, 2002), all of which prevent an athlete from excelling. In addition, adaptation to training in favour of one system in the body is often to the decrement of another, which may in the long term have serious health implications, such as the development of e.g. autoimmune diseases or chronic fatigue syndrome, which are equally daunting to a career in sports. For this reason, researchers have been searching for markers to monitor training load and to issue a timely warning when any of these possibly pathological conditions are imminent, so that the

necessary precautionary steps may be taken. Such markers would allow athletes to train optimally, thereby also improving performance maximally.

The earliest reported, and probably the most widely known, marker/indicator of increased stress is the hormone cortisol. However, the theory of general adaptation suggested by Hans Selye - that increased cortisol concentration is a central and general response to stress which could explain all stress related illnesses (Selye, 1978; Viru, 2002) - has been considerably modified over the years. One of the reasons for this is that stress is not an easily defined condition with clear causes and effects. Rather, "stress" is the general term describing any demand that is outside the norm, be that physical or psychological. A second reason is that different individuals may experience the same stressor at different levels of perceived intensity, thereby causing a great variation in the response of individuals to a specific stressor. Thirdly, the stress response is a cascade of events involving several organs and systems, so that differences between individuals, or adaptation, may be the result of differences or changes that may occur at many different sites. Fourthly, while one individual may be able to adapt and cope with an ongoing stress, so that in effect it becomes a lesser stressor in the long term, another individual may not be able to adapt to it and may then suffer a chronic negative effect as a result.

Therefore, since the postulation of Selye's theory, it has become clear that stress is a complex condition which does not only affect the catabolic endocrine system, and that the body's response to it requires interaction of several additional systems. The purpose of this thesis is therefore to further elucidate the physiological response and adaptation to stress, and to investigate associations between two systems involved in the acute and long-term processes, namely the immune system and the endocrine stress-axis.

In this chapter I will give a basic overview of these two systems, which are both intimately involved in and influenced by the stress response. Since these systems are in themselves also complex, I will indicate which sub-aspects are particularly relevant to the scope of this thesis. In the next chapter I will provide an overview of the available literature on the interaction of these systems in the short-term response and longer-term adaptation to stress, with specific focus on the athletic population and exercise-induced stress. However, due to

the additive nature of various stressors, the effects of psychological stress, which accompanies high-level sport, will also be addressed.

1.2 Overview of the immune system

The immune system may be divided into three parts, namely the immune organs (bone marrow, spleen, and lymph nodes), the cellular compartment (white blood cells) and the messenger system (cytokines). The latter two are of importance for the purposes of this thesis, and brief overviews of the most relevant components of each part are given below, as well as the ways in which they interact to mount an inflammatory response to enable the body to resist the challenge of pathogenic invasion. I will also discuss the principles of assays most frequently used to investigate and assess these functions of the immune system.

1.2.1 White blood cells

White blood cells (WBCs), or leukocytes, may be functionally divided into two groups, namely phagocytes and immunocytes. Phagocytes include granulocytes (mainly neutrophils, but also eosinophils and basophils) and monocytes, while the different types of lymphocytes makes up the circulatory immunocyte population. Since the role of the non-specific immune system, and in particular that of neutrophils, in the response to stress has been extensively investigated and reported on (more detail in Chapter 2), this thesis will focus on mainly monocytes and lymphocytes, but a short overview of all three cell types is provided below.

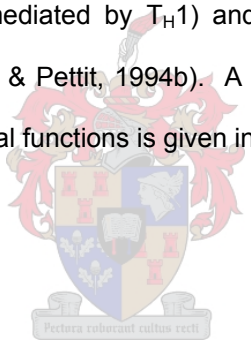
Monocytes originate in the bone marrow, where they differentiate and mature for 16 – 26 hours. After leaving the bone marrow, monocytes remains in circulation for up to 7 days, after which they move into tissues, where they are known as macrophages, which have a lifespan of several months or even years. Macrophages have the ability to proliferate to a small extent at sites of inflammation. Although dendritic cells are known to play a pivotal role as antigen presenting cells, monocytes and macrophages have the ability to phagocytose bacteria and other larger particles, and also play a cardinal role in immunity because of their

ability to present ingested antigens on their surfaces so that they may be recognised by lymphocytes, and the cell-mediated antigen-specific immune response may be initiated. In addition, monocytes contain large quantities of lipase, that can degrade bacteria that have a lipidic capsule (Hoffbrand & Pettit, 1994a), since these bacteria (which includes e.g. *Haemophilus influenzae* and *Streptococcus pneumoniae*) are not susceptible to destruction by lysozymes or the complement pathway (Bester, 1991). Of specific interest to the stress response, is the ability of monocytes and macrophages to secrete the pro-inflammatory cytokines IL-1 and IL-6 (Baumann *et al.*, 1984). Given the long lifespan and variety of immune functions of monocytes, their adaptative response to stress is of great significance for the maintenance of immune competency.

Neutrophils originate in the bone marrow, from the same stem cell type as monocytes. In peripheral circulation, neutrophils account for more than 90 % of all **granulocytes**, and more than 60 % of all leukocytes. Their main function is phagocytosis of foreign substances in tissues following their migration from the blood compartment. The ingested particle is then destroyed by release of intracellular granules, containing amongst others, enzymes and substances such as myeloperoxidase, acid phosphatase, collagenase, lactoferrin and lysozyme. The time mature neutrophils spend in circulation is about 10 hours, and their lifespan is limited to 1-3 days. They do not seem to have the ability to recharge their killing mechanism once they have reacted to a challenge (Hoffbrand & Pettit, 1994a). Therefore, although important role players in the acute non-specific response to stress, this cell type is probably less important in the longer-term adaptive processes of the immune system in response to stress.

Lymphocytes make up about 25 – 35 % of all circulating leukocytes and originate from the general stem cells in the bone marrow, as well as from the thymus. Maturation takes place in the peripheral lymphoid organs (lymph nodes, tonsils, spleen, appendix, Peyer's patches in the gut), after which the mature cells enter the circulation again. Different subpopulations exist, which may be distinguished by the characteristic markers on the cell membrane, called cluster of differentiation (CD) markers (Hoffbrand & Pettit, 1994a). **T cells** (originating from the thymus) make up the largest portion of all circulating lymphocytes – 66 to 88 %, while about 12 to 24 % are made up by **B cells** (originating from the bone marrow). While B cells

only survive in the circulation for a few days, T cells may live from 4 to more than 20 years. T and B cells cannot be distinguished morphologically, but only by specific laboratory techniques, such as immunohistochemistry and flow-cytometry (Hoffbrand & Pettit, 1994a). However, these two subgroups of lymphocytes have very different roles in immunity. **B lymphocytes**, on stimulation, will differentiate further to form plasma cells, which secretes antibodies, and are thus important for humoral (antibody-mediated) immunity. **T lymphocytes**, on the other hand, are important role-players in cellular immunity, delayed sensitivity reactions and graft rejections. T cells can be divided into subpopulations, including helper T, suppressor T and cytotoxic T cells. Helper T cells are further divided into a type I (T_H1, initiates the cell-mediated immune response), type II (T_H2, initiates the humoral immune response) (Mosmann & Coffman, 1989; Vander *et al.*, 1998a) and type III (T_H3, produces the inhibitory cytokines IL-10 and transforming growth factor (TGF)-β) (Fukaura *et al.*, 1996). The distribution of the different types of helper T cells influences the balance between the cell-mediated (mediated by T_H1) and antibody-mediated (mediated by T_H2) immune responses (Hoffbrand & Pettit, 1994b). A summary of the main subpopulations of lymphocytes with their individual functions is given in Figure 1.1.



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1.2.2 Immune cell function

Although immune cells can be divided into phagocytes and immunocytes, as mentioned above, a different broad classification is the cell-mediated and the antibody-mediated arms. Cells involved in the cell-mediated arm of the immune system are monocytes/macrophages, NK cells, granulocytes, cytotoxic T lymphocytes and T_H1 cells. These cells respond to invading pathogens by recognising general molecular patterns, and attack and destroy anything that appears foreign to the body, by processes such as phagocytosis and degranulation. The main functions of this non-specific first line of defense are to contain foreign invasions until a more specific immune response can be launched, and to activate the appropriate specific immune response. The antibody-mediated arm of the immune system provides a more targeted response against individual invading pathogens. Monocytes, B lymphocytes and T_H2 lymphocytes are the cells most commonly associated with this arm of the immune system. Immune competence has been defined as a proper balance between the humoral and cellular components of the specific immune system (Hässig *et al.*, 1996), and changes in the balance of cells initiating these types of responses should therefore be considered in assessments of immune function.

1.2.3 Cytokines

These intercellular mediators were first named on the basis of their immune system function. When it became clear that these mediators were not only produced by lymphocytes and monocytes, but also a variety of other cell types, and that one specific mediator may have several functions, it was decided in 1979 to call these mediators “interleukins”, which literally means “between cells” (Mackinnon, 1999). Cytokines are involved in regulation of the immune, haematopoietic, endocrine and nervous systems (Vander *et al.*, 1998a). Although all the physiological interactions are, as yet, incompletely identified and the interactive implications ever more poorly delineated, a basic overview of the origin and functions of the main cytokines are discussed below.

Interferons (IFN) are a group of cytokines released to coat uninfected cells in a non-specific manner, in order to prevent them from becoming infected (Vander *et al.*, 1998a). In this way,

viral replication may be inhibited. Apart from its antiviral properties, interferon may also enhance the immune response, depending on the subgroup of IFN: IFN-α is produced by virus-infected monocytes and lymphocytes, IFN-β is mainly produced by virus-infected fibroblasts, and IFN-γ is produced by stimulated T-lymphocytes and natural killer (NK) lymphocytes (Mackinnon, 1992). While IFN-α and IFN-β bind to the same receptor, IFN-γ has its own specific receptor. All interferons induce cell growth, increase expression of major histocompatibility complex (MHC) class I, and activate cytotoxic T-cells and NK-cells. In addition, IFN-γ also increases expression of MHC class II, activates macrophages and neutrophils, activates the vascular endothelium to promote T and B cell differentiation, and increases secretion of IgG2, IL-1 and IL-2 (Vander *et al.*, 1998a). These functions all form part of the T-helper 1 (T_H1) response of the immune system. What has only become apparent more recently is that cytokines such as IFN-γ also interact with the endocrine stress-axis to increase secretion of ACTH and cortisol (de Metz *et al.*, 1999).

Interleukin-1 (IL-1) is produced mainly by macrophages (Solomon *et al.*, 1990), but may also be secreted by other stimulated immune cells, such as type I $CD4^+$ lymphocytes (T_H1 cells) (Mosmann & Coffman, 1989). IL-1 stimulates cytokine (TNF, IL-6) and cytokine receptor (in particular IL-2 receptor) production by T cells, and also stimulates proliferation of B cells (Roitt, 1994). Two forms of IL-1 exist, IL-1α and IL-1β, with the same basic function, but with quite different structures. IL-1α seems to usually be membrane-associated, while IL-1β may also circulate in its free form. Both forms bind to the same receptors, which occur mostly on blood and bone marrow cells, but also, of importance to the stress response, to brain cells and adrenal cells. IL-1 seems to be the most potent inducer of corticotrophin releasing hormone (CRH) (Watkins, 1994), and may also be able to directly release adrenocorticotrophic hormone (ACTH) from the pituitary (Sapolsky *et al.*, 2000), thus stimulating the hypothalamic-pituitary-adrenal (HPA)-axis to respond to a stressor. IL-1 production is inhibited by the corticosteroids, such as cortisol. Furthermore, its ability to bind to vascular endothelial and smooth muscle cells (Roitt, 1994) allows IL-1 to play a major role in characteristics of inflammation, such as vasodilation, fever and cramps. It was also recently reported to contribute to the wasting syndrome (cachexia), by inducing adhesion molecule expression on vascular endothelium (Tisdale, 2001).

Interleukin-2 (IL-2) is secreted by some T cells after stimulation, as a result of antigen binding to receptors present on the T cell. Within one to two days, these T cells start to secrete IL-2 and/or express high affinity receptors for IL-2. Binding of IL-2 to these T cells initiates T cell proliferation, and complex changes in morphology, metabolism, receptor expression and production of cytokines. Therefore, once IL-2 has activated a cell, that cell can promote clonal expansion of itself, as well as other T cells that cannot secrete IL-2, such as cytotoxic T cells and suppressor T cells. However, it cannot act directly on unstimulated cells, since they do not express the IL-2 receptor (Roitt, 1994). IL-2 also leads to increased secretion of IFN, activation of NK cell cytotoxicity and monocytes, and proliferation of B cells (Vander *et al.*, 1998a).

Interleukin-4 (IL-4) was shown to be a B cell proliferation cofactor in 1982 (Mackinnon, 1999). It may act as 1) activation factor, inducing resting B cells to increase in size and to express MHC class II, 2) proliferation factor, increasing replication of B cells, and 3) differentiation factor, inducing production of the immunoglobulin subclasses IgE and IgG1. However, apart from effects on B cells, IL-4 also plays a major role in T cell development (Vander *et al.*, 1998a). IL-4 secretion promotes differentiation of helper T cells into type 2 (T_H2) cells, which are themselves the major source of IL-4, during an immune response. Its action is inhibited in the presence of IFN-γ, which is secreted by T_H1 cells. This interaction is an example of why the proper balance between T_H1 and T_H2 cells is so important.

Interleukin-6 (IL-6) is secreted by a wide variety of cells, such as fibroblasts (May *et al.*, 1988), endothelial cells (May *et al.*, 1989), keratinocytes (Baumann *et al.*, 1984; Fujisawa *et al.*, 1997) and peripheral blood mononuclear cells (PBMCs) (Baumann *et al.*, 1984), more specifically monocytes and T_H2 cells. IL-6 is beneficial to the immune response by enhancing B cell differentiation into plasma cells for antibody secretion, by increasing NK cell cytotoxicity and promoting the inflammatory response (Vander *et al.*, 1998a). The main functions of IL-6 are pro-inflammatory and include increasing T cell proliferation and activating the release of other pro-inflammatory cytokine and acute phase proteins. On the other hand, IL-6 was also reported to exert an indirect anti-inflammatory action by a) down-regulating TNF release by negative feedback (Nukina *et al.*, 1998), b) stimulating release of IL-1 receptor antagonist (IL-1ra) (Jordan *et al.*, 1995), c) stimulating release of CRH and

ACTH from the hypothalamus and pituitary gland, resulting in the release of cortisol, and d) directly stimulating the adrenal glands to produce and secrete cortisol, which is a potent anti-inflammatory hormone. In this way, the inflammatory response can be both initiated and controlled. Metabolic effects of IL-6 include promotion of liver glygogenolyis (Vaartjes *et al.*, 1990) and adipose tissue lipolysis (Petersen *et al.*, 2004).

Two types of **tumor necrosis factor** (TNF) have been identified to date: TNF-α (cachectin) and TNF-β (lymphotoxin). Both types have cytotoxic activity directed against tumour cells. TNF-β is produced by activated T-cells and exerts both cytostatic and cytotoxic activity against tumour cells (Vander *et al.*, 1998a). TNF-α is produced by most peripheral blood mononuclear cells (PBMCs, monocytes and lymphocytes). Its main functions are antiviral activity and activation of macrophage killing of tumour cells (Vander *et al.*, 1998a). Prolonged high concentrations of TNF-α may have harmful effects like chronic inflammation and cachexia (Alvarez *et al.*, 2002; Costelli *et al.*, 1993; Llovera *et al.*, 1993). Similar to IL-1, TNF also exhibits interaction with the endocrine system, since the hypthalamic-pituitary-adrenal (HPA-) axis (more specifically ACTH and corticosterone) was reported to down-regulate production of both types of TNF in rats (Fantuzzi *et al.*, 1995). New functions of TNF-α are still being elucidated. Recent reports indicate a role in stress-related neurodegeneration by up-regulation of inducible nitric oxide synthase expression via nuclear factor kappa B (NF-kappa B) activation in the brain cortex (Madrigal *et al.*, 2002).

1.2.4 Interaction between WBCs and cytokines

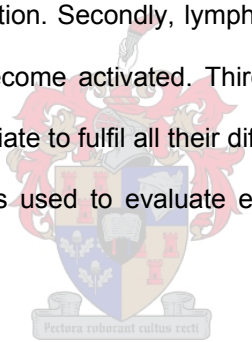
The balance between processes mediated by different immune cells (T_H1 or T_H2 lymphocytes, neutrophils and monocytes/ macrophages) and cytokines in the response to stress will determine: 1) whether the response is of short-duration or a longer-term adaptation, 2) the speed of the response (e.g. non-specific response is faster than the specific response) and 3) whether a shift in favour of one particular response will result in another response being compromised. A summary of the interaction between cytokines and the various immune cells involved in the different types of immune responses are illustrated in Figure 1.2.

Pectora roborant cultus recti

1.2.5 Immune functional tests

Cell counts give an indication of the availability of immune cells that can possibly react to an immune insult. However, cell counts do not provide information on the ability of the cells that are present, to fulfil their functions. In order to draw accurate conclusions regarding immune system competence, it is therefore necessary to consider changes in both immune cell count and function.

Since the humoral stress response is the particular immune focus of this thesis, I will limit the overview of functional evaluation to that relevant to this part of the immune system. The humoral response is dependent on proper functioning of three consecutive steps. Firstly, after binding of an antigen to the antigen presenting cells (in peripheral blood predominantly monocytes), these cells must be able to react to this insult by producing cytokines and secreting them into the circulation. Secondly, lymphocytes must be responsive/ sensitive to these cytokine signals and become activated. Thirdly, the activated lymphocytes must be able to proliferate and differentiate to fulfil all their different functions, as discussed earlier. A brief description of the assays used to evaluate each stage of these humoral responses follows below.



Cellular production and secretion of cytokines: From the overview above, it is clear that cytokines are usually secreted by more than one cell type. Measurement of changes in cytokine concentrations in plasma or serum is therefore not useful for the evaluation of cell function, since the specific source of the increased cytokine is unclear. To enable evaluation of type-specific cellular function, cell culture techniques are used (Alvarez *et al.*, 2002; Pool *et al.*, 2002; Tantak *et al.*, 1991). In short, the cell type to be investigated is cultured *in vitro*, and challenged with a standardised antigen that will stimulate cytokine production. The concentration of one or more cytokines is then measured in the culture supernatant of a stimulated *vs*. an unstimulated culture. The difference between the two measurements is the concentration of cytokine secreted specifically in response to the antigen. This value may be compared to that of control samples to determine abnormalities, or to previous samples from the same individual to monitor changes over time. While this method has been in use since the 1970's, more recent advances in technology, such as the flow cytometer, enable

researchers to also determine the concentration of cytokines produced intracellularly before being secreted, pinpointing the origin of these cytokines more specifically. However, since the technology required for the latter is so expensive, the former technique is used quite commonly.

Cell responsiveness: All circulating immune cells have already undergone primary differentiation, resulting in different subpopulations. When these cells are stimulated by cytokine action, and they are sensitised sufficiently to become activated, they are capable of secondary proliferation. Cells committed to secondary proliferation express the cellular surface marker CD69, which has been linked to their activation, proliferation and cytotoxic functions (Borrego *et al.*, 1999a; Borrego *et al.*, 1999b; Werfel *et al.*, 1997). CD69 is reported to be the earliest specific activation antigen expressed on the surface of T-cells in the circulation (Llera *et al.*, 2001), and to be undetectable or present in very low concentrations in unstimulated lymphocytes (Werfel *et al.*, 1997). The cytoplasmic domain of CD69 was reported to induce TNF-α production in rat mucosal mast cells in culture (Sancho *et al.*, 2000), but does not appear to provide information on events downstream, such as proliferation (Krowka *et al.*, 1996). Nonetheless, it is an excellent screening tool to assess lymphocyte responsiveness, and specifically impaired responsiveness. It correlates well with the ^{3}H-thymidine assay for lymphocyte proliferation (see below), with the added advantage of requiring only a 4-6 hour incubation period, compared to the 72 hour incubation period of the ^{3}H-thymidine assay (Mardiney, III *et al.*, 1996). CD69 expression may be determined for each lymphocyte subpopulation separately by flow cytometry. See Appendix A for a description of the principle of flow cytometric analysis of whole blood and interpretation of results.

Lymphocyte proliferative response: This assessment of immune cell function determines the rate of secondary proliferation in lymphocytes, by measuring the rate by which radioactively labelled precursors (^{3}H-thymidine) are incorporated into lymphocyte DNA after *in vitro* stimulation. Different synthetic stimuli (mitogens) have been developed that are specific to certain lymphocyte subpopulations, to enable differentiation between proliferation rates of the different subgroups. The mitogens most frequently used in exercise-related studies are concanavalin A (ConA) and phytohaemagglutinin (PHA), which stimulate T cells,

pokeweed mitogen (PWM), a stimulator of B cell proliferation and lipopolysaccharide (LPS) which stimulates B cell proliferation in non-human species (Mackinnon, 1992). This assay is however unable to distinguish between T cell subpopulations.

1.3 Overview of the endocrine stress-axis

1.3.1 The hypothalamic-pituitary-adrenal (HPA-) axis

The hypothalamic-pituitary-adrenal (HPA-) axis is a major endocrine role-player in the stress response. The hypothalamus plays a key role in the regulation of pituitary function. It receives, sorts and integrates signals from a variety of sources, and directs them to the pituitary gland (Genuth, 1983b; Vander *et al.*, 1998b). Afferent nerve impulses to the hypothalamus originate from the thalamus, the limbic system, the eyes and remotely from the neocortex, largely via the neurotransmitters nor-epinephrine, acetylcholine and serotonin. This input of sensations (of e.g. pain, sleep or wakefulness, emotion, stress, olfactory awareness, light and even thought), stimulates efferent impulses (via neurotransmitters dopamine, acetylcholine, gamma-aminobutyric acid (GABA), and beta-endorphin) which stimulate the release of releasing or inhibitory hormones. One of these hormones is corticotrophin releasing hormone (CRH), a peptide hormone with 41 amino acids. CRH stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH) via the cAMP second messenger system. ACTH is a peptide with 39 amino acids and its concentration shows a diurnal pattern, with peak values just before awakening, and the nadir just before or after falling asleep. ACTH circulates in plasma in an unbound form and has a half-life of only 15 minutes. The main function of ACTH is to stimulate the secretion of cortisol by the adrenal cortex. It also promotes growth (in cell size rather than cell number) of zones in the adrenal cortex that are responsible for the secretion of steroid hormones. The major hormones secreted by the adrenal cortex are 1) the mineralocorticoid, aldosterone, which is vital to maintenance of sodium and potassium balance, 2) precursors to the sex steroids, estrogens and androgens, which play important roles in establishing secondary sexual characteristics as well as being anabolic agents, and 3) the glucocorticoids, cortisol and corticosterone. It is the latter two, androgens and glucocorticoids, which are of particular importance in the response to stress and the downstream effects of this stress response.

The synthesis of glucocorticoids occurs mainly in the zona fasciculata, but also to a small extent in the zona reticularis of the adrenal cortex (Genuth, 1983a; Vander *et al.*, 1998b). In humans, cortisol is the dominant glucocorticoid, while corticosterone is dominant in rodents. Cortisol is not stored in the adrenocortical cell, but rapidly released after production. Therefore, an acute need for increased circulating cortisol requires rapid activation of the entire synthesis and release sequence.

Although not of direct relevance to the aims of this thesis, it is of interest to mention the role of catecholamines in the endocrine stress response. These hormones, mainly epinephrine and norepinephrine, which are secreted by the adrenal medulla, are responsible for the early sympathetic changes seen in response to stress exposure, such as increased heart rate and blood pressure, increased glucose release from the liver, and increased blood flow to the heart, brain and skeletal muscle – the so-called "fight-or-flight" response. These hormones are secreted by direct nervous activation, so that secretion occurs more quickly after exposure to a stressor than the glucocorticoids, which are only secreted at the end of a multi-step endocrine response pathway. On the other hand, the clearance rate of catecholamines from the circulation is also much faster than that of the glucocorticoids, which therefore exert a much longer lasting effect on target cells.

1.3.2 Cortisol receptors and binding globulins

Although it is common practice to measure total serum cortisol concentration as an indicator of stress, this parameter alone may not be ideal, since several other peptides and hormones may influence its biological activity. For example, an earlier theory of heterogeneity in glucocorticoid receptors (GR) (Kahn *et al.*, 1978) was recently confirmed by identification of two types of GR: type I, a high-affinity receptor which is also a mineralocorticoid receptor (MR), and type II, a more abundant low-affinity receptor that is more specific to both endogenous and synthetic glucocorticoids (Devenport *et al.*, 1991; Devenport *et al.*, 1993; Kellendonk *et al.*, 2002; Spencer *et al.*, 1996). Binding of glucocorticoids to MR is associated with anabolic effects, such as increased appetite and weight gain in rats (Devenport *et al.*, 1991), while binding to type II GR is thought to have a catabolic effect (Devenport *et al.*,

1993; Spencer *et al.*, 1996). Therefore, since the type of GR is important in determining the effect of the cortisol bound to it, GR concentrations may be a desirable parameter to measure in conjunction with cortisol concentration. Unfortunately, this requires invasive procedures to obtain tissue samples for analysis, which is not ideal for competitive athletes. The previous finding of no relationship between the serum corticosterone concentration at rest and hepatic glucocorticoid receptors (Dellwo & Beauchene, 1990) could possibly be due to heterogeneity in receptor type. GR (type II) has two isoforms, GR-α and GR-β, in humans and GR-β is thought to have a negative effect of GR-α transcriptional regulation, which may implicate glucocorticoid responsiveness, e.g. as illustrated in neutrophils (Strickland *et al.*, 2001). However, Spencer *et al*. subsequently reported no correlation between serum corticosterone level and splenic type II receptors (Spencer *et al.*, 1996). Therefore, our knowledge of the relationship between stress, cortisol concentration, GR receptor number and type is too incomplete to warrant tissue sampling in competitive athletes. More preliminary work in other mammalian models is required, and is indeed proceeding, albeit slowly.

In the same rat study by Spencer *et al*. (1996) however, there was a correlation between the splenic type II receptor and plasma **corticosteroid binding globulin (CBG)** concentrations, but not between GR and corticosterone concentration. This may indicate that CBG plays a larger role than GR in regulation of the biological activity of corticosterone. CBG is synthesised in the liver (Feldman *et al.*, 1979), endometrium (Misao *et al.*, 1994) and lungs (Hammond *et al.*, 1987), but the reason for this widespread distribution is not clear. Neither is it clear whether or not other tissues are also involved. About 80 % of total cortisol in circulation is transported bound to CBG (Brien, 1981), which renders the cortisol biologically inactive by inhibiting its binding to receptors. Cortisol concentration positively correlated with CBG concentrations in humans at rest ($r = 0.88$; $P < 0.0001$) and after exposure to a stressor ($r = 0.64$; $P < 0.001$) (Dhillo *et al.*, 2002). This suggests that the net effect of an increased cortisol concentration may be misinterpreted or overestimated, unless the parallel increases in CBG concentration are also considered. This has major implications for studies investigating sensitivity to glucocorticoids, or function of the HPA-axis. It may therefore be more useful to express the combination of cortisol and CBG concentrations as a ratio to enable more accurate interpretation of catabolic status. A finding that has never been

confirmed but is also of relevance to the theme of this thesis, is that elastase, expelled during the degranulation of activated granulocytes in the inflammatory process, has the ability to cleave CBG and release cortisol from CBG, rendering the glucocorticoid biologically active (Hammond *et al.*, 1990). Therefore, elastase could be considered a pro-cortisol immune agent. Of more general importance is that cortisol action is promoted or inhibited by several other agents that on first consideration have other primary functions.

1.3.3 Endocrine anti-glucocorticoid agents

Testosterone is produced by the Leydig cells in the testes and its primary function is related to reproduction, which is not within the scope of this thesis. However, another function, which is of interest, is the anabolic effect of testosterone, which counteracts in part the catabolic effects of cortisol (Genuth, 1983a). This anabolic effect of testosterone is mainly achieved by stimulation of growth hormone and insulin-like growth factor release, both of which are essential for protein synthesis not only in growth, but also in repair of bone and skeletal muscle. However, more recently additional beneficial effects of testosterone have become evident, such as its role in the limitation of the extent of muscle catabolism after severe stress. An example of this is the finding that although testosterone administration to trauma patients with severe burns did not affect protein synthesis rate, it was associated with a 2-fold decrease in protein catabolism (Ferrando *et al.*, 2001). The exact mechanism(s) by which testosterone has its anabolic effect on target cells or organs are not clear yet. However, a recent review postulated that testosterone promotes the commitment of pluripotent stem cells into the myogenic lineage and inhibits their differentiation into the adipogenic lineage (Bhasin *et al.*, 2003), a theory that could possibly explain the testosterone-induced decrease in fat mass (Wang *et al.*, 2004) and increase in myonuclear and satellite cell number (Sinha-Hikim *et al.*, 2003) recently reported. Similar to cortisol, testosterone may also circulate either in its free form, or bound to a binding globulin, namely sex hormone binding globulin (SHBG). However, while ≈ 80 % of cortisol in circulation is bound to CBG, only ≈ 44 % of testosterone in plasma is bound to SHBG (Hackney, 1996), so that SHBG likely plays a relatively smaller role than CBG in the control of bioactive hormone concentration.

Although it is clear that testosterone has functions opposing the effect of the glucocorticoids, the regulation of testosterone production may also influence its anti-glucocorticoid effect. Synthesis of sex steroid precursors occurs mainly in the zona reticularis. The main androgen produced in the adrenal gland, is dehydroxyepiandrosterone (**DHEA**). DHEA circulates in human blood in the sulphated form, DHEAs, at a higher concentration than any other steroid hormone. It is metabolised to its active form (DHEA) by the enzyme steroid sulphatase (Vander *et al.*, 1998b). DHEA serves as prehormone for the biosynthesis of androgens and is converted to e.g. testosterone in peripheral tissues. Both DHEA and glucocorticoids are synthesised from cholesterol, with a common intermediate - 17-hydroxy-pregnenolone (Genuth, 1983a). Therefore, their synthesis is initially similarly controlled, but then separately after this branch point in the shared synthetic pathway.

Since an increase in DHEA synthesis may result in subsequent decreased glucocorticoid synthesis, DHEA should be regarded as an indirect antagonist to glucocorticoids. For example, while elevated corticosterone concentration was shown to be essential in maintaining the overweight nature of Zucker rats (Alarrayed *et al.*, 1992), another study in these rats illustrated that short-term DHEA administration increased the mitochondrial respiratory rate in the livers of both lean and obese Zucker rats (Mohan & Cleary, 1988) to create a negative energy balance. This report suggests a direct anti-glucocorticoid, anti-obesity action of DHEA. The exact mechanism of DHEA's anti-glucocorticoid action remains unclear, since DHEAs alone was reported to be unable to prevent the activation of glucocortiocoid-inducible enzymes such as tyrosine aminotransferase (TAT) in Zucker rat liver and kidney (Wright *et al.*, 1992). However, given the recent reports of an imbalance in the relationship between cortisol and DHEA in various pathological conditions related to the endocrine and immune systems, such as HIV and depression (Christeff *et al.*, 2000; Gallagher & Young, 2002; Valenti, 2002), it may be of interest to consider changes in both the DHEA and glucocorticoid concentrations, or changes in the ratio between the two, when investigating the stress response.

1.4 Involvement of the endocrine stress-axis and immune system in the general stress response

1.4.1 Immune response

Monocytes and macrophages carry a common receptor (CD14) for different bacterial components on their surface membrane (Kreutz *et al.*, 1997). When an antigen, such as endotoxin, binds to this site, the cell becomes an antigen presenting cell (APC), presenting the antigen to helper T lymphocytes (which cannot bind directly to an antigen, since they lack the CD14 site) (Kreutz *et al.*, 1997). These helper T cells then become activated and secrete a range of cytokines: Type 1 helper T cells (T_H1 cells) secrete IL-2, IL-12 and IFN-γ, causing a positive feedback to activate more monocytes and macrophages, while T_H2 cells secrete IL-1, IL-10, IL-4, IL-5, IL-13 and a small amount of IL-6. IL-1 activates more monocytes to secrete large quantities of IL-6. IL-6 has several functions, both pro- and anti-inflammatory. Pro-inflammatory actions include stimulating the release of acute phase proteins which result in fever, stimulating T cell proliferation and B cell differentiation into immunoglobulin-secreting plasma cells. On the other hand, IL-6 was reported to also have anti-inflammatory actions, e.g. controlling the level of pro-inflammatory cytokines such as TNF-α, but not that of the anti-inflammatory cytokines such as IL-10 (Xing *et al.*, 1998), reducing the neutrophilia commonly associated with inflammation (Xing *et al.*, 1998), and acting on both the hypothalamus and pituitary gland in the HPA-axis to increase ACTH and cortisol secretion (Vander *et al.*, 1998a), and so increases the neutrophilia associated with exercise. Therefore, the inflammatory response to a bacterial stimulus results in a general stress response. However, the stress response may also be activated in the absence of an infectious agent. Exposure to a psychological stressor may also directly activate the interactions of cytokines and the HPA-axis. Perception of stress stimulates the hypothalamus to secrete corticotrophin releasing hormone (CRH), which in turn stimulates the anterior pituitary to secrete ACTH, initiating the general stress response.

1.4.2 Anti-inflammatory response

Cortisol has several anti-inflammatory functions, namely a) to inhibit prostaglandin-mediated vasodilation and increased vascular permeability, to prevent swelling in the area of damage or infection, b) to inhibit the margination and migration of white cells from the circulation to injury sites, c) to inhibit the leukotriene-facilitated phagocytic and bactericidal burst of neutrophils and d) to decrease the number of circulating helper T cells (Genuth, 1983a). Apart from these actions aimed at reversing inflammatory processes, cortisol also plays other roles in limiting the magnitude of the inflammatory response by inhibiting the action of TNF (Fantuzzi *et al.*, 1995), IL-1, IL-2 and IFN-γ, and causing arrest of lymphocyte proliferation in cell stages G_0 and G_1 , and lymphocyte apoptosis (Vander *et al.*, 1998a).

Although the anti-inflammatory functions of cortisol are required to prevent reactions that may seriously harm the organism, such as autoimmune reactions, chronically elevated levels of cortisol may in turn result in increased susceptibility to infection. Furthermore, cortisol has a number of other metabolic functions (see 1.4.3), which, although necessary in a stress situation, may in the long run not be beneficial to the organism.

1.4.3 Other stress-associated effects of glucocorticoids

Cortisol increases the conversion of amino acids (predominantly alanine and glutamine) to glucose in the liver. The increased gluconeogenesis helps to maintain liver glucose output and prevent hypoglycaemia during prolonged exercise. However, a chronically elevated cortisol concentration may lead to increased muscle breakdown above that required for conversion to carbohydrates, which may lead to cachexia, which itself has been linked to increased morbidity and mortality in various chronic diseases (Anker *et al.*, 1997; Anker & Sharma, 2002; Kotler *et al.*, 1989; Kotler, 1994; Zinna & Yarasheski, 2003). In human subjects, cortisol infusion was recently reported to result in prolonged changes in skeletal muscle amino acid patterns, similar to those reported early in protein catabolism (Hammarqvist *et al.*, 2001), illustrating a direct link between cortisol and skeletal muscle breakdown.

Another debilitating effect of chronically elevated cortisol is its down-regulation of osteoblast proliferation and collagen type I synthesis, which results in osteoporosis (Delany *et al.*, 1995). IL-6 was recently reported to up-regulate cortisol receptors in osteoblast-like cell lines, suggesting an interaction between cortisol and IL-6 in the progression of osteoporosis (Angeli *et al.*, 2002). Glucocorticoid-induced osteoporosis is a common complication of pathological conditions characterised by long-term sustained hypercortisolaemia, such as Cushing's syndrome (Di Somma *et al.*, 2003), or malnutrition, e.g. anorexia nervosa (Misra & Klibanski, 2002). However, osteoporosis may also be a secondary condition after long-term glucocorticoid replacement therapy, e.g. in Addison's disease (Jodar *et al.*, 2003). Bone loss of up to 15 % may occur within the first 3 to 6 months of chronic glucocorticoid therapy (Saag, 2004). Although female athletes, specifically those in sports requiring low body mass, are known to have an increased risk for suffering from osteoporosis, its cause in this population is most likely eating disorders, rather than chronically elevated cortisol (Warren & Goodman, 2003).

According to a recent review (Peters *et al.*, 2004), the mechanisms by which these negative long-term effects of increased circulating glucocorticoids occur is likely to be related to a stress-related "resetting" of the balance, or setpoint, of the limbic-hypothalamic-pituitary-adrenal axis, which is determined by the balance between high-affinity MR and low-affinity GR in the system. Therefore, development and progression of chronic diseases such as NIDDM and the metabolic syndrome, may be as a result of chronic stress-related disruption of the balance between the different types of glucocorticoid receptors.

1.5 Summary

It is clear that a situation of chronic stress, such as endurance training, may have severe consequences to many physiological systems. It is therefore imperative to study the mechanisms of and associations between systems involved in these responses, to limit long-term damage to an athlete's body. Although the stress response has been researched extensively, the concept of exercise as more than simply a metabolic stressor is relatively new, and many questions are still unanswered. Since a large part of the stress-related literature reports on studies performed in individuals with an underlying pathology or disease

state, results cannot always be extrapolated to a healthy, athletic population. Thus, population-specific and stressor-specific investigations should allow a more comprehensive understanding of the stress that exercise places on physiological systems in athletes.

However, since many of the long-term effects of exercise may be obscured by the great variability introduced by every-day life stressors, some studies using protocols of more extreme stress are warranted. In order to control the daily environment, most of these studies are done in animal models of stress (e.g. inescapable tail shock). However, the results obtained in these studies cannot necessarily be extrapolated to situations of exercise stress, since the severity of the stressor may override the influence of coping mechanisms which may be sufficient under physiological conditions of stress. Therefore, it is necessary to use mild stressors if the purpose of the investigation is to model the exercise stress response.

Chapter 2

Literature review

An acute stressor results in physiological changes that are, at least with regard to the immune system and endocrine stress-axis, usually only transient. Changes in immune competence for example, return to baseline after a few hours of recovery, rendering the body fully able to react to a new onslaught. However, when repeated stressors occur in short succession without sufficient recovery time, which is characteristic of a strenuous exercise training regimen or a continuously stressful occupation, these transient changes in immune and hormonal parameters do not always have enough recovery time to return to baseline between challenges. This may result in transient effects becoming more chronic, thereby rendering the body either unable to react competently to additional challenges for prolonged periods of time, or unable to down-regulate the stress response, leading to other chronic diseases. At least to a certain extent, the number and intensity of previous stressors will determine whether the body has adapted to cope with such an acute stressor, or whether previous onslaughts have resulted in a pathologically altered stress response.

This chapter will provide an overview of the available literature on the responses of the specific immune system and endocrine stress-axis to acute stressors, and their adaptation to chronic stressors. Particular attention will be paid to exercise as a stressor, but since the additional effects of psychological life stress cannot be excluded in a human population, I will also provide a brief overview of relevant literature pertaining to psychological stress. The effects of stress on the immune system and endocrine stress-axis will first be discussed separately, followed by an integration to point out interactions between these systems.

2.1 Immune and cytokine system responses to exercise stress

2.1.1 Responses to acute exercise stress

Cytokine response: Acute, long-duration, strenuous exercise such as a marathon or distance triathlon, has been shown to result in systemic endotoxaemia, due to leakage of intestinal *E.coli* lipopolysaccharide (LPS) into the circulation (Bosenberg *et al.*, 1988; Jeukendrup *et al.*, 2000). This occurs when the vascular wall loses integrity due to the ischaemia associated with strenuous exercise, when blood flow to non-vital systems is decreased in favour of skeletal muscle and skin. The endotoxin in circulation binds to monocytes and results in increased release of IL-6. Increases in plasma IL-6 concentration of up to 128-fold have been reported after exercise (Jeukendrup *et al.*, 2000; Ostrowski *et al.*, 1999; Steensberg *et al.*, 2001b), but the site of increased cytokine production remains a point of debate. Possible sources of exercise-induced IL-6, other than in response to endotoxaemia, will be discussed below.

In endurance athletes, monocytes isolated from blood collected before, immediately after and 20 minutes after a VO_{2max} test (cycling) were incubated in a cell culture (Rivier *et al.*, 1994). Spontaneous secretion of TNF-α and IL-6 was increased immediately after exercise, and that of IL-6 was still increased from baseline after 20 minutes of recovery, indicating that monocytes are a source of IL-6 during and immediately after exercise. A deficiency in this study design was that serum IL-6 concentration was not measured. Therefore, it was not possible to assess whether the magnitude of the contribution of monocyte IL-6 could be significant for the total increase in serum IL-6 seen after exercise in other studies (Jeukendrup *et al.*, 2000; Pedersen *et al.*, 2001; Steensberg *et al.*, 2001b). Spontaneous release of both TNF-α and IL-6 were positively correlated with maximum oxygen consumption (VO_{2max}), maximum ventilation and peak power output (PPO), suggesting that for monocytes, the stimulus to release these cytokines is dependent on the intensity of an exercise effort.

Muscle damage was previously named as another contributing factor to the increased plasma IL-6 concentration seen after exercise, with eccentric exercise resulting in a significantly greater increase in plasma IL-6 concentration compared to concentric exercise in healthy males (Bruunsgaard *et al.*, 1997). However, a subsequent study in rats indicated that contracting muscle exhibited an increase in IL-6 mRNA, independently of whether the muscle performed concentric or eccentric exercise (Jonsdottir *et al.*, 2000). While both concentric and eccentric muscle contraction in the rat study were controlled by electrical stimulation, that of the human study (forward and backward pedaling on a cycle ergometer) may not have represented stressors of equal magnitude, which may explain the inconsistency in results obtained by Bruunsgard *et al.* (1997) and Jonsdottir *et al*. (2000).

Another study in human subjects (Steensberg *et al.*, 2000) indicated that during one-legged concentric exercise, the femoral artery-femoral vein (a-v) difference in IL-6 concentration in the exercising leg paralleled the net increase in mixed venous circulating IL-6 concentration, while no a-v difference was observed in the non-exercising leg. This led to the conclusion that muscle contraction, and not endotoxemia, was responsible for the post-exercise increase in IL-6 concentration. I propose that since these subjects were exercising only one limb at 40 % of maximum effort, the intensity of exercise was probably not enough to result in gut ischaemia with endotoxaemia or muscle damage, or to result in a significant endocrine-immune stress response. The study design does not make it possible to consider these confounding factors and the results should be considered conclusive only for low intensity exercise. Although another, earlier study is in agreement with the suggestion that PBMCs are not responsible for IL-6 increases (Ullum *et al.*, 1994), on the grounds of finding no change in PBMC mRNA concentrations for TNF-α, IL-1α, IL-1β and IL-6 after a 1-hr cycle at 75 % VO_{2max}, there are several confounding factors contributing to the conclusions of this study. Firstly, the method used, detection of IL-6 mRNA in purified PBMCs by Northern blotting analysis, may not have been sensitive enough for detection of very small changes. In comparison, the highly-sensitive quantitative competitive PCR techniques used more recently, measured increased IL-6 mRNA in skeletal muscle after exercise in rats (Jonsdottir *et al.*, 2000). Secondly, while electrical stimulation of skeletal muscle, as used by Jonsdottir *et al*. (2000), results in homogenous stimulation of muscle cells, exercise does not necessarily activate IL-6 production in all monocytes and T_H2 lymphocytes, and a sample of

isolated PBMCs will also contain cells which are not capable of secreting IL-6, such as NK cells and T_H1 lymphocytes. Therefore, the availability of mRNA would be much lower in the PBMC sample compared to a skeletal muscle sample, which makes detection of small changes in response to exercise less likely. Thirdly, the analyses for the various cytokine mRNAs were performed for only 1-3 of the 17 athletes participating in the study by Ullum *et al*. (1994), which further decreases the likelihood of detecting stress-induced changes in mRNA. These results therefore do not conclusively exclude PBMC involvement in the IL-6 release seen after exercise. On the other hand, more recent studies, using flow cytometry, more clearly illustrated that prolonged strenuous exercise results in decreased monocyte production of IL-6 (Starkie *et al.*, 2001a; Starkie *et al.*, 2001b). Therefore, we conclude that acute, strenuous exercise is likely to suppress monocyte-mediated inflammatory capacity directly post-exercise.

Oxidative stress was recently suggested as the major stimulus for exercise-induced cytokine production (Vassilakopoulos *et al.*, 2003), since a blunted plasma cytokine response (TNF-α, IL-1β and IL-6) to exercise was observed in healthy non-athletes after administration of oral antioxidants for 2 months. In addition, the authors argued against a role for monocytes in exercise-induced cytokine release, since they found no effect of either their exercise protocol (45 minutes cycling at 70 % VO_{2max}) or antioxidant administration on monocyte count or monocyte intracellular cytokine production from pre- to post-exercise. However, monocyte counts reported tended to be increased immediately after exercise. It is possible that statistical significance was not achieved as a result of the low subject numbers (n = 6), combined with the high variability in monocyte counts reported and the short duration of the exercise protocol. A significantly increased post-exercise monocyte count was reported previously after longer-duration exercise in both athletes and non-athletes, using larger subject numbers (n = 9 to 17 per study) (Rhind *et al.*, 2001; Rohde *et al.*, 1996; Ullum *et al.*, 1994). Also, insufficient data is provided on the performance or fitness level of subjects at the two time points, or their participation in exercise in-between, to enable assessment of changes in fitness levels, which may have been a confounding factor. Although the blunting effect of antioxidants on plasma cytokine concentration is a valid and important finding, which may be useful in precautionary supplementation regimens, more investigation is

necessary before monocytes can be excluded as modulators in the cytokine response to exercise.

Considering the results of all studies mentioned in this section, muscle damage seems unlikely as a sole cause of post-exercise circulating serum IL-6 concentration, while endotoxaemia-stimulated monocytes and contracting muscle have now both been established as contributors to IL-6 secretion, and therefore initiation of inflammation during exercise. The relative importance of these contributors may depend on the exercise protocol itself. Further investigation is warranted to determine the role of oxidative stress and muscle damage in the changes in circulating cytokine concentrations after exercise.

The cytokine response to an acute exercise bout is only transient, with both pro-inflammatory cytokines, particularly IL-6, and anti-inflammatory cytokines, such as IL-10, reported to reach peak plasma concentrations within 2-3 hours after starting an exercise bout and then to decline steadily during recovery (Ostrowski *et al.*, 1999). The magnitude of IL-6 secretion was also shown to be dependent on the mode of exercise, fitness and nutritional state of the athlete (for a recent review, refer to Shephard, 2002).

White cell quantitative response: Immune cells are able to undergo rapid demargination and redistribution between compartments, resulting in changes in the number of cells in circulation even without any proliferation. This process is the result of mechanical sheer stress on vascular walls (Bierman *et al.*, 1952; Foster *et al.*, 1986) and neuroendocrine signals (McCarthy & Dale, 1988) associated with exercise. The latter signals are mediated mainly by the activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis, i.e. via release of catecholamines and glucocorticoids (Miller *et al.*, 1998). Although heat has been suggested as the cause of exercise leukocytosis, thermal clamping studies enabled researchers to exclude a core temperature rise as a critical mediator of lymphocyte redistribution (Rhind *et al.*, 1999).

Prolonged endurance exercise of moderate intensity (60 – 80 % VO_{2max} for 2 – 2.5 hours) is associated with a leukocytosis during or immediately after exercise, depending on the duration of the bout (McCarthy & Dale, 1988; Shephard & Shek, 1996). Although

lymphocytes show greater relative (percentage) increases, neutrophils are the biggest absolute contributors to this exercise-induced leukocytosis. Exercise of shorter duration and higher intensity elicits a similar response. For example, one study reported an average increase of 114 ± 20 % in total lymphocyte count after incremental treadmill running to exhaustion, while neutrophil count increased by a relatively moderate 34 ± 7 % (Gleeson *et al.*, 1995).

The extent of exercise-induced lymphocytosis was shown to be related to both exercise intensity (Nieman *et al.*, 1993) and exercise duration (Natale *et al.*, 2003). For example, after 45 minutes of exercise at either 50 % or 80 % VO_{2max}, the higher intensity exercise was associated with a greater increase in the proportion of NK cells from pre- to post-exercise (Nieman *et al.*, 1993). Also, while both long duration endurance exercise (2 hours cycling at 60 % VO_{2max}) and short-duration high-intensity exercise (5 minutes cycling at 90 % VO_{2max}) resulted in an increased lymphocyte count, the effect of the longer-duration exercise was of greater magnitude when compared to the short-duration exercise (Natale *et al.*, 2003). Not all lymphocyte subpopulations contribute equally to this lymphocytosis. Despite the fact that increased recruitment of NK cells into the circulation is the main contributor (Mazzeo *et al.*, 1998; Nieman *et al.*, 1993), relatively greater increases occur in B compared to T cells, resulting in a lowered T:B cell ratio (McCarthy & Dale, 1988; Shephard & Shek, 1996). Immature T lymphocytes are more cortisol-sensitive than mature T cells (Hoffbrand & Pettit, 1994a; Vander *et al.*, 1998a). The relative decrease in T cells reported in stress situations may be the result of increased sequestration of these cells to the bone marrow (Hässig *et al.*, 1996). Furthermore, greater increases occur in $CD8^+$ than in $CD4^+$ T cell counts (Natale *et al.*, 2003; Shephard & Shek, 1996), so that the $CD4^+$:$CD8^+$ ratio decreases with exercise. The magnitude of this change in ratio was shown to be independent of exercise type, since similar results were obtained after eccentric (Pizza *et al.*, 1995b), aerobic concentric (Nehlsen-Cannarella *et al.*, 1991a; Nieman *et al.*, 1995) and anaerobic concentric (Deuster *et al.*, 1999) exercise. Resistance exercise (3 sets of 10 repetitions at 60 – 70 % of 1-RM) and high-intensity cycling (5 minutes at 90 % VO_{2max}) were shown to have a longer-lasting effect on $CD4^+$:$CD8^+$ ratio when compared to submaximal endurance exercise (2 hours cycling at 60 % VO_{2max}) (Natale *et al.*, 2003). Therefore, the decreased $CD4^+$:$CD8^+$ ratio may be one of the most consistent immune system responses to acute exercise.

Given the controversy around plasma cytokine concentration increases in response to acute exercise, it has become more important in the recent past to consider results of *in vitro* cell culture studies to assess the influence of exercise on the ability of immune cells to secrete cytokines. In order to assess whether non-specific and specific immune functions are affected similarly, some studies specifically assessed changes in T cell cytokine production. Variable results have been obtained, depending on the study design. A study in mice investigated possible links between increased incidence of infection after strenuous exercise and changes in the specific T cell cytokine response (Kohut *et al.*, 2001). Mice were subjected to acute exhaustive treadmill running, after which they were infected with a herpes simplex virus (upper respiratory infection), and sacrificed on days 2 and 7 post-infection. Two days post-infection, spleen cell cultures exhibited suppressed cytokine production of both T_H1 (IL-2, IFN-α and IL-12) and T_H2 (IL-10) cytokines, as well as decreased NK cell cytotoxicity, when compared to non-exercising control mice. All parameters were similar to controls on day 7. Similarly, in humans, exhaustive exercise decreased the capacity of white blood cells in a whole blood culture to secrete IFN-γ, IL-1β and TNF-α immediately post-exercise in response to LPS (Baum *et al.*, 1997), again suggesting general inhibition of T cell function. Also, IFN-γ, IL-6, TNF-α, IL-1 and IL-2 release after *in vitro* stimulation with LPS or PHA in whole blood culture were reduced after 68 minutes of exhaustive exercise in triathletes (Weinstock *et al.*, 1997). On the other hand, a single session of high intensity cycling exercise (8x 10-second maximum effort cycling bouts separated by 5 minutes) was reported to result in inflammation (increased IL-6 and CRP concentration), with some biological markers of inflammation (increased CRP) persisting for more than 24 hours after cessation of exercise (Meyer *et al.*, 2001). Similarly, *in vitro* studies using whole blood culture techniques reported increased mitogen-induced cytokine (IFN-γ and IL-2) secretion after moderate intensity exercise (30 minutes cycling at 70% anaerobic threshold) (Baum *et al.*, 1997). Therefore, while acute exhaustive exercise seems to suppress both T_H1 and T_H2 cytokine secretion, acute moderate or intermittent exercise appears to have the opposite effect.

Although the above-mentioned *in vitro* studies provide indirect information on possible changes in cytokine concentrations in circulation, it is unclear whether such changes could

be a result of changes in individual cell function, or changes in cell count. Some studies have investigated these issues *in vivo* by assessing cell type-specific cytokine production after exercise, in order to more fully explain the effect of exercise on immune cells. For example, an acute bout of strenuous exercise (2.5 hours at 75 % VO_{2max}) resulted in a decreased $CD4^+$ T cell count during recovery, which was accounted for by a decrease in mainly T_H1 cell numbers, and thus decreased capacity for cell-mediated immunity (Steensberg *et al.*, 2001a). Similar results were subsequently reported after 1.5 hr running at 70% VO_{2max}, with an unaltered T_H2 response (IL-2 and IL-4), but decreased T_H1 response (IFN-γ) (Ibfelt *et al.*, 2002). Both the cytokine and endocrine systems seem to contribute to these changes, since IL-6 correlated positively with the number of T_H2 cells secreting IL-4 in the first study (Steensberg *et al.*, 2001a), thus enhancing T_H2 lymphocyte-mediated immunity, while epinephrine correlated negatively with the number of T_H1 cells secreting IL-2 (Steensberg *et al.*, 2001b; Steensberg *et al.*, 2001a), which may indicate a suppressive role of epinephrine on cell-mediated immunity.

Taken together, these results imply that while moderate duration, moderate intensity exercise seems to enhance immune function, prolonged, strenuous exercise has the opposite effect, with the T_H1 lymphocyte-mediated immune system suffering the greatest negative effect. A recently published review (Smith, 2003) supports this conclusion.

During recovery after exercise, the leukocytosis of exercise is reversed, with the $CD4^+$ cell count recovering to resting concentrations, while $CD8^+$ cell counts are usually lower than baseline. This was suggested to be the result of migration of these cells to either injured muscle or lymphoid tissue (Shephard & Shek, 1996), but it has not been substantiated. While the neutrophilia may persist for up to 24 hours post-exercise (Nieman *et al.*, 1989a), 2 – 3 hours of recovery is usually sufficient for most lymphocyte subpopulation counts to return to baseline, in a manner seemingly independent of the duration of the exercise bout, since similar time frames for lymphocyte recovery have been reported after 45 minutes, 1.5 and 3 hours of endurance exercise (Natale *et al.*, 2003; Nieman *et al.*, 1989a; Nieman *et al.*, 1994).

There is still some controversy surrounding the influence of muscle microdamage after exercise, on immune parameters in circulation during recovery. For example, a reduced

circulating cell count may be due to infiltration of these cells into injured muscle. A recent study showed that the immunological changes in skeletal muscle after delayed onset muscle soreness (DOMS)-inducing eccentric cycling exercise were characterised specifically by increased macrophage and neutrophil infiltration into muscle, illustrated by immunostaining of biopsy samples, with a peak infiltration evident at 24 hours post-exercise (Malm *et al.*, 2000). No change from baseline was evident in muscle total T or B cell concentrations. One possible explanation for this result is that muscle microdamage only impacts on the non-specific immune system. On the other hand, baseline biopsy samples had very low concentrations of lymphocytes, and changes in T cell distribution are exercise-intensity dependent. Therefore, the moderate-intensity, short duration protocol followed in this study (250-300 W for 30 minutes), although of eccentric nature and sufficiently severe to result in DOMS, may not have represented a stressor severe enough to result in muscle lymphocyte infiltration of sufficient magnitude to allow for changes in cell counts to be detected in needle biopsy samples. The former opinion is most likely, since changes observed in the circulating immune cell counts support the theory of an effect only on the cell-mediated immune system: neutrophil, monocyte, suppressor/cytotoxic T cell and NK cell counts were all increased immediately post-exercise, while $CD4^+$ T cell and B cell counts were not affected. Therefore, I suggest that skeletal muscle microdamage is not a significant contributor to the activation of the specific immune system after exercise. This is supported by previous studies (discussed earlier) which together illustrated no additional effect of eccentric exercise on exercise-induced changes in $CD4^+$:$CD8^+$ ratio (Deuster *et al.*, 1999; Nehlsen-Cannarella *et al.*, 1991a; Pizza *et al.*, 1995b).

Although the reported post-exercise changes in total white cell counts and subpopulation distribution give an indication of the availability of cells which may react to a pathogenic challenge during recovery, these data are not sufficient to draw conclusions on changes in actual responsiveness of immune cells, and changes in cell function should also be considered.

T cell functional response: High intensity exercise (45 minutes level running at 80 % VO_{2max}), but not moderate exercise (45 minutes graded treadmill walking at 50 % VO_{2max}), resulted in a 21 % decrease in ConA-stimulated (T cell) lymphocyte proliferative response at

1 hour post-exercise (Nieman *et al.*, 1994). Similarly, 45 minutes of walking at 60 % VO_{2max} did not affect the T cell lymphocyte proliferative response in fit females (Nehlsen-Cannarella *et al.*, 1991b), while 2.5 hours of running at 75 % VO_{2max} decreased T cell proliferative response in experienced marathon runners (Nieman *et al.*, 1995). This depressed function lasted for more than 3 hours post-exercise. These different studies clearly indicate that endurance exercise is not detrimental to T cell functional response unless the intensity and/or duration are quite extreme. The observed continuation of this depressed function into the recovery period has led to the "open window" theory for increased infection risk (Nieman & Pedersen, 1999).

Although endurance athletes or endurance exercise is most frequently studied, other types of exercise have also been reported to affect immune function. Acute supra-maximal interval training sessions (25 x 1-minute bouts of running or kayaking, with 2-minute rest in between bouts) in athletes varying from recreational to high-performance level, were shown to decrease T cell proliferative response post-exercise, but only transiently, with values returning to baseline by 2 hours post-exercise (Fry *et al.*, 1992). Lymphocyte proliferative response therefore seems to be negatively affected by various types of extreme exercise bouts, i.e. either continuous or intermittent high intensity, or long duration bouts.

Although assessment of change in the lymphocyte proliferative rate is an accepted, useful functional test to assess changes in immune ability, it cannot differentiate between T cell subpopulations, and therefore provides information about total T cell function only. However, it is possible that some subpopulations with opposing functions, such as $CD4^+$ and $CD8^+$ T cells, are affected differently by exposure to a stressor, so that one cell type has depressed function and another enhanced function. Although this would return a net result of "no change" in the lymphocyte proliferative response assay, it clearly does not provide the full picture. An alternative test, assessing cell activation by measurement of the activation marker CD69, a more upstream event, is able to differentiate between T cell subpopulations, enabling more comprehensive analysis and interpretation of changes in immune function. Few studies have investigated changes in T cells' reactivity, as indicated by CD69 expression, in response to exercise. Gabriel *et al*. investigated the effect of a 240 km cross-country cycle race or an ultra-triathlon in 12 endurance athletes, by measuring the CD69

response at rest and after acute exhaustive exercise two weeks before and 8-9 days after the racing event (Gabriel *et al.*, 1993). No changes were apparent in NK and non-NK cell CD69 expression in response to the racing event. This was the first exercise study to use CD69 expression as a functional test, and there were several factors in the protocol making it difficult to fully interpret results. Firstly, follow-up samples were taken 8-9 days after the event, at which time CD69 expression was similar to baseline, but this does not illuminate whether a change in CD69 expression may have occurred at an earlier post-race time point. Secondly, results reported seem to be pooled data for resting and post-exercise samples, which may mask changes. Thirdly, T cells were divided into only 2 subgroups, namely NK and non-NK, which may mask alterations in relative activation between e.g. $CD4^+$ and $CD8^+$ cells. These results are therefore not useful in determination of T cell activation status in response to strenuous exercise.

A recent report by the same research group investigated both CD69 expression and lymphocyte proliferation rate in response to acute exercise (Green *et al.*, 2003). Similar counts for $CD4^+CD69^+$ and $CD8^+CD69^+$ cells were reported at rest and after 1 hour of moderate intensity treadmill running (95 % of ventilatory threshold). Lymphocyte proliferative response was decreased after exercise, but was similar to the resting response when corrected for changes in lymphocyte count. This resulted in the conclusion that this type of exercise did not influence T cell function. However, it is doubtful whether the moderate exercise intensity protocol used would have elicited a stress response in the well-trained subjects who participated in the study, given the results of studies discussed on the previous pages. Therefore, these findings still do not elucidate whether or not CD69 expression is a useful marker of immune status in athletes who are used to high-intensity training.

Studies by other groups do report suppressive effects of acute bouts of exercise on lymphocyte activation. One of these studies reported decreased CD69 expression on $CD4^+$ and $CD8^+$ cells after acute exhaustive exercise (Vider *et al.*, 2001). Of importance is that the T lymphocyte proliferative response immediately after exercise was also decreased in this study. Similarly, repeated bouts of cycling on the same day (75 minutes at 75 % VO_{2max}, with 3 hours recovery between bouts) were reported to have a cumulative suppressive effect on the percentage of NK cells expressing CD69 post-exercise (Ronsen *et al.*, 2001c). The

report of no significant change in the percentage of $CD4^+$ and $CD8^+$ cells expressing CD69 in the same study is not inexplicable, since the exercise protocol used in the latter study was less strenuous than the one used by Vider *et al.* (2001). Unfortunately, absolute $CD69^+$ cell counts were not provided in the study by Ronsen (2001b), so that the influence of exercise-induced changes in cell count cannot be integrated in my interpretation of their results. A subsequent study investigated the effect of 3 hours *vs*. 6 hours of recovery between acute bouts of endurance exercise (again 75 minutes cycling at 75 % VO_{2max}) (Ronsen *et al.*, 2002). Although no significant differences in proportions of either NK, $CD4^+$ or $CD8^+$ T cells expressing CD69 were found between the two recovery protocols, the longer recovery protocol was associated with a smaller exercise-induced increase in the absolute number of $CD8^+$ cells expressing CD69 when compared with the shorter recovery protocol. This finding stresses the importance of assessing cell counts in conjunction with functional assays, since the net effect of changes in cell count and function should be interpreted for a comprehensive understanding of immune changes. These reports suggest that the immune system may not recover to baseline levels for several hours after an acute bout of strenuous exercise, stressing the importance of sufficient recovery time between strenuous training sessions to ensure maintenance of immune competency. In addition, the longer recovery protocol was also related to a smaller catabolic hormone response to the second bout (see 2.3.1), indicating the necessity for combined assessment of the immune and stress hormone systems.

2.1.2. Responses to chronic exercise stress (training)

Susceptibility to infection: Earlier investigations suggest progressively increased susceptibility to infection with chronic training at intense levels over the course of a season (Tomasi *et al.*, 1982), with ultra-marathon athletes reportedly suffering a significantly greater number of upper respiratory tract infections (URTI) than a control group (Peters & Bateman, 1983; Shephard & Shek, 1993), and faster athletes reporting more episodes of illness than slower runners. On the other hand, participation in moderate exercise does not seem to precipitate illness, but rather to shorten the duration of illness, and to increase the resistance to infection (reviewed by Shephard and Shek, 1993). Although there is general acceptance of this “J-shaped hypothesis” of infection risk (Mackinnon, 1992; Nieman, 1994) – according

to which, light to moderate exercise decreases URTI risk, while extreme exercise increases risk for URTI – not all researchers are in agreement. Some groups argue that athletes adapt to their chronic training regimens, since an epidemiological study has shown that elite skiers suffer a similar number of episodes of infectious disease (of which 74 – 93 % were URTI) when compared to non-trained individuals (Berglund & Hemmingsson, 1990).

Reports on the effect of training on immunoglobulin release and mucosal immunity are varied. Some research groups report a steady decline in salivary immunoglobulin concentrations with chronic exposure to endurance exercise (running, cycling, swimming and cross-country skiing), with resting concentrations of IgA, IgG and IgM decreasing significantly over time, as well as decreased IgA secretion after acute exercise bouts in elite swimmers (Gleeson *et al.*, 2000; Gleeson, 2000). However, in elite female rowers, IgA concentrations at rest were reported to be 77 % higher when compared to a non-athlete control group, with no effect of acute exercise (2 hours of rowing training) on salivary IgA concentrations or secretion rates measured before, immediately after and 1.5 hours after exercise in the elite athletes (Nehlsen-Cannarella *et al.*, 2000). In addition, the same group reported 20% increases in resting IgA, IgG and IgM concentrations in sedentary subjects after moderate training (5 sessions of 45 minutes walking per week) for 6 weeks, but which was attenuated after 15 weeks of training (Nehlsen-Cannarella *et al.*, 1991a). Other studies showed no differences in salivary concentrations of IgA, IgG and IgM of endurance athletes at rest, after graded maximal exercise or during recovery, or compared to sedentary controls (Kajiura *et al.*, 1995; Nieman *et al.*, 1989c). Although hydration status may influence results, the results obtained from saliva samples in the mentioned studies seem to be dependent on the research group reporting it, suggesting that values obtained were most likely influenced by inter-laboratory differences in the technique of sample collection, resulting in the varied findings reported. For these reasons, I have not been able to draw a conclusion from the available literature on the effect of training on immunoglobulin secretion. Therefore, I would not recommend measurement of salivary immunoglobulin concentrations as an indicator of immune status.

Cytokine response: During the early stages of acute local inflammation, macrophages and fibroblasts secrete cytokines and chemokines which result in changes in the expression of

adhesion molecules and inflammatory receptors on vascular endothelial cells supplying the inflamed tissue. This results in recruitment of large numbers of leukocytes to the injured tissue (Butcher *et al.*, 1999). However, for tissue repair to take place, the redundant or dead cells need to be cleared. A second effect of chemokine release is activation of dendritic cells to migrate with the antigen (and antigen presenting cells) to draining lymph nodes, where a specific immune response takes place. This transition from the inflamed area to the lymph nodes coincides with tissue repair and leads to immune memory (Buckley *et al.*, 2001). However, during chronic local inflammation, this transition does not take place due to failure of fibroblasts to stop the production of chemokines, resulting in increased chemokine release and thus increased recruitment and prolonged retention of leukocytes in tissue, preventing repair (Buckley *et al.*, 2001). This in turn results in chronic systemic inflammation, which was recently proposed to be the major cause of the overtraining syndrome (Smith, 2000).

Furthermore, monocytes of endurance trained individuals were reported to be more sensitive to LPS at rest than the monocytes of untrained individuals, so that they secreted similar IL-6 concentrations compared to the monocytes of untrained individuals (168.4 $\pm$ 29.9 pg/ml *vs*. 160.1 $\pm$ 34.4 pg/ml), but in response to much lower concentrations of added LPS in culture (0.003 μg/ml for endurance athletes vs. 0.03 μg/ml for untrained subjects) (Duclos *et al.*, 1999). In addition, PBMCs of endurance athletes also exhibited increased sensitivity to stimulation by exogenous glucocorticoids at rest. These results suggest an upregulation of the immune response to stress after exercise training, possibly to cope with a chronic low-grade exposure to LPS during training and racing. However, this increased sensitivity at rest was abolished after exercise in the same study. I suggest that this may be due to some counter-regulatory mechanism, which prevents “overshoot” of the immune response in response to an additional acute stressor. Although such a protective mechanism may be beneficial in preventing severe inflammation after e.g. competitive events, the long term effect of this “immune suppression” remains to be elucidated. Also, it is possible that this capacity for counter-regulation is not always successful. This issue should be further investigated, as it may support, at least in part, the hypothesis of increased IL-6 release as cause of the overtraining (underperformance) syndrome (Robson, 2003).

White cell response: Both short-term (Pizza *et al.*, 1995a; Verde *et al.*, 1992) and longer term training (Baj *et al.*, 1994; Shore *et al.*, 1999) were reported to significantly decrease resting total T cell count, $CD4^+$ T cell count and $CD4^+$:$CD8^+$ ratio. In a study on rats (Hoffman-Goetz *et al.*, 1986), chronic exercise stress was shown to reduce splenic T lymphocyte proliferation in response to mitogen stimulation, which suggests that the decreased resting $CD4^+$ cell counts reported in human training studies, may be the result of decreased release of cells from the spleen into circulation. In elite runners, 3 weeks of training at 138 % of usual training volume, also had suppressive effects on resting $CD4^+$ counts and the $CD4^+$:$CD8^+$ ratio, but not on the lymphocyte proliferative response, when compared to baseline (Verde *et al.*, 1992). However, an acute bout of exercise (30 minutes high-intensity) after the training period resulted in a transient depression of lymphocyte proliferation rate that was not apparent after a similar acute exercise test at baseline. Similarly, in runners, a 40-day period of increased training load (increased volume, or increased intensity, or both) resulted in a decrease in $CD4^+$ T cell count and the $CD4^+$:$CD8^+$ ratio at rest, the magnitude of which was more dependent on training intensity than training volume (Kajiura *et al.*, 1995). After a period of taper, the $CD4^+$:$CD8^+$ ratios had returned to baseline values in both studies (Kajiura *et al.*, 1995; Verde *et al.*, 1992), suggesting reversibility of training-induced changes in immune competence. However, these studies do not enable a conclusion with regard to the combined effects of long-term training and insufficient recovery time on immune competence.

Not all published papers are in agreement with the results obtained in the above studies. Recently, ten weeks of training in competitive cyclists, which included volume-building (6 weeks), high-intensity (18 days) and unloading (10 days) phases, were reported to have no effect on resting total T cell count, T cell subpopulation counts, relative distribution of T cell subpopulations, serum concentrations of IL-1β, IL-6 and TNF-α or urinary excretion of cortisol and testosterone (Dressendorfer *et al.*, 2002). This led the authors to conclude that immune status is maintained during training. However, 36-44 hours of recovery were allowed after each phase, prior to each resting immune assessment. My interpretation of the available literature is that during intense training, the main reason for chronically altered immune parameters, is the fact that athletes do not allow themselves sufficient recovery periods between strenuous bouts. The results of the latter paper suggest that immune

parameters may recover to baseline after 1.5 – 2 days of recovery from a short-term increased training program (10 weeks). However, since few or no elite athletes afford themselves this much rest on a regular basis, the results of this study cannot be seen as the typical effect of training stress, but they do show that athletes could benefit from two days of rest prior to competition, and worked into their within-season periodisation.

It is important to note, once again, that the decreased T cell counts reported after training do not necessarily indicate decreased immune function. Although the resting $CD4^+$ cell count was shown to decrease after training (Baj *et al.*, 1994), *in vitro* stimulation of white blood cells with PHA and ConA revealed an increased T lymphocyte proliferation rate in endurance trained athletes at rest in the same (Baj *et al.*, 1994) and other studies (Fry *et al.*, 1992; Verde *et al.*, 1992). I propose that this may be a compensatory mechanism for the decreased $CD4^+$ cell numbers. Therefore, changes in cell count and cell function should preferably be considered in combination, to enable accurate assessment of immune competency. Although it has been established that T cell proliferation rate increased in response to training, it is unclear whether $CD4^+$ and $CD8^+$ T cell subpopulations are affected similarly, or whether these reported changes are subpopulation specific, since measurement of the lymphocyte proliferative response does not allow distinction between T cell subpopulations. Although these distinctions could be made with assessment of CD69 expression in T cells in response to training, this has not been investigated. This parameter could provide more information regarding the specific site of training-induced adaptation, i.e. whether it is at the level of cell activation (CD69) or downstream (cell proliferation), as well as which subpopulations are affected most, or which may be unresponsive to training stress. Since $CD8^+$ cells are either immune suppressive (suppressor cells) or part of the cell-mediated immune response (cytotoxic cells), while $CD4^+$ cells have their function in the antibody-mediated immune response, investigating the effect of training on responsiveness of these two parameters separately (i.e. using CD69 expression instead of lymphocyte proliferative response) could provide more useful information pertaining to the balance between the different sections of the immune system and its implication for overall immune competency.

2.1.3. Responses to overreaching and overtraining

Before considering the symptoms of the overtraining syndrome (OTS), it is necessary to clarify some terms that are commonly confused. Overtraining is a short-term overworking of an athlete, during which training is at abnormally high volume or intensity, with insufficient recovery time between bouts, leading to a decline in work capacity (Brown *et al.*, 1983; Ketner & Mellion, 1995). Overtraining is very common in athletes, who often try to correct the decline in performance by training harder, rather than allowing themselves rest. Several reports of athletes giving their best performances after a period of illness or forced rest, supports this contention (Brown *et al.*, 1983). Overreaching is a controlled form of overtraining that often forms part of a specific phase in a training program (Ketner & Mellion, 1995), with the aim of supercompensation during a planned phase of decreased training following the overreaching. While the symptoms of both overreaching and overtraining can be reversed if a sufficient recovery and regeneration period is allowed, the overtraining syndrome (also called the unexplained underperformance syndrome) is the final stage of increasingly severe chronic fatigue, which develops as a result of long-term overtraining (Ketner & Mellion, 1995). This syndrome has many long-lasting symptoms, including decreased performance (Urhausen & Kindermann, 2002), disturbances in mood state (Urhausen & Kindermann, 2002), chronic lymphadenopathy and immunosuppression (Barron *et al.*, 1985; Fry *et al.*, 1991b; Kuipers & Keizer, 1988), resulting in an increased frequency of infection and illness. This syndrome requires several months of recovery (Fry *et al.*, 1991b; Kuipers & Keizer, 1988; Lehmann *et al.*, 1998). Apart from the obvious problems associated with illness, frequent interruptions in training or the inability to compete in competitions also have negative financial and competitive ranking implications for an athlete. It is therefore vital to understand the sequence of events leading to the overtraining syndrome, so that it can be recognised and managed in its early stages.

Cytokine response: The OTS has been likened to a “chronic acute phase response” (Eichner, 1995). Cytokines, in particular the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, have been suggested to be key role players in the development of the OTS (the cytokine hypothesis of overtraining) (Robson, 2003; Smith, 2000). However, resting serum IL-1 and IL-6 concentrations were reported to be similar in overtrained athletes compared to

controls (Parry-Billings *et al.*, 1992). Resting concentrations of plasma IL-6 and TNF-α were recently reported to remain unchanged after overreaching in endurance-trained cyclists (Halson *et al.*, 2003), which does not support these hypotheses of the mechanisms for overtraining. However, the overreaching period in this study lasted only two weeks, and was preceded by baseline training. This relatively short period of overreaching, after a period of relatively moderate training, may simply have been too short to be sufficiently stressful to result in chronic cytokine elevations at rest.

White cell response: In a study on 10 overtrained athletes from various sporting backgrounds, total white cell count and differential counts at rest were within the normal ranges, with the only significant exception a high monocyte count in one of the athletes (Rowbottom *et al.*, 1995). Lymphocyte subpopulation distribution was within the normal range, suggesting no direct effect of overtraining on the immune cell counts. Markers of immune cells' functional capacity have been determined in only two studies of overtrained endurance athletes. These athletes, who exhibited no differences in lymphocyte counts or distribution, also did not differ for lymphocyte activation at rest or after an acute bout of exercise, when compared to non-overtrained controls (Gabriel *et al.*, 1998). However, in the latter study, CD45RO expression on T lymphocytes was increased in the overtrained athletes, which may suggest that these athletes were suffering from infections. Therefore, no conclusion can be drawn from these results.

Most studies are of cross-sectional nature and report white blood cell counts and function within the normal range, rather than change over time. This is understandable since the OTS is relatively rare, has a long aetiology and cannot be ethically inflicted for research purposes. However, this may explain the contradictory results reported, since more subtle changes in the white blood cell count, while staying within the normal range, may nevertheless significantly impact on an individual's ability to mount an immune response.

Another problematic issue which complicates the interpretation of studies investigating the effects of exercise stress in humans, is that these individuals may also be exposed to other additional stressors, such as occupation-related stress, or the constant psychological pressure to excel. Given the general nature of the stress response, various different

stressors may impact on the immune or endocrine system fairly similarly, and thus additively, if present at the same time. Since psychological stress may contribute significantly to the overall stress status of athletes, it is therefore necessary to also consider the effect of psychological stressors on these systems.

2.2 Immune and cytokine responses to psychological stress

As mentioned in the previous paragraph, many scenarios in life may elicit a stress response, including various forms of psychological stress. For the purposes of this thesis, I will first provide a brief description of typical models of psychological stress used for research, followed by an overview of the results obtained in studies using these models.

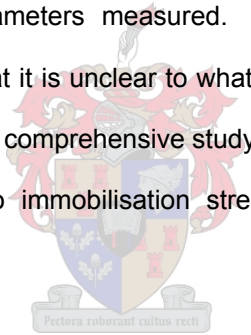
In human subjects, stress prior to examinations is the model most commonly used to investigate the effect of psychological stress on immune function. However, mental stress may also be experimentally induced, by subjecting participants to mental tests, such as the Stroop colour-word interference task, mirror tracing tasks, or mental arithmetic in front of an audience. In rats, immobilisation stress and social stress models are most commonly used. With the models for immobilisation stress, various levels of severity may be achieved by either prone restraint (a severe stressor), where the animal is taped down on a wooden board, or immobilisation in a tube or small chamber (a mild stressor). For the model of social stress, male-female pairs of rats are housed together (one pair per cage) for a few weeks. When taking one of these males from his female and placing him with another male-female pair, social confrontation for dominance results between the intruder and the resident male. Indicators of subdominance (e.g. retreat, flight and a full defensive posture) are noted for a specific time period. A dominance index is calculated, by scoring the number of subdominant events the intruder male has displayed himself, or triggered in the resident rat. If this score is <0.5, the intruder rat is named a loser (subordinate) rat, otherwise it is named a winner (dominant) rat. The effect of stress in loser rats may be assessed by comparison of results to baseline values for the same rats, or by comparison to control rats, which were left undisturbed with their females, or by comparison to winner rats. In the next section, I will provide a brief overview of results from representative studies using these models.

Cytokine response: With regard to the physiological response to psychological, work-related stress in human subjects, most previous studies have focused on mental health itself and cardiovascular disease risk (Spurgeon *et al.*, 1997). Other potential conditions related to psychological stress, such as musculoskeletal symptoms and immune suppression, have received very little attention. However, a few studies are available on the acute, as well as the more long-term, effects of examination stress in students. The psychological stress of academic examinations was reported to result in increased circulating concentrations of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ, as well as the T_H1-cell inhibitor, IL-10 (Maes *et al.*, 1998). In addition, students with higher stress perception had significantly higher concentrations of TNF-α and IFN-γ when compared to students with lower perceived stress close to the examination (one day before), but not at baseline one month before. In a similar study, neither circulating concentrations of IL-1β, nor mitogen-stimulated IL-1β secretion changed in response to the stress of an oral academic examination (Lacey *et al.*, 2000). However, no other plasma cytokine concentrations were determined in this study. It is possible that, similar to the study by Maes *et al*. (1998), plasma IL-6 concentrations were increased, which would down-regulate IL-1β secretion. A recent review suggested that brief (minutes) or short-term (< 24 hours) psychological stress may cause a shift in favour of a T_H1 response, while longer term stress may result in a cytokine production shift in favour of a T_H2 response (Matalka, 2003). This suggestion is, in part, supported by the findings of Maes *et al*. (1998). More directly supporting the theory of Matalka (2003), acute experimentally induced psychological stress resulted in a transient, but significant increase in NK cell count immediately after the experimental stress tests (Owen & Steptoe, 2003). However, IL-6 concentrations were significantly increased from baseline at 45 minutes post-stress, arguing against the theory that a shift is a longer-term response. Therefore, it is still unclear whether the acute and long-term responses of the cytokine system to stress are similar or not.

The results from the studies above are varied, with the different models yielding different results. There is more than one reason for this: Firstly, similar to variations in the intensity of exercise stress, psychological stress also varies in severity. Unfortunately, it is much more difficult to categorise psychological stress, since individuals also have different levels of stress perception to a similar stressor. Secondly, differences in the duration of stressors and the timing of blood samples are varied. Therefore, the only conclusion that can be made is

that, similar to exercise stress, psychological stress in human subjects also results in an inflammatory cytokine response. However, due to the lack of availability of more studies using similar protocols, a more comprehensive interpretation of results is not possible. Some researchers have turned to animal models of stress to more easily control the stress intervention. Also, in these models experimental tools such as "cytokine-blockers" are available.

In mice, acute immobilisation stress in conjunction with anti-IL-6 antibody treatment, resulted in increased TNF-α concentrations compared to non-treated immobilised rats immediately after, and one hour after cessation of 2 hours of acute immobilisation stress (Nukina *et al.*, 1998). Treatment with anti-IL-6 however did not change the ACTH or corticosterone responses to stress. This led to the conclusion that IL-6 down-regulates TNF-α concentrations to restore homeostasis after exposure to an acute stressor, without having an effect on the endocrine parameters measured. However, IL-6 concentration was not determined in this study so that it is unclear to what extent IL-6 function was blocked by the administered antibody. A more comprehensive study is needed to elucidate the role of IL-6 in the inflammatory response to immobilisation stress in both acute and more long-term (chronic) exposure.



White cell response: In studies investigating the effects of acute (2 hours) and chronic (48 hours) social stress in rats (Stefanski, 2000; Stefanski & Engler, 1999), after 2 hours of confrontation all rats had highly increased concentrations of granulocytes, decreased $CD4^+$ and $CD8^+$ cell counts with resultant elevated $CD4^+$:$CD8^+$ and T:B cell ratios, lowered T cell proliferative response and reduced NK cell cytotoxicity when compared to control rats. Although chronic social stress again resulted in increased granulocyte and decreased $CD4^+$ and $CD8^+$ cell counts, $CD4^+$:$CD8^+$ ratio was unaffected, and T:B cell ratio was decreased. T cell proliferative response was also lowered after 48 hours, although to a lesser extent when compared to the acute response. These results suggest a similarity in the habituation to stress between psychological and physiological stress: in both situations, adaptation to the stressor occurs which results in a blunted immune response.

Furthermore, in a study of two groups of graduate students, T cell proliferative response was reduced when compared to the control group 6-8 weeks before an oral examination. However, this immune suppression was not evident 1 hour before, although cortisol concentrations indicated greater stress closer to the oral examination (Lacey *et al.*, 2000). I speculate that the T cell proliferative response was down-regulated in chronic stress, similar to the down-regulation seen in PBMCs' reactivity to mitogen activation at rest in trained individuals. This would effectively prevent chronic activation of the immune system despite ongoing stress. However, as seen for PBMCs after additional exogenous challenge, this down-regulation is lifted in the presence of an acute stressor, i.e. close to the examination.

In summary, although these studies are quite varied with regard to protocol and results, together they appear to indicate many similarities in the immune responses to exercise stress and to psychological stress. Therefore, everyday life stress is likely to contribute to total stress in athletes through the same immune and endocrine mechanisms. However, more studies are needed to fully substantiate this.

2.3 Responses of the endocrine stress-axis to exercise stress

2.3.1 Responses to acute exercise stress

A number of studies have been designed to investigate some of the most important questions regarding the endocrine response to exercise stress, such as a) whether the stress response is more sensitive to exercise intensity or exercise duration, or both, b) whether a similar exercise stress test will affect all individuals to the same extent, c) whether repeated exercise bouts have a cumulative stress effect and whether this may be influenced by the duration of recovery time, and d) whether the anabolic:catabolic balance is disturbed in response to exercise stress, or whether the concentrations of endogenous anti-catabolic agents also increase in parallel to that of cortisol. In this section, I will review the most important findings from these studies.

The most consistent change reported in earlier studies investigating the endocrine response to an acute exercise stressor, is that of increased circulatory cortisol (Kuoppasalmi *et al.*,

1980; Nieman *et al.*, 1994; Pestell *et al.*, 1989), the magnitude of which, according to some papers, appears to be dependent mainly on the intensity of exercise rather than the duration (Kuoppasalmi *et al.*, 1980; Nieman *et al.*, 1994). However, some groups report greater increases in cortisol concentration after long duration low intensity exercise (≈ 3 hr at 55% VO_{2max}) compared to shorter duration higher intensity exercise (on average 37±19 minutes at 80% VO_{2max}) (Robson *et al.*, 1999). Although these results seem to be contradicting, the shorter duration exercise in the latter study showed an increase in cortisol concentration of about 33% from baseline to post-exercise. This is similar to that observed in response to the two shorter duration exercise protocols used in the study by Kuoppasalmi *et al*. (Kuoppasalmi *et al.*, 1980), who reported 27% and 43% increases after 90 minutes and 45 minutes respectively. Therefore, these results suggest that for shorter duration exercise (up to 90 minutes), intensity may play a larger role than duration in the stress response. However, for exercise of very long duration (e.g. 3hr), the cortisol response duration is greater than for any of the shorter protocols: an 73% increase in cortisol concentration from baseline was observed after 3 hours of exercise (Robson *et al.*, 1999). This may have been due to added metabolic stress, or simply a longer duration for cortisol to accumulate.

Not all reports agree with the assumption that the cortisol response to exercise is similar in all athletes after a standardised exercise test. The possibility of one or more different typical profiles for HPA-axis responsiveness to stress was tested in healthy, moderately trained men. Subjects could be classified as either low or high responders on the basis of their ACTH secretion in response to strenuous treadmill exercise after administration of a mild dose of the synthetic glucocorticoid dexamethasone (Petrides *et al.*, 1997). High responders exhibited significantly greater ACTH and cortisol responses to strenuous exercise (90 % VO_2max) when compared to low responders. In another study without dexamethasone, an acute exercise bout (10 min running at 70% VO_2max) after a prolonged endurance exercise bout (2 hours of running) resulted in increased serum concentrations of cortisol in 50% of participants, while the other 50% showed either no change or a decrease from baseline concentrations (Viru *et al.*, 2001). Given the greater magnitude of change in hormone concentrations in the first *vs*. the second group, it is possible that the first group may be similar to the high responders to exercise stress in the study by Petrides *et al.* (1997). A third test, a 1-minute anaerobic test, was performed before the 2 hours running and after the

post-exercise acute aerobic test, to assess anaerobic muscle power. This showed that anaerobic muscle power in the first group was higher after the 2 hour run than at baseline, while it was significantly lower in the second group. Together, these results suggest that, although the acute aerobic exercise test was set relative to each individual's maximum, the two groups responded differently to this fatiguing stressor. The fact that the group who were still able to mount a cortisol response to increased exercise stress after 2 hours of endurance exercise, could also perform better in a 1-minute anaerobic test, suggests that the less active pituitary-adrenal axis is abnormal and cannot respond appropriately to the second stressor. Whether this is an innate inability or an acute down-regulation, or a pre-pathological condition as a result of prior chronic stress exposure is unknown. More investigation is needed to gain additional information about this divergent response to stress, since ability to identify high- and low responders amongst athletes early could impact hugely on individualised approaches to training.

In contrast to that of cortisol, serum testosterone concentrations may remain unchanged during exercise, but decrease in the recovery period (Kuoppasalmi *et al.*, 1980), and may take up to 24 hours of recovery to return to baseline concentrations (Fry *et al.*, 1991a). Therefore, while the activity of the pituitary-adrenocortical system during exercise appears to be a good indicator of the capacity to respond to stress, pituitary-testicular system changes occurring during the recovery period may more accurately reflect the effort expended.

The effects of repeated bouts of exercise, and the influences of recovery duration between successive bouts, were recently investigated in elite endurance athletes. Repeated bouts of exercise (separated by 3 hours) were shown to have an additive stress effect, with increases in cortisol concentrations in response to the second bout of exercise that were much greater than those reported after the first bout (Ronsen *et al.*, 2001a). In a similar study by the same group, the additive effect of exercise was influenced by the duration of recovery allowed between bouts. Subjects had smaller increases in cortisol, ACTH, epinephrine and norepinephrine concentrations in response to a second exercise bout after 6 hours of recovery between bouts than after only 3 hours of recovery (Ronsen *et al.*, 2002).

In contrast to this cumulative effect of repeated exercise bouts on the catabolic stress hormone response, concentrations of anabolic hormones such as growth hormone and testosterone were reportedly unaffected by the number of bouts per day or the recovery time between bouts (Ronsen *et al.*, 2002). However, these anabolic agents were determined only immediately after exercise, and not during recovery, which was earlier established to be more useful (Kuoppasalmi, 1980), so it is not possible to make such a clear-cut conclusion regarding the anabolic response to multiple bouts of exercise.

Although total cortisol concentration is commonly used as an indicator of stress, the total concentration of cortisol in the circulation does not necessarily reflect the concentration of bio-available (unbound) cortisol at tissue level. Futhermore, the effect of anti-catabolic agents may also contribute to lessen the net effect of cortisol released in response to stress. Very few studies have investigated these issues in athletes. In highly trained athletes, cortisol concentrations were increased after an ultramarathon as expected, but CBG concentrations remained unchanged from baseline, indicating that the net effect was that of increased free cortisol (Pestell *et al.*, 1989). In addition, SHBG was reported to be unaffected by either short, high-intensity, or longer, moderate-intensity exercise, so that changes in total testosterone concentrations were associated with parallel changes in the fraction of free testosterone in the circulation (Kuoppasalmi, 1980). However, very little is known about the role of CBG in the response to different exercise intensities or more chronic exercise stress. These issues should be investigated more thoroughly, since CBG may have important regulatory functions in the response to stress.

Another anti-catabolic agent which may have a regulatory role in the cortisol response to stress is DHEA. In rats, administered DHEA was shown to have anti-glucocorticoid action, by blocking the dexamethasone-mediated activation of the glucocorticoid-inducible enzymes tyrosine aminotransferase (TAT) and ornithine decarboxylase (ODC), but this blocking effect was not effective after additional experimentally-induced stress (dexamethasone injection) in rats (Wright *et al.*, 1992). It is therefore possible that DHEAs does not increase in response to glucocorticoids. However, since it is difficult to assess the overall, integrated response in the dexamethasone model, this result is difficult to interpret. In elite female athletes, an acute bout of exercise (simulated handball match) did not alter DHEA concentrations (Filaire

& Lac, 2000), suggesting that DHEA is not responsive to a whole body stressor, but there is not enough data available to substantiate this theory. Also, the recent finding that an acute endotoxin injection increased the serum cortisol:DHEAs and cortisol:17OH-progesterone ratios in a dose-dependent manner in patients suffering from chronic inflammatory disease (Straub *et al.*, 2002), suggests that in response to a stressor, cortisol production may be favoured at the expense of the adrenal androgens. More investigation is needed to determine if these parameters are possible regulators of the net effect of cortisol and testosterone in response to exercise stress. Taken together, the results of the studies mentioned above, suggest that expression of the concentration of cortisol as a ratio to either CBG or DHEAs concentrations, may possibly be better indicators of changes in catabolic status than measurement of cortisol concentration alone, and should be investigated more comprehensively in exercise stress and other stress models.

2.3.2 Responses to chronic exercise stress (training)

Moderate exercise training regimens should not influence the endocrine system negatively, since enough time for the physiological recovery (and adaptation to the exercise) should be allowed between bouts. In a study comparing the effect of endurance, sprint and mixed training protocols lasting 10 weeks each (Kraemer *et al.*, 1989), the results indicated that higher intensity endurance training with bouts of short duration (80% VO_2max for 30 minutes, 3 times per week) did not alter resting cortisol concentrations, or the cortisol response to maximal exercise. However, both interval sprint training (2x4 20-second sprints at 96% of maximum heart rate, 3 times per week) and a combination of the two (alternating endurance and interval sprint protocols, 6 times per week) resulted in increased resting cortisol concentrations (baseline: ≈ 330 *vs*. post-training: ≈ 450 nmol/L for both groups). This would suggest that higher intensity and higher volume training, especially that which includes sprint training on several days per week, results in a higher stress condition at rest. However, when comparing pre-training mean resting cortisol concentrations, those of the interval sprint group and the combined protocol group were significantly lower than that of the endurance group (≈ 330 *vs*. 540 nmol/L), while post-training resting cortisol concentrations were similar in all groups. Since the subjects used in this study were healthy active individuals randomly divided into the three training categories, previous training experience should not have been

a confounding factor. However, the higher initial resting cortisol concentrations reported in the endurance protocol group may have been a confounding factor, leading to this group appearing to be the only group unresponsive to training. The fact that ACTH concentrations remained similar at rest and in response to an acute exercise test (VO_{2max} test) in all groups, while there were significant training-induced increases in cortisol concentrations at rest in at least two of the groups investigated, may suggest that 10 weeks of training is not sufficient in duration, or not of strenuous enough intensity, for ACTH adaptation to take place In addition to these results after a 10-week training protocol, a training study of shorter duration also reported unchanged resting concentrations of cortisol and ACTH after a 100% increase in training volume for ten days in cyclists (both exercise type specific and cross-training protocols were used) (Pizza *et al.*, 1995a), which may indicate that 10 days is not sufficient time to result in chronic changes in cortisol concentration at rest.

The net effect of cortisol does not only depend on factors playing a role in its release, such as ACTH, but also on agents with an opposite effect to that of cortisol, such as DHEA and testosterone. It is therefore necessary to take into account changes in the concentrations of these parameters relative to that of cortisol, after training. Results obtained in human studies investigating this issue are varied. For example, 10 days of increased training volume (100 % increase) in well-trained distance runners resulted in a significant decrease in DHEAs concentration at rest, although resting cortisol concentration was not affected (Flynn *et al.*, 1997). Similarly, resting salivary DHEA concentrations were shown to be lower in elite athletes when compared to sedentary controls (Filaire & Lac, 2000). A third study that also reported decreased resting DHEAs concentration after 3 months of endurance training in previously sedentary females, also showed a decreased resting testosterone concentration post-training (Keizer *et al.*, 1987). On the other hand, in previously sedentary subjects, three months of exercise that induced a negative energy balance resulted in a significant increase in resting DHEAs and testosterone concentrations, although again with no effect on resting cortisol concentration (Pritchard *et al.*, 1999). This result was also true for elite female athletes, whose resting salivary DHEA concentrations were increased significantly after 16 weeks of training, while salivary cortisol concentrations remained similar. This resulted in an increase of more than 30 % in the DHEA:cortisol ratio (Filaire *et al.*, 1998). The fact that both sedentary and elite athletes showed a similar response in the latter two studies,

suggests that the DHEAs response is not related to fitness level. Rather, I propose that the results from all the above studies indicate that the initial DHEAs response to unaccustomed training workloads, as in the studies of Pritchard et al. (1999) and Filaire et al. (1998), is an increased DHEAs concentration that could counteract the effects of repeated transient elevations in the circulating cortisol concentration in response to the repeated training bouts. However, DHEAs concentrations decrease over time in response to chronic exercise training, which may suggest that changes in the DHEAs concentration at rest may be a more accurate marker of exercise stress than cortisol concentration itself. This interpretation of the exercise literature is in support of the earlier suggestion in the medical literature that the cortisol:DHEAs ratio may be a useful indicator of risk of long-term stress-related pathology (Hechter *et al.*, 1997).

Another marker of anabolic potential, namely resting testosterone concentrations, also decreased in response to 10 days of training at 200 % of normal training volume, while resting cortisol concentration remained similar (Pizza *et al.*, 1995a). These data are indirect evidence that doubling of training volume may lead to an inability of the endocrine system to maintain a net anabolic effect, which may be detrimental to exercise performance. It may therefore also be useful to calculate the ratio of cortisol to testosterone at rest, to give a better indication of changes in the anabolic-catabolic balance in response to a training programme.

The usefulness of measuring the cortisol:testosterone ratio in response to exercise training was investigated in professional cyclists (Hoogeveen & Zonderland, 1996). Three months of performance-enhancing intensive training with progressively increasing volume (1 month each of cycling 405 km/wk, 510 km/wk and 740 km/wk) resulted in a 26 % increase in the cortisol:testosterone ratio at rest in these athletes. This was the result of both a 39 % increase in resting cortisol and a 15 % decrease in resting testosterone concentrations. However, training did not have a significant effect on either the cortisol or the testosterone response to an acute bout of exhaustive exercise. An earlier study reported that more than 24 hours of rest may be required for hormone concentrations to return to baseline concentrations after an acute bout of exercise (Fry *et al.*, 1991a). Furthermore, in endurance athletes, repeated bouts of acute exercise on the same day were shown to have cumulative

effects on the neuroendocrine response, with higher increases in epinephrine, norepinephrine, ACTH, cortisol and growth hormone concentrations during, and larger decreases in testosterone concentration after the second bout of exercise (Ronsen *et al.*, 2001a). In a subsequent study, this cumulative effect was reported to be inversely related to the recovery time allowed between bouts (Ronsen *et al.*, 2002). Therefore, the discrepancy in the results from the studies discussed on the previous 2 pages may be the result of cumulative increases in cortisol and decreases in testosterone concentrations, after repeated exercise bouts with insufficient recovery in between bouts. Although not enough information regarding the rest periods before each blood sampling is provided in all the above studies to enable critical assessment in this regard, these results highlight the importance of obtaining proper resting blood samples to prevent confounding effects of prior acute exercise bouts, to ensure accurate interpretation of data regarding the effects of chronic exercise.

In summary, the significance of an increased resting DHEAs concentration after chronic unaccustomed increase in exercise training load remains to be elucidated. However, these results supports the earlier theory that there is a shift away from androgen and towards glucocorticoid production at times of increased physiological or psychological stress (Parker *et al.*, 1985).

2.3.3 Responses to overreaching and overtraining

Due to ever-increasing competitive standards set in professional sports today, training workloads may be detrimental to performance in the long run, due to inability of the endocrine system to cope with this chronic stressor. An overtraining syndrome may then develop, with symptoms such as failure to improve performance, lethargy, weight loss, elevated resting heart rate and blood pressure and insomnia (Barron *et al.*, 1985; Brown *et al.*, 1983; Kuipers & Keizer, 1988). The diagnosis of OTS appears to be done by investigating symptoms and excluding other possible causes, rather than using specific objective indicators. Very few studies are available with data generated on actual OTS sufferers. Most information available originates from interpretation of, and extrapolation

from, short-term overreaching studies, which will be discussed in the next few paragraphs, along with the few studies available on truly overtrained athletes.

Increased cortisol concentrations were proposed to indicate acute high physiological strain during normal training (Kraemer *et al.*, 1989; Kuoppasalmi *et al.*, 1980; Urhausen *et al.*, 1987). The author of a review on overtraining suggested that decreased cortisol concentrations may indicate long-duration chronic strain and overtraining (Lehmann *et al.*, 1998). In a study searching for an objective marker for overtraining, 10 previously diagnosed overtrained athletes had an average resting cortisol concentration of 396 ± 74 nmol/L, which is within the normal range of 220 – 750 nmol/L (Rowbottom *et al.*, 1995). However, there are more than one possible explanation for this finding: firstly, by a counter-regulatory mechanism, as a protective measure against the negative effects of a chronically elevated cortisol concentration. The mechanism by which this may occur is not clear. Possibilities include decreased adrenal sensitivity to ACTH and catecholamines, which is reported in overtrained individuals, as reviewed earlier (Lehmann *et al.*, 1998), or a shift from cortisol to androgen (e.g. DHEA) production. The latter option is also likely, given the finding of increased DHEAs concentrations after chronic severe exercise stress, as discussed in the previous section. However, insufficient data is available to enable a clear-cut conclusion. A second possibility is that these athletes may have decreased their performance and became symptomatic as a result of the inability of their HPA-axis to keep pace with the stress of exercise, by increasing cortisol secretion during training sessions. Hence the tested cortisol concentration was not abnormal. Alternatively, cortisol production may have been decreased earlier on in the development of the overtraining syndrome, but was no longer outside the normal range by the time of diagnosis. However, this parameter alone is not sufficient to make a diagnosis, especially since many available studies were recently reviewed to show that results regarding the cortisol response at rest and after exercise in overtrained endurance athletes were very varied (Urhausen & Kindermann, 2002). The study by Rowbottom *et al*. (1995) clearly illustrates the difficulties in interpretation of data obtained in already overtrained athletes. Therefore, in order to be able to prevent the occurrence of the OTS by early identification, it is crucial to monitor changes in resting hormone concentrations, even within the normal range, to allow early detection of a

decrease or lack in endocrine responsiveness to stress. The latter would indicate inability to cope with the stressor, even before the other characteristic symptoms of OTS develop.

In another study, not only plasma concentrations of cortisol, but also that of epinephrine and norepinephrine were reported to remain unchanged over the course of a training season in 10 elite swimmers (Hooper *et al.*, 1993). Nevertheless, epinephrine concentration was positively correlated to exercise volume in these athletes. Three swimmers were diagnosed with OTS during the course of this season, and exhibited higher epinephrine levels compared to the other swimmers from mid-season onwards, which may indicate a possible role for epinephrine as indicator of the OTS.

In a study in cyclists subjected to an overreaching training protocol (2 weeks each of normal training, intensified training and recovery) (Halson *et al.*, 2002), no change was reported in the resting plasma catecholamine concentration in response to training, although performance in maximal tests, intermittent tests and time trial tests were reduced. (Blunting of the cortisol and adrenaline response to acute exhaustive exercise was reported after short-term overreaching in the same study.) More studies are required to investigate the possibility of using epinephrine as a marker for overtraining, but this study indicates that performance decrements may precede changes in the endocrine response. This is further supported by a study in resistance trained men (Fry *et al.*, 1998). No changes were reported in resting cortisol, testosterone and growth hormone concentrations after participation in a 2-week resistance training protocol designed to induce overtraining, although performance decreased significantly, suggesting a state of overtraining, or at least overreaching. In addition, these athletes showed no significant training-induced change in the responses of cortisol, testosterone, growth hormone or ratios between testosterone and cortisol, to an acute bout of exercise (Fry *et al.*, 1998). These results suggest that blunting of the endocrine response to exercise stress may occur late in the development of OTS, while decreases in performance occur earlier, possibly in a phase where symptoms are still reversible. Therefore, early detection of a persistent blunted endocrine response may enable timely intervention (e.g. changes to the training workload) to prevent the development of the full-blown syndrome.

In order to determine whether the endocrine adaptation to overreaching or overtraining is dependent on the type of exercise, a 2-week overreaching training protocol in runners was followed (Flynn *et al.*, 1997). Runners were required to either perform running exercise only (200% of usual training volume), or to cross-train (running at 100 % of usual training volume plus cycling to yield an added 100 % of energy expenditure as per usual running training). Similar to results from other more moderate training studies, resting ACTH and cortisol concentrations remained unchanged during both training protocols. However, resting testosterone concentrations decreased significantly by day 5. There was no difference in this measure between the two protocols, indicating that the endocrine response to excessive training is not exercise type specific, at least not when both types of exercise are endurance exercises. The decreased testosterone concentration indicates the possibility that, although cortisol concentration was not increased, overreaching may coincide with a decreased anabolic status, which may in part explain the decrement in exercise performance seen in the OTS.

Psychological stress elicits a similar endocrine stress response when compared to exercise, at least pertaining to the HPA-stress axis. The results of the previous study show that two different moderate exercise stressors together have similar effects on the endocrine stress response to that of one severe exercise stressor. Since athletes are under severe psychological pressure to perform, even when training, this chronic psychological stressor may contribute to the effects of exercise stress in increasing their overall stress status. It is therefore also important to take into account the added effects of psychological stress in an athlete population, especially since it may be more prolonged than the exercise training sessions themselves.

2.4 Endocrine responses to psychological stress

Inducing psychological stress in human subjects in an experimental situation is not as simple as in laboratory animals, since the effects of prior exposure to stress in human subjects are difficult to control and may have confounding effects on the results obtained. Therefore, animals are often used as a more standardised model to investigate the physiological responses to psychological stress.

Studies in rats determined that immobilisation stress of different severities (tube restraint *vs*. prone restraint on wooden board) yielded different magnitudes of corticosterone response (Garcia *et al.*, 2000). Furthermore, peak corticosterone concentrations were only achieved after stressors of more than 1 hour in duration (Garcia *et al.*, 2000; Hu *et al.*, 2000), after which these peak concentrations were maintained for the duration of the immobilisation protocol (Hu *et al.*, 2000). With chronic intermittent stress protocols, the corticosterone concentration after acute stress increased with each successive episode, but recovery to baseline concentrations was more efficient over time (Garcia *et al.*, 2000; Marti *et al.*, 2001), indicating both enhanced responsiveness and enhanced clearance. However, this habituation of recovery was shown to be stressor-specific, since a novel stressor elicited a typical "acute" stress response again (Garcia *et al.*, 2000; Marti *et al.*, 2001). To summarise, these reports suggest that the magnitude of the corticosterone response to immobilsation stress is sensitive to both the severity and the duration of each episode, as well as the number of repeated episodes, and that a chronic, stressor-specific response is characterised by quicker return of corticosterone concentrations to baseline after removal of the stressor. Therefore, the speed of recovery of corticosterone concentrations to baseline may be a good indicator of adaptation to a psychological stressor.

Similar to the results obtained in rodent models (Garcia *et al.*, 2000; Marti *et al.*, 2001), repetition of experimentally-induced psychological stress in healthy humans (2 sessions, including the Stroop Color Word Interference task, public speaking and mental arithmetic in front of an audience, separated by 7 days) was recently reported to result in habituation, with smaller increases in ACTH and cortisol concentrations in response to the stress on day 8, while epinephrine and norepinephrine did not habituate (Gerra *et al.*, 2001). The ACTH and cortisol responses to both acute experimentally-induced and chronic work-related psychological stress could be divided into two distinct patterns, one of low-response and one of high-response, with the high-responders showing a highly variable diurnal response with high early morning cortisol concentrations and several peaks during the morning, with a much lower evening cortisol concentration (the typical normal diurnal curve), and low-responders showing low diurnal variability and low morning cortisol concentrations (a flattened diurnal curve) (Gerra *et al.*, 2001; Rosmond *et al.*, 1998). This was observed

despite similar values for perceived stress in the two responder groups. The implication of these two studies for our understanding of exercise-stress is that it may not be possible for training studies to group all athletes together without confounding the results. Indeed, high- and low-responders have also been reported in response to acute exercise stress (Petrides *et al.*, 1997).

In a study comparing 19 patients suffering from the chronic fatigue syndrome (CFS) to 10 healthy controls (Scott *et al.*, 2000), basal DHEA and cortisol concentrations, as well as the cortisol:DHEA ratio, were found to be similar in the two groups at rest. However, 30 minutes after administration of 1 ug of ACTH, there were some significant differences in the adrenal responses. While there was no significant difference in the DHEA response (average increase of 28 ± 7 ng/L in CFS *vs*. 27 ± 10 ng/L in controls), there was a significant difference in the magnitude of change in cortisol concentrations between the two groups (average increase of 272 ± 31 nmol/L in CFS *vs*. 360 ± 27 nmol/L in controls; P = 0.05). The differences between groups were also reflected in the cortisol:DHEA ratio, which had a tendency to be decreased in CFS patients when compared to controls, in response to the ACTH challenge. Long duration stress seems to be characterised by decreased adrenal sensitivity to ACTH, similar to the response reported after chronic exercise stress in the early stages of overtraining (Lehmann *et al.*, 1998).

Although the cortisol:DHEA ratio may give an indication of the availability of “anti-catabolic” precursors relative to the cortisol concentration, the net biological activity of cortisol may also be dependent on the concentration of CBG. In a study investigating the effects of social stress in rats (model discussed in 2.2) on the endocrine and immune systems, no correlation was reported between CBG and corticosterone concentrations after 7 days of social stress (Stefanski, 2000). However, while corticosterone concentrations were stable in both dominant and subordinate rats, CBG concentration was decreased in subordinate rats, but not in dominant rats, resulting in a relative increase in free corticosterone concentrations in subordinate rats. Another study on chronic social stress in rats reported decreased CBG concentrations in subordinate (-70 %) as well as in dominant (-40 %) rats after 2 weeks when compared to controls, while only subordinate rats had significantly higher corticosterone concentrations after 2 weeks when compared to the control group (Spencer *et*

al., 1996). In addition, CBG concentrations were correlated positively with type II corticosteroid receptors in the spleen, so that lower CBG concentrations (during higher stress) were associated with lower receptor numbers. Furthermore, in a study on the effect of inescapable tailshock in rats, CBG concentrations were decreased and corticosterone concentrations increased (Deak *et al.*, 1999), suggesting a similar CBG response to a variety of non-exercise related stressors. Together, these results suggest that chronic stress results in greater increases of free glucocorticoid, which may impact on vital organs.

The significance of a parallel decrease in splenic corticosteroid receptors is difficult to interpret, but may be the result of down-regulation in response to increased binding in the presence of high free cortisol concentrations. It is of interest to note that CBG concentrations in all these studies were determined using a competitive binding procedure. However, during inflammation, serine proteases on the surface of activated neutrophils are able to cleave the CBG molecule into a form with lower affinity for corticosteroids (Hammond *et al.*, 1990; Pemberton *et al.*, 1988). Therefore, it is unclear whether the CBG concentrations were in fact decreased, or whether chronic stress resulted in a decreased capacity for binding of corticosteroid to its binding globulin. Although the mechanism remains debatable, the end result is clearly that of an increased free glucocorticoid concentration after chronic stress. Similar to the studies mentioned above, serum CBG concentrations (also measured by competitive binding assay) were decreased after acute exposure to ether anaesthesia (Tinnikov, 1999). In addition, the decrease in CBG concentration was greater when this stressor was combined with cold exposure or fasting (i.e. conditions requiring mobilisation of spare fuel). This suggests that the mechanism for glucocorticoid regulation of fuel mobilisation may not always be to change cortisol concentration itself, but rather to regulate free glucocorticoid concentration by manipulation of CBG concentration. Few studies of stress within a physiological rather than an extreme range have added CBG to the analyses.

2.5 Relationships between the immune system, endocrine stress-axis and anti-catabolic agents in the stress response

In the previous sections, it has been established that exercise stress results in a shift away from androgen production, in favour of cortisol production. Since cortisol has direct negative effects on some immune cells (e.g. decreasing $CD4^+$ cell count), it is possible that a disturbance or shift in the anabolic:catabolic balance may also result in a shift within the immune system, so that some parts of it may become either more activated or compromised. In the next section, I will discuss studies that have investigated the relationships between parameters of the endocrine and immune systems, in response to both acute and chronic exercise stress.

2.5.1 Relationships measured in the response to acute stress

Changes in immune capacity may be demonstrated by changes in cell availability (either cell count, or relative distribution, or both), or changes in cell function (such as cytokine production and/or secretion). In the next section, I will discuss previous studies which have reported associations between stress-induced changes in the concentration of cortisol or anti-catabolic agents, and changes in immune cell availability and function.

In marathon runners, 2.5 hours of running resulted in increased post-exercise plasma cortisol concentrations for more than 3 hours into recovery, when compared to non-exercising controls (Nieman *et al.*, 1995). At 3 hours, cortisol concentrations were reported to be positively correlated with the neutrophil:lymphocyte ratio, which suggests that increased stress results in a shift in the availability of immune cells in favour of the non-specific immune component. Unfortunately, the correlations between these parameters at the other time points were not provided, so that a more comprehensive interpretation, including e.g. also the relationship immediately after exercise or at rest, is not possible. The results of this study were therefore not conclusive evidence of a consistent association between shifts favouring non-specific immunity and stress. However, a subsequent study reported higher percentages of $CD8^+$ and NK cells and lower percentages of $CD4^+$ cells at

rest in subjects who were previously identified as cortisol high-responders when compared to low-responders (Deuster *et al.*, 1999). Together these two studies suggest that it is informative to assess the immune response to severe stress at rest and that there is likely to be i) an association between the cortisol response and the immune response, and ii) a shift in the lymphocytes in circulation even in a resting condition, to those that are mostly non-specific. This may imply a relatively decreased capacity for the antibody-mediated specific immune response, which may impact on the resistance of athletes such as those in the study by Deuster *et al.* (1999) to chronic illnesses, but certainly indicates that immune system function does not remain unaltered in the face of stress.

Post-exercise increases in cortisol concentrations were also reported to be positively correlated with the peak amounts of IL-6 produced by lymphocytes in response to an ionomycin and PMA challenge *in vitro* (Steensberg *et al.*, 2001a). Similarly, a cloned T cell culture capable of secreting both IL-2 and IL-4, secreted reduced concentrations of IL-2 and increased concentrations of IL-4 after exposure to cortisol (Daynes *et al.*, 1990). Although these assays do not fully distinguish the cell types responding, the implication seems to be that the lymphocytes available after acute stress have increased functional capacity to activate the antibody-mediated immune response, which in turn results in a relative inhibition of activation of the cell-mediated immune response. However, several reports of altered cytokine profiles *in vivo* exist, which suggest a shift away from a T_H1 cytokine response, in association with increased cortisol concentrations. HPA-axis high-responders were reported to have lower IL-6 concentrations at rest when compared to low-responders (Deuster *et al.*, 1999). Also, in a study investigating the acute effects of mild psychological stress (Kunz-Ebrecht *et al.*, 2003), the inflammatory cytokine response (IL-6 and IL-1ra) to acute psychological stress was inversely related to the cortisol response, with cytokine concentrations in response to stress reported to be much higher in the low-responder group compared to the high-responder group. Thus, while in cortisol high-responders the enhanced cortisol response decreased the inflammatory cytokine response by negative feedback, this was not the case in the low-responders. It was recently hypothesised that this dual response to stress may be the result of stress-exposure at critical times during development, which may induce structural and functional changes in the nervous, immune and endocrine systems, leading to altered responses to subsequent stressors (Hurwitz &

Morgenstern, 2001), but this remains to be substantiated. Unlike acute exercise stress, chronic stress may down-regulate the cortisol response, thus allowing an increased inflammatory response.

In addition to these effects of cortisol on immune function, testosterone was also reported to enhance $CD8^+$ cell function (Tanriverdi *et al.*, 2003) and to increase $CD8^+$ cell count, with a parallel decrease in CD4:CD8 ratio (Yao *et al.*, 2003). A suppressed T cell proliferative response was also reported in response to testosterone administration in the latter study, which seems to contradict this finding. However, measurement of T cell proliferation rate does not allow differentiation between subpopulations. Therefore, it is not clear whether the suppressed response was due to suppressed function of $CD8^-$ cells, such as $CD4^+$ cells.

The altered cytokine profile in response to stress may also be linked to changes in the concentration of DHEA. Macrophages were shown to be, at least in part, responsible for conversion of DHEAs to its metabolically active form, DHEA (Hennebold & Daynes, 1994). This acitvation of DHEA was further shown to be inhibited by T_H1, but not T_H2 cytokines, in the same study. Therefore, when the cytokine response shifts in favour of T_H2 cytokines in response to acute exercise, an increased concentration of DHEAs will be converted to DHEA, so that a larger anti-catabolic effect is possible. Furthermore, a number of *in vivo* and *in vitro* studies reported that both testosterone and DHEA enhanced the T_H1 response, e.g. by enhancing secretion of IL-2 (Daynes *et al.*, 1990; Hässig *et al.*, 1996; Malkin *et al.*, 2003; Regelson *et al.*, 1994; Straub *et al.*, 2002), with resultant enhanced immune resistance to viral and bacterial infections. This may suggest a role for DHEA in reversing the cortisol-induced shift away from a T_H1 response after exercise. Also, testosterone was reported to increase monocyte secretion of IL-10, indicating an enhanced anti-inflammatory function, in addition to suppression of secretion of inflammatory cytokines such as IL-6 and TNF-α (Malkin *et al.*, 2003). Therefore, a decrease in testosterone or DHEA after training may contribute to a greater net inhibitory effect of cortisol on the T_H1 immune response.

Epinephrine was also shown to be an important regulator of total leukocytes, lymphocytes, and $CD3^+$, $CD4^+$, $CD8^+$, and $CD3^-CD16^+/56^+$ subpopulation redistribution after acute exercise in humans (Rhind *et al.*, 1999). While epinephrine has been shown to play a role in

acute exercise-induced changes in lymphocyte number and function, epinephrine infusion alone could not mimic the changes in plasma IL-6 observed after exercise (Steensberg *et al.*, 2001a). Therefore, it is unlikely that epinephrine is a major regulator of immune function in response to acute exercise, but it may have been indirectly associated in the study of Rhind *et al.* (1999).

Taken together these results indicate important roles for both DHEA and testosterone in reversing the negative effects of cortisol on immune competence. Therefore, it is important to consider changes in the availability of these parameters in the circulation, in conjunction with changes in the cortisol concentration, not only for a more comprehensive assessment of endocrine stress status, but also as indirect indicators of changes in immune system functional capacity.

2.5.2 Relationships measured in response to chronic stress

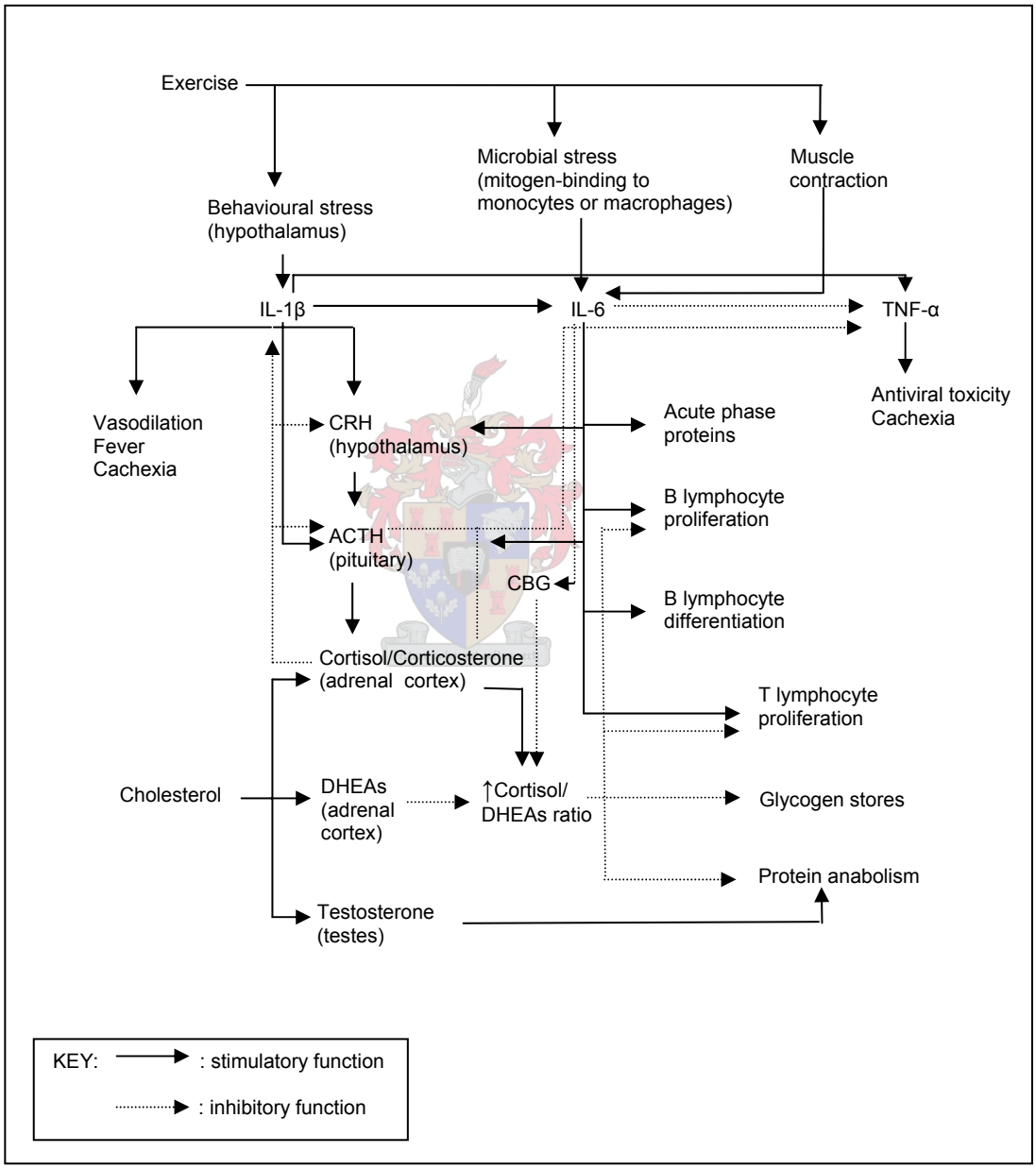
In HIV-infected patients, cortisol concentrations have been shown to be chronically elevated at rest, while that of DHEA is decreased, leading to the hypothesis that the imbalance in these hormones may be responsible for the progression of the disease from HIV infection to full-blown AIDS (Clerici *et al.*, 1997). Subsequent to this hypothesis, changes in cortisol:DHEAs ratio have been shown to correlate negatively with $CD4^+$ count in this population (Christeff *et al.*, 2000; Grinspoon *et al.*, 2001). The changes in the cortisol:DHEAs ratio were mainly due to reductions in DHEAs concentration, which were previously also shown to correlate positively and independently with $CD4^+$ counts (Wisniewski *et al.*, 1993). Thus decreased DHEAs concentration is associated with decreased specific immune function at least in this population. It was established earlier that glucocorticoids enhanced *in vitro* secretion of T_H2 cytokines by directly stimulating secretion of IL-4 and inhibiting IL-2 secretion (Daynes & Araneo, 1989). The progression of HIV infection to AIDS is also characterised by a shift in production away from T_H1 (cell-mediated immunity: IFN-γ, IL-2, IL-12) toward T_H2 cytokines (humoral immunity: IL-4, IL-5, IL-6 IL-10)(Clerici & Shearer, 1993; Clerici & Shearer, 1994). While T_H2 cytokines have been shown to render $CD4^+$ lymphocytes more susceptible to programmed cell death (Clerici *et al.*, 1994), IL-6 specifically was shown to up-regulate the production of the HI-virus (Shephard, 2002). Also in combination with

TNF-α, IL-6 can induce the production of the virus in latent infected cells (Shephard, 2002). These studies suggest that changes in the balance between endocrine parameters contribute at least in part to the chronically decreased immune ability observed in HIV-infected individuals, possibly through increased activation of programmed cell death in $CD4^+$ cells. Furthermore, from these reports it is clear that the chronic effects of exercise and psychological stress are in many ways, although much milder, similar to HIV-induced changes in both the endocrine stress-axis and immune system. Therefore, although this disease is pathogen-induced, and results obtained in this model can therefore not directly be extrapolated to other non-pathogenic stress conditions, results obtained in HIV-related studies may provide additional information useful in other areas of stress research.

2.6 Summary

A schematic presentation of the interactions between cytokines, the immune system and the endocrine stress-axis in the response to exercise stress is given in Figure 2.1. A brief summary of the reviewed literature is provided in the next section.

Figure 2.1 Interaction of immune, cytokine and endocrine systems in the response to exercise stress.



2.6.1 Immune and cytokine system responses to stress

From the available literature, both acute psychological stress and acute exercise stress increase pro-inflammatory cytokine production. In addition, excessive exercise training seems to result in an increased pro-inflammatory capacity. Cytokines, and in particular IL-6, have been named as key role players in the development of the OTS (Robson, 2003; Smith, 2000), although no concrete evidence has been presented to prove this in an overtrained subject population. However, the magnitude of the role played by the immune system in facilitating the increase in circulating cytokines, specifically IL-6, remains debatable. This may be in part because IL-6 is produced by immune and non-immune cells, but also because the immune and endocrine response to exercise is complex and not yet fully understood. In particular, an integrated understanding of *in vitro* and *in vivo* cytokine data has not been achieved.

Acute exercise stress results in a lowered T:B lymphocyte ratio, as well as a lowered $CD4^+$:$CD8^+$ ratio, the magnitude of which is dependent on both exercise intensity and duration. Training has been shown to result in a chronically decreased $CD4^+$:$CD8^+$ ratio at rest, which is more dependent on training intensity than volume, but T cell numbers in athletes suffering from the OTS are similar to those of non-sufferers. Psychological stress elicits similar responses to exercise stress on immune cell number. Recent technological advances now also make it possible to assess the functional capacity of immune cells.

White cell proliferation rate was shown to be enhanced by acute moderate exercise, but depressed by high-intensity or long-duration bouts of acute exercise. Moderate training appears to enhance lymphocyte proliferative responsiveness – making it possible to compensate for chronically decreased cell numbers. However, since the lymphocyte proliferative response assay is not T cell subpopulation specific, it does not give sufficient information to draw conclusions on downstream effects of this change in immune capacity. Measurement of CD69 expression is T cell subpopulation specific; however, very few research groups are assessing this parameter. Similar to results obtained for lymphocyte proliferation, acute strenuous exercise was shown to depress $CD4^+$, $CD8^+$ and NK cell expression of CD69. In addition, the depressed CD69 expression exhibited a cumulative

effect of successive bouts of exercise when insufficient recovery time was allowed. Unfortunately not enough reliable data is available on the CD69 response to training or overtraining, to understand whether chronic training induced changes in lymphocyte function are T cell subpopulation specific. Furthermore, no conclusive results are available on the effect of psychological stress, either acute or chronic, on white cell function.

2.6.2 Responses of the endocrine stress-axis to stress

Psychological and physiological stressors seem to elicit a similar response from the endocrine stress-axis. Cortisol concentration increases after an acute stressor, in a manner dependent on both intensity/severity and duration, with cumulative effects of repeated bouts. However, the cortisol response adapts to a moderate stressor over time, with cortisol concentrations reaching lower peak values and returning to baseline concentrations quicker after each successive stress exposure. This effect was shown to be stressor-specific, so that a new type of stressor will again increase the cortisol response. However, a severe stressor does not seem to allow habituation, with cortisol concentrations remaining elevated after subsequent exposures. In addition, a divergent response of cortisol to a stressor was described after both physical and psychological stress exposure, that of low- and high-responders. Highly elevated cortisol levels seem to indicate high strain, while low or decreased cortisol levels may indicate long-duration chronic strain, and possibly overtraining or the chronic fatigue syndrome. This warrants further investigation, particularly since the separation of adaptation and maladaptation to exercise stress on the basis of blood measurements is not currently possible.

In contrast to cortisol, testosterone concentrations only change after cessation of the stress exposure, and decrease during recovery. Testosterone concentration may be a useful parameter to measure as indicator of recovery status, since it returns to baseline after sufficient recovery, but remains decreased after severe stress exposure with insufficient recovery.

Available literature suggests that binding globulins may play a significant role in regulating the amount of metabolically active hormones available in circulation. Investigation of the role

of CBG and SHBG in the stress response may therefore be useful to determine the net change in biologically active cortisol and testosterone in these situations. Also, stress seems to increase glucocorticoid production at the expense of androgen production – both testosterone and DHEAs. Therefore, measurement of the ratios between concentrations of cortisol and testosterone at rest, or between that of cortisol and DHEAs, may prove more useful than measurement of cortisol alone.

2.6.3 Relationship between the immune system, endocrine stress-axis and anti-catabolic agents in the stress response

From the literature consulted, increased cortisol concentration is associated with decreased circulating lymphocyte counts, as well as increased IL-6 production in PBMCs in culture. However, despite increased PBMC production of IL-6 *in vitro*, circulating IL-6 concentration was negatively correlated to cortisol concentration, possibly due to rapid negative feedback by cortisol.

Both increased testosterone and DHEAs concentrations seem to enhance the T_H1 response, which results in increased resistance to viral and bacterial infection. In addition, testosterone has been associated with suppression of the inflammatory response by regulating the actions of both pro- and anti-inflammatory cytokines, and DHEAs was negatively correlated with circulating IL-6 concentration, suggesting a possible down-regulatory effect of androgens on the inflammatory response. In addition, testosterone was reported to increase both $CD8^+$ cell count and activity, and to decrease the $CD4^+$:$CD8^+$ ratio. Testosterone was also shown to suppress the lymphocyte proliferative response, which seems to contradict the previous statement. However, this assay cannot distinguish between T cell subpopulations, so that the target cells of the suppressive action of testosterone remain unclear.

In HIV patients, an increase in the cortisol:DHEAs ratio was associated with decreased $CD4^+$ count and progression of disease. Since this change in ratio was mainly due to a decreased DHEAs concentration, DHEAs may be important for maintenance of immune function. Similarly, IL-6 (which may be down-regulated by DHEAs) was shown to increase

susceptibility of $CD4^+$ cells to apoptosis. However, these relationships between cytokines, T cells and androgens have not been investigated in non-HIV-related stress conditions.

2.7 Current challenges for immune-endocrine exercise physiologists

Assessment of changes in absolute white blood cell counts in response to stress is insufficient to draw conclusions pertaining to changes in immune status. Different types of immune cells do not function independently of each other. For example, suppressor T lymphocytes inhibit the overall activation of the helper T lymphocyte-mediated immune response. Although research on patients with e.g. HIV or leukaemia, emphasises the importance of the ratio between $CD4^+$ and $CD8^+$ cells, the literature on exercise and stress does not.

Another reason not to rely only on assessment of cell counts is that their functional capacities may also be affected by exercise or stress. Although new methods are being developed to assess functional capacity, we still don't fully understand the interpretation of the results obtained with these assays. This is, in part, because some of the assays are quite non-specific and in part, because some of the assays have not been used to comprehensively assess the more chronic effects of exercise.

The functional responses of the non-specific immune cells, such as neutrophils and NK cells, to exercise have been extensively investigated. Also, the proliferation rate of the general T cell population in response to a stressor has been investigated. However, the latter cannot differentiate between subpopulations with potentially opposing effects, and does not provide information about activation of monocytes, which occurs upstream from T cell activation. Recently, new technology was developed to allow for assessment of immune cell functional capacity (responsiveness to a mitogenic stimulus) in a T cell subpopulation-specific manner, by determination of CD69 expression on activated cells. Assessment of PBMCs capacity to secrete pro-inflammatory cytokines *in vitro* is another rather non-specific assay. However, it is the only functional assay available that allows for assessment of changes in the responsiveness of the inflammatory response to stress.

Although it has been established that PBMCs exhibit decreased ability to secrete IL-6 after exercise, the longer term effects of exercise training on PBMC function is not known. It is unclear whether exercise training-induced increased PBMC activity at rest may render PBMCs less capable of reacting to an additional, exogenous stressor. This possibility may have a significant effect on the risk of athletes for infections in the "open window" period after exercise, during which the specific immune response is also suppressed. However, an alternative possibility is that exercise primes the PBMCs so that an excessive response results.

The endocrine system may also impact on immune system functionality, but the effects of different endocrine parameters on immune function are often interpreted independently of each other. Therefore, the net effects of several role-players with opposing functions, such as catabolic (e.g. cortisol) *vs*. anti-catabolic (e.g. testosterone and DHEAs), are not clear. Assessing changes in the ratios of these different parameters in response to stress, and investigating relationships between these ratios and immune parameters will allow for a more integrated interpretation and application of results.

Communication between the endocrine and immune systems is influenced in part by the messenger system, the cytokines. IL-6 has a central role in activation of both the pro-inflammatory and subsequent anti-inflammatory responses to stress. However, these processes are characterised by many interactions and feedback mechanisms between the various role players. This may explain why controversy exists about the importance of IL-6 in the response to exercise stress and promotion of chronic disease. These controversies are also the motivation for exercise physiologists to focus investigations on this cytokine.

Some of the confounding factors alluded to above include varying levels of every-day psychological stress, large day-to-day intra-individual variation, as well as substantial inter-individual variation in parameters commonly used to assess stress and immune status. Therefore, many investigators have turned to protocols of extreme stress, many of these using animal models, in order to more clearly study the effects of stress. Although these studies provide valuable information, results obtained cannot necessarily be extrapolated to

a more physiological level of stress. Therefore, in order to more accurately model the physiological response to stress, models of mild stress should be used.

In order to investigate and attempt to answer some of these questions, transient changes in and/or adaptations of the various relevant endocrine and immune parameters to stress were assessed in various models. These include a) a model of extreme acute stress in humans (Chapter 3), b) a high-intensity training intervention in cyclists (Chapter 4) and c) a model of chronic psychological stress in sedentary male subjects (Chapter 5). We also developed an animal model of mild, psychological stress (Chapter 6) and confirmed its effectiveness in the assessment of altered stress status (Chapter 7). This model was also implemented in a mechanistic study to study the role of IL-6 in the inflammatory stress response (Chapter 8).

Chapter 3

Determination of the functional ability of peripheral blood mononuclear cells to secrete interleukin-6 using a whole blood culture technique, in samples from athletes participating in an ultra-distance triathlon

3.1 Introduction

Interleukin-6 (IL-6) is a pro-inflammatory cytokine and is released into the circulation in larger quantities than any other cytokine in response to exercise (Pedersen & Hoffman-Goetz, 2000). This previously led to the hypothesis that the increased blood IL-6 concentration post-exercise was an immune response to exercise-induced processes, including muscle damage, which require an inflammatory response to be resolved. However, no correlation has been reported between peripheral blood IL-6 levels and indicators of muscle damage (Jonsdottir *et al.*, 2000); rather IL-6 was shown to be released during exercise as a result of skeletal muscle contraction itself (Ostrowski *et al.*, 1998). Also, another group (Moldoveanu *et al.*, 2000) have shown that IL-6 mRNA concentrations in peripheral blood mononuclear cells (PBMCs) were not influenced after 3 hours of moderate intensity exercise, despite increased plasma concentrations of IL-6. These lines of evidence lend the conclusion that a large percentage of the increased plasma IL-6 concentrations seen immediately after exercise can be accounted for by increased release of IL-6 from exercising muscle, independent of an inflammatory response (Jonsdottir *et al.*, 2000; Ostrowski *et al.*, 1998; Steensberg *et al.*, 2000). It follows that measurement of plasma IL-6 concentrations do not provide accurate information on the immune cell-mediated inflammatory response of an athlete to a specific bout of exercise, or the ability of an athlete to mount an inflammatory response during recovery from exercise.

Jeukendrup *et al*. (2000) recently studied elite athletes who completed an Ironman long distance triathlon and reported a 27-fold increase from baseline in mean plasma IL-6 concentration immediately post-race. An increase in circulatory lipopolysaccharide (LPS) concentration was reported immediately post-exercise, peaking at 1 hour post-exercise.

Also, a decrease in serum IgG anti-lipopolysaccharide levels was reported 2 hours post-exercise, which gradually decreased up to 16 hours post-exercise. From these results it was concludedthat lipopolysaccharide (LPS) enters the circulation of athletes during an ultra-endurance event and causes an immune response. Therefore, both immune cells' responses to LPS entering the circulation and muscle contraction may contribute to the highly increased plasma IL-6 concentrations reported by Jeukendrup *et al*. (2000). The magnitude of exercise stress and gut ischaemia in individuals completing an Ironman triathlon may be much greater compared to that induced by controlled moderate intensity laboratory exercise and this difference may explain the seemingly conflicting results reported by Jeukendrup *et al*. (2000) and Moldoveanu *et al*. (2000). Although Jeukendrup *et al.* established that participants in ultra-endurance events may suffer endotoxaemia, no study has investigated the effect of such an endurance event on the ability of cytokine-secreting immune cells, *in vitro*, to respond to additional stress, although one group previously reported decreased cytokine release by monocytes after participation in a marathon (Starkie *et al.*, 2001a). Therefore the purpose of this study was to investigate the functional response of PBMCs *in vitro* before and after an ultra-endurance event.

The ability of PBMCs in a whole blood culture to release IL-6 spontaneously and also to release IL-6 in response to an endotoxic stimulus (*E.coli* LPS) added *in vitro* was determined. While the former measurement serves as indication of the spontaneous state of excitation of PBMC, the latter provides information regarding changes in the ability of these immune cells to react to mitogens. This is of importance to ultra-endurance athletes, since a decrease in immune cell reactivity after a competitive event may weaken host defence and add significantly to their risk of infection.

3.2 Methods

Subjects: Twelve triathletes (nine male and three female) took part in the study. Written informed consent was obtained from all volunteers prior to the study. This study was granted ethical approval by the University of Stellenbosch Sub-Committee C ethics committee.

Sample collection: Whole blood samples were obtained on three occasions from twelve triathletes participating in the South African Ironman competition (3.8 km swim, 180 km cycle, 42.4 km run): one day prior to the race, within 30 minutes of completing the race, and one week post-race. Subjects were instructed not to exercise for 24 hours prior to resting blood sampling, i.e. the pre- and one-week post-race samples. At each sample time point, 4.5 mL blood was obtained by venepuncture from an antecubital vein, into a pyrogen-free lithium heparin Vacutainer tube (BD Vacutainer Systems, Plymouth, UK). Samples were kept at room temperature and analysed within 4 hours after collection.

Whole blood culture IL-6 assay: Heparinised whole blood was cultured 1:4 in either RPMI 1640 medium (Highveld Biologicals, South Africa) only (unstimulated culture), or in RPMI 1640 medium containing 4.8 units/mL LPS (stimulated culture). After incubation at 37 ºC, in 98 % humidity and in the presence of 5 % CO_2 for 18 hours, according to the method of Pool *et al.* (Pool *et al.*, 2002). After incubation, the supernatant was drawn off and analysed for IL-6 concentration using an in-house ELISA, which was previously validated by comparison to results obtained using a commercially available IL-6 ELISA kit (Amersham)(Pool, 1999). For a detailed description of the ELISA assay, please refer to Appendix B.

Nomenclature of in vitro samples: Samples analysed from wells incubated in medium only are termed 'spontaneous', whereas samples analysed from wells incubated in LPS-containing medium are termed 'activated'. Endotoxin-induced release of IL-6 was calculated by subtracting spontaneous concentrations from activated concentrations. (Although the values obtained in this assay, and specifically the 'spontaneous' value, include the amount of IL-6 present in plasma prior to the incubation period, this amount of IL-6 is negligibly small in comparison to the amount of IL-6 secreted during the 18 hour incubation period.)

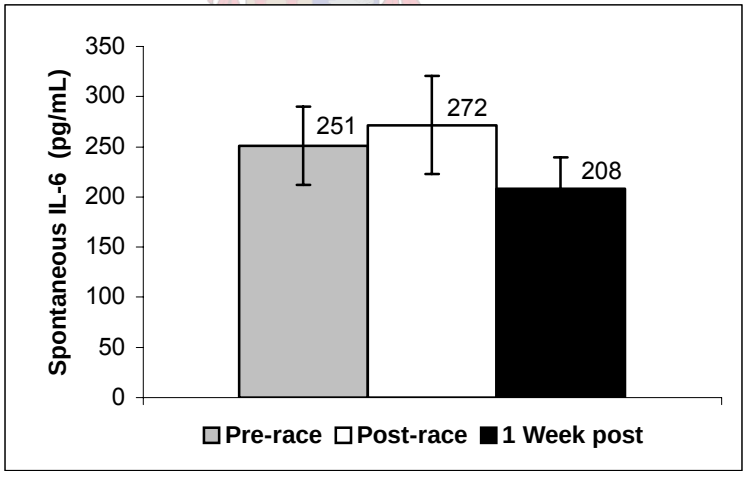
Data analysis: Data were analysed using repeated measures one-way analysis of variance (ANOVA), with a Tukey *post hoc* test, and Pearson's correlations (Statistica 5.5, StatSoft Inc, OK, USA). The accepted level of significance was $P < 0.05$. All results are reported as mean $\pm$ standard error of the mean (SEM), unless otherwise indicated.

3.3 Results

Physical characteristics of the subjects were as follows (mean ± SD): age, 34 ± 5 years, height, 179 ± 12 cm and body mass, 77 ± 13 kg.

No significant differences were found for the spontaneous release of IL-6 *in vitro* between any time points (Figure 3.1). All spontaneous IL-6 concentrations were elevated (above 200 pg/mL) when compared to the results obtained for sedentary subjects at rest (6 ± 8 pg/mL) in a previous study using the same methods (Pool *et al.*, 2002). Spontaneous release of IL-6 by PBMC at rest before the race correlated with spontaneous IL-6 release at rest one week after the race ($r = 0.57$, $P < 0.05$), but neither was correlated with immediately post-race spontaneous IL-6 release ($r = -0.13$ and $r = 0.40$ respectively).

Figure 3.1 *In vitro* spontaneous PBMC IL-6 release obtained before, immediately after and one week after an ultra-endurance triathlon.



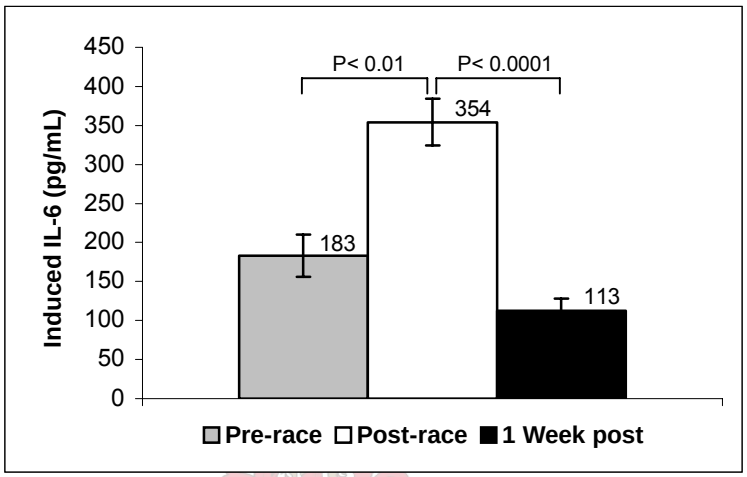
Error bars indicate SEM.

The first and final samples were taken in a properly rested condition at least 24 hours after the previous exercise bout, whereas the middle sample was within 30 minutes post-race.

LPS-induced release of IL-6 was not different pre-race and one week post-race (Figure 3.2). However, induced release of IL-6 after *in vitro* stimulation with *E.coli* LPS was significantly

higher in samples taken immediately post-race compared to both pre-race ($P < 0.01$) and one week post-race ($P < 0.0001$) samples (Figure 3.2).

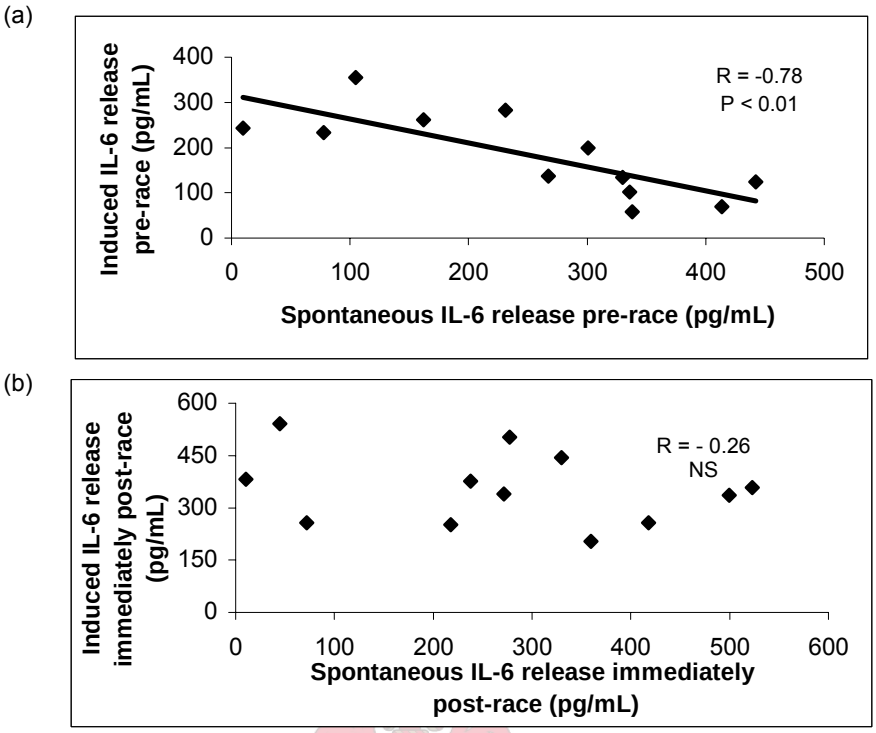
Figure 3.2 LPS-induced IL-6 release by peripheral blood mononuclear cells (PBMC) before (rested condition), immediately after (within 30 minutes) and one week after (rested condition) an ultra-endurance triathlon.



Error bars indicate SEM.

Pre-race spontaneous IL-6 release correlated negatively with induced IL-6 release at the same time point (Figure 3.3a) and again one week post-race spontaneously released IL-6 tended to correlate negatively with induced IL-6 release at the same time point ($r = -0.56$, $P = 0.06$). However, the induced release of IL-6 *in vitro* immediately post-race was not correlated with spontaneous IL-6 release *in vitro* at the same time point (Figure 3.3b).

Figure 3.3 The relationship between spontaneous IL-6 release by PBMC and mitogen-induced IL-6 release by PBMC, (a) pre-race and (b) immediately post-race.



3.4 Discussion

The main findings of this study were 1) chronically elevated spontaneous activity of PBMC *in vitro* in highly trained triathletes that was not affected by participation in an ultra-endurance race, and 2) transiently enhanced ability to respond to a LPS challenge *in vitro*, immediately post-race.

In vitro spontaneous production of IL-6 from blood samples taken at all time points was elevated in comparison to concentrations previously reported for sedentary subjects at rest (Pool *et al.*, 2002). Even one week post-race the comparative elevation in athletes was still more than 300-fold higher than previously reported in sedentary subjects. This indicates chronic excitation of white blood cells in endurance athletes during periods of high training volume and competitive events. This finding supports the findings of Sprenger *et al*., who reported raised concentrations of γ-interferon, IL-1β, tumour necrosis factor (TNF)-α and IL-6 in the urine of well-trained endurance runners pre-race (Sprenger *et al.*, 1992). They

speculated that regular endurance training might achieve a “training of leucocytes”, resulting in chronically increased circulating concentrations of various cytokines.

Studies in rats and humans have shown that a balance exists between pro-inflammatory cytokines, such as IL-6, and their inhibitors (including but not exclusively the anti-inflammatory cytokines), so that the magnitude and duration of an inflammatory response may be restricted (Ostrowski *et al.*, 1999; Xing *et al.*, 1998). In marathon runners, for example, TNF-α, IL-1β and IL-6 release were shown to reach peak plasma concentrations within one hour after the race. At the same time, concentrations of cytokine inhibitors such as soluble TNF-α receptors and IL-1ra, as well as the anti-inflammatroy cytokine IL-10, were also significantly increased, to maintain a balance between the pro- and anti-inflammatory responses to exercise (Ostrowski *et al.*, 1999). We hypothesise that the lack of change in spontaneous IL-6 production as a result of the ultra-endurance event itself may have been due to the presence of a protective counter-regulatory mechanism that may also be chronically active in athletes doing so much endurance training.

In rats, administration of exogenous endotoxin resulted in secretion of both pro-inflammatory IL-6 and anti-inflammatory IL-10 into the circulation (Xing *et al.*, 1998). We further hypothesise that the secondary effect of simultaneous anti-inflammatory adaptation may be sufficient to restrict the effect of further exercise stress (in the form of continued training or a competitive event) on the inflammatory response. This hypothesis is substantiated by our observation of no further influence of the race itself on spontaneous PBMC IL-6 release despite the likelihood of elevated PBMC counts post-exercise, as well as by the negative correlation that existed between spontaneous and mitogen-induced PBMC IL-6 release at rest, which indicated that a greater spontaneous activity could inhibit the mitogen-induced activity in PBMCs.

However, a significant increase in *in vitro* LPS-induced IL-6 release by PBMC was observed immediately after the ultra-endurance event itself (159 ± 50 % higher than pre-race). The absolute values obtained were similar to that reported in sedentary individuals at rest, in a previous study by our group (Pool *et al.*, 2002), but since a control group was not included in this study, no direct comparisons in this regard is possible. Moldoveanu *et al*. reported no

significant effect of exercise on IL-6 mRNA expression in circulating mononuclear cells after three hours of moderate intensity exercise, despite an increase in circulating IL-6 levels post-exercise (Moldoveanu *et al.*, 2000). Therefore, one possible reason for the increased IL-6 secretion measured in this study could be an increase in circulating monocytes rather than increased IL-6 release by existing monocytes. Increased circulating monocyte numbers were previously reported after exercise (Espersen *et al.*, 1990; Nieman *et al.*, 1989a). However, since we did not measure monocyte count in this study, we cannot substantiate this specifically. Of importance is that the chronic counter-regulatory effect was lost directly after the race, indicating that either the extent of the stress (either intensity, or duration, or both) overrode the level of adaptation, or that the newly circulating monocytes did not have this adaptation.

3.5 Conclusion

The spontaneous IL-6 results reported in the current study support the idea of Sprenger *et al*. (Sprenger *et al.*, 1992), of a training effect on the chronic level of excitation of cytokine secreting immune cells. Our data provides sufficient evidence to hypothesise that such chronic spontaneous elevations may limit an exercise-induced pro-inflammatory response by activating endogenous counter-regulatory mechanisms to prevent further increases in circulating cytokine concentrations during training or racing, unless a severe mitogenic stimulus is experienced. Our results indicate that the proposed counter-regulatory mechanisms *in vivo* are not sufficient to prevent an ultra-endurance race from eliciting a post-race response to an exogenous stimulant *in vitro* in excess of the response typically elicited to the same exogenous stimulant at rest. Further investigation is warranted to clarify the different mechanisms responsible for the chronic counter-regulation and the post-race attenuation of this counter-regulation.

3.6 Limitations

A limitation of this study is that total and differential white cell counts were not measured. Determination of total WBC counts would have enabled expression of LPS-induced IL-6

secretion normalised on a per cell basis. This would have allowed for interpretation of changes in counts as a result of exercise, as well as changes in cell function relative to cell number.

Furthermore, measurement of plasma IL-6 concentration may provide information with regard to the contribution of immune cells to post-exercise changes in IL-6 concentration. In addition, in the PBMC assay, the dosage of LPS used followed convention of published studies. Therefore, the assay design does not allow for assessment of possible changes in PBMC sensitivity to LPS over time or as result of exercise. Since monocytes were previously shown to be more sensitive to LPS stimulation in athletes post-exercise when compared to pre-exercise values (Duclos *et al.*, 1999), more than one concentration of LPS should be used at each time point in future studies to correct for such changes in sensitivity.

Chapter 4

Effect of performance enhancing high-intensity cycling training on selected endocrine and immune parameters

4.1 Introduction

The ideal exercise training programme allows an athlete sufficient rest to recover between bouts of exercise. Athletes should be able to start each training session sufficiently well-rested, to be able to train at the desired intensity and with the desired perceived effort. However, due to the ever-increasingly high standards set in professional sports today, moderate training programmes are not sufficient for athletes to excel at their sport. Therefore athletes must include in their weekly programmes days of very strenuous high-intensity training, interspersed with sessions of longer duration, both of which may take their toll on, amongst others, the neuroendocrine-immune axis.

Early epidemiological surveys in marathon athletes showing an increase in the incidence of upper respiratory tract symptoms after exercise (Nieman *et al.*, 1989b; Nieman *et al.*, 1990; Peters & Bateman, 1983), have stimulated interest in the effect of exercise on the non-specific immune system. Since these surveys, many studies have investigated and reviewed the suppressive effect of exercise stress on the non-specific immune response (Berk *et al.*, 1990; Blannin *et al.*, 1996; Gleeson *et al.*, 1998; Heath *et al.*, 1992; Nieman, 1995; Nieman, 2000; Robson *et al.*, 1999; Walsh *et al.*, 2000) and these studies are in agreement that the exercise-induced changes in the non-specific immune function are only transient. However, much less information exists on the effect of stress on the specific, T cell-mediated immune response. Since proper functioning of T cell-mediated immunity is vital to prevent long-term illnesses, which may be linked to either overactivation (e.g. auto-immune disease) or underactivation (which may predispose an individual to suffer from e.g. cancer), it is important to understand the influence of chronic stress on this subsection of the immune system.

Lymphocyte counts, and more specifically T lymphocyte subpopulation counts, are reported to increase during acute exercise and to decrease to either baseline (Hoffman-Goetz *et al.*, 1990; Mazzeo *et al.*, 1998; Ronsen *et al.*, 2001b) or below baseline (Kajiura *et al.*, 1995; Natale *et al.*, 2003; Shore *et al.*, 1999) following recovery after the exercise bout. The magnitude of change in T lymphocyte subpopulation counts may be influenced by fitness or exercise intensity, or both, with long duration aerobic exercise resulting in the biggest change (Mackinnon, 1999; Natale *et al.*, 2003). However, since most acute exercise-induced changes in lymphocyte counts have been shown to be transient, resulting in only a short "open window" period of altered immune status, the chronic response to the cumulative stress of training regimens may be more accurately reflected by changes in immune status assessed under rested conditions.

Literature on the effect of training on resting immune cell counts is not consistent. While experimentally controlled mild cycling training lasting 12 weeks was reported to decrease resting total T lymphocyte counts in previously sedentary male subjects, moderate training (same intensity, but more sessions per week) did not result in a decrease in resting T lymphocyte counts in another group. Neither of the two regimens affected $CD4^+$:$CD8^+$ T cell ratio (Shore *et al.*, 1999). On the other hand, other research groups reported that increased training volume in experienced or elite athletes either had no effect on total and subpopulation T-cell counts (Dressendorfer *et al.*, 2002), or decreased the number of $CD4^+$ and $CD8^+$ cells in circulation (Baj *et al.*, 1994; Verde *et al.*, 1992). It therefore remains unclear how and to what extent T cell counts respond to changes in training.

Furthermore, immune competency relies not only on the number of cells present, but also on their functional capacity. A number of studies investigated the lymphocyte proliferative response to either acute exercise bouts or training, or both (Baj *et al.*, 1994; Fry *et al.*, 1992; Shore *et al.*, 1999; Verde *et al.*, 1992), but again the results varied. Acute anaerobic kayaking or running to fatigue, in both recreational and high-performance athletes, were reported to result in a transient decrease in mitogen-induced lymphocyte proliferative response *in vitro* (Fry *et al.*, 1992). All exercise-induced changes returned to baseline after 2 hours of recovery. Similarly, the lymphocyte proliferative response that was measured in resting samples from previously sedentary male subjects before and after cycling training

(mild or moderate volume) lasting 12 weeks, was not affected by the training regimen (Shore *et al.*, 1999). These data seem to imply that only acute high-intensity exercise may be transiently detrimental to specific immune function. However, in trained subjects, an increase in training volume and intensity resulted in an increase in the lymphocyte proliferation rate assessed in resting samples (Baj *et al.*, 1994; Verde *et al.*, 1992), indicating a more chronic effect.

Furthermore, it is unclear whether the reported exercise-induced changes in lymphocyte proliferation rate are due to responsiveness of cells to mitogenic activation, or whether the regulation of proliferative capacity occurs downstream from activation. An early step in activation of lymphocytes is expression of CD69, which was previously investigated before and after acute bouts of exercise (Gabriel *et al.*, 1993; Green *et al.*, 2003; Ronsen *et al.*, 2001c; Ronsen *et al.*, 2001b; Ronsen *et al.*, 2002; Vider *et al.*, 2001). Four of these studies investigated mitogen-stimulated CD69 expression by helper and suppressor T cells in well-trained or elite athletes, but results reported in the different studies are inconsistent: one study showed decreased proportions of PHA-stimulated $CD4^+$ and $CD8^+$ cells expressing CD69 after acute incremental exercise to exhaustion (Vider *et al.*, 2001), and the others reported no effect of a single bout (Ronsen *et al.*, 2001b; Ronsen *et al.*, 2002) or repeated bouts (Ronsen *et al.*, 2001c; Ronsen *et al.*, 2002) of longer duration strenuous exercise on these cells' activation status. The seemingly contradictory results suggest that CD69 expression may only be sensitive to very high exercise intensities, but that this can result in compromise of both $CD4^+$ and $CD8^+$ cell responsiveness. These studies investigated only the acute effects of exercise, and the longer-term effect of exercise training on responsiveness of these cells at rest is still unknown. However, since typical training programmes for performance enhancement include high intensity training and Vider *et al*. (2001) showed that acute high intensity exercise had a suppressive effect on lymphocyte CD69 expression, we hypothesise that the training programmes of competitive athletes are likely to have a cumulative negative effect.

The acute immune response to exercise is hypothesised to be mainly a "side-effect" of the exercise-induced changes in cardiac output, controlled by catecholamine action (Foster *et al.*, 1986; Malm, 2002) and exercise-induced fuel mobilisation controlled by e.g. cortisol

(Newsholme *et al.*, 1993; Newsholme, 1994). Therefore, if any chronic effects of training on the immune system are observed, they may also be mediated by the endocrine system. It is well-established that (in the absence of overtraining or negative energy balance) endurance training has no effect on resting blood cortisol concentration (Kraemer *et al.*, 1989; Urhausen & Kindermann, 1992), whereas testosterone concentrations are decreased during recovery for a period which may differ in duration from a few hours to several days (Urhausen & Kindermann, 1992). Since these changes are still only transient, it was suggested that hormonal monitoring of training stress is not useful (Urhausen *et al.*, 1995). However, since it is now known that white blood cells carry receptors to both androgens and glucocorticoids (Benten *et al.*, 1999a; Benten *et al.*, 1999b; Grasso *et al.*, 1997; Vorobiev *et al.*, 1996), the relationships between these parameters of the endocrine system and the chronic immune response to training should still be assessed.

The purpose of this study was therefore 1) to determine whether a laboratory-based, controlled high-intensity training intervention, which is sufficiently strenuous to result in increased performance, would negatively influence the responsiveness of the specific immune system in recreational cyclists, and 2) to investigate whether increased training intensity may alter relationships between the endocrine and immune systems.

4.2 Methods

Subjects: Seven recreational, competitive male cyclists took part in the study, after giving their written, informed consent. Ethical approval was gained from the Stellenbosch University ethics committee. Mean (± SD) age, height and weight were 22.6 ± 4.7 years, 180 ± 10 cm and 73.1 ± 6 kg respectively, and average cycling peak power output (PPO) and VO_{2max} on entrance into the study was 354 ± 13 W and 56.1 ± 4.7 mL/min/kg .

Training and performance testing intervention: The PPO test was performed on an electronically braked cycle ergometer, and consisted of incremental workloads to voluntary exhaustion, with initial workload determined by multiplying the subject's body mass by 1.33, and subsequent workloads increasing by 30 watt each (protocol according to ACSM

guidelines). VO_{2max} was defined as the maximum oxygen consumption during the PPO test. The 6-week intervention entailed a 4-week period of controlled high intensity (HI) cycle training, with individualised load adjustment according to the change in PPO after 2 weeks. HI training consisted of 2 laboratory-based training sessions per week: 1) 5-8 bouts (increased by 1 bout each week) of cycling for 5 minutes at 80 % PPO, with 1 minute at 50 % PPO between bouts, and 2) 2 bouts of cycling for 5 km at 90 % of 5 km time trial (5TT) speed, with 20 minutes cycling at 50 % 5TT speed between the two bouts. This phase was followed by 2 weeks of controlled supra-maximal intensity (SMI) cycle training. SMI training consisted of 3 laboratory-based training sessions per week: 1) 8 bouts of cycling for 5 minutes at 80 % PPO, with 1 minute at 50 % PPO between bouts, 2) 10 bouts of cycling for 90 seconds at 110% PPO, followed by 3.5 minutes cycling at 50 % PPO, and 3) 3 bouts of cycling 2.5 km at 110 % 5TT speed, followed by 15 minutes cycling at 50 % 5TT speed. Subjects were instructed to maintain their usual outdoor training load during HI, to reduce outdoor training by only one session per week during SMI, and to refrain from exercising for 24 hours prior to performance testing sessions. Each subject kept a daily training diary from 2 weeks prior to the study and throughout the study protocol, to enable validation of compliance. PPO was performed on an electronically braked cycle ergometer (Bikerace, Technogym, Italy), at baseline (B), post-HI and post-SMI training. Performance was assessed by 5TT and 40 km time trial (40TT) performed at B, post-HI and post-SMI, using the subject's own bicycle on a time-trial ergometer (Spintrainer, Technogym, Italy) as previously described (Myburgh *et al.*, 2001).

Sample collection: Subjects were instructed not to exercise for at least 24 hours prior to collection of resting blood samples. Whole blood samples were taken fasted and at rest, at 08h00 in the morning at B, post-HI and post-SMI. Blood samples were obtained from an antecubital vein by venepuncture, and collected into 4.5 ml EDTA Vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) for determination of full blood count (FBC) and differential white cell count (diff), and into 4.5 ml lithium heparin Vacutainer tubes for determination of lymphocyte subpopulation distribution and CD69 expression on helper- and suppressor T-lymphocytes. Blood was also collected into 4.5 ml SST Vacutainer tubes for determination of serum concentrations of cortisol, cortisol binding globulin (CBG), testosterone, sex hormone binding globulin (SHBG), and dehydroepiandrosterone-sulphate

(DHEAs). All blood samples collected in SST tubes were allowed to clot at room temperature for 10 minutes and were subsequently centrifuged at 3000 rpm for 10 minutes at 4 °C, before being aliquoted into eppendorf tubes and frozen at –80 °C until subsequent batch analysis.

Sample analysis: All blood samples for immune analyses were analysed within three hours after collection. EDTA-anticoagulated whole blood samples were analysed for FBC and differential white blood cell count (Coulter STKS, Beckman/Coulter, Fullerton, CA). Using heparinised whole blood, monoclonal antibodies (Multitest, SA Scientific Products, Jhb, SA) and three-colour flow cytometry, lymphocytes were divided quantitatively, into the following subpopulations: total T-cells ($CD3^+$), T-helper cells ($CD4^+$), T-suppressor/cytotoxic cells ($CD8^+$), total B-cells ($CD19^+$) and natural killer (NK) cells ($CD16^+/CD56^+$). The activation of $CD4^+$ and $CD8^+$ cells in response to a stressor was determined by measuring the expression of the cellular activation marker, CD69, after *in vitro* exposure to a stimulant, as previously described (Breytenbach *et al.*, 2001). Briefly, this entails incubation of whole blood in the presence of the synthetic stimulant phorbol-12-myristate-13-acetate (PMA) and ionomycin (1 ug/mL). After 4 hours of incubation, the cells are labeled with a fluorescence-labelled monoclonal antibody specific for CD69 and fluorescent cells quantified using standard flow cytometry procedures (FACSCalibur, Becton Dickinson, New Jersey, USA).

Chemiluminescent analytical procedures were performed for determination of cortisol (Access B81600, Beckman/Coulter, Fullerton, CA, USA), DHEAs and SHBG concentrations (Immulite I, Diagnostic Products, Los Angeles, CA, USA). Testosterone concentration was determined by immunoassay (Advia Centaur, Bayer Diagnostics, Leverkusen, Germany) and CBG concentration by radioimmunoassay (CBG-RIA-100, Biosource, Nivelles, Belgium). Free testosterone concentration is derived from the law of mass action at equilibrium using a specially designed computer program containing a complex mathematical formulation (Rinaldi *et al.*, 2002; Vermeulen *et al.*, 1999). This method was not available, so instead the free androgen index (FAI), which is commonly used by clinicians, was calculated using the formula:

$$FAI = \frac{\text{Testosterone concentration}}{\text{SHBG concentration}} \times 100$$

Serum cortisol concentration, CBG concentration and CBG binding affinity constants were used to calculate the concentration of biologically active (unbound) cortisol, using the following previously validated equation (Coolens *et al.*, 1987):

$$U = \sqrt{0.0167 + 0.182 * (T - C)^2 + 0.0122 * C} - 0.0167 + 0.182 * (T - C),$$

where U = unbound cortisol concentration, T = CBG (transcortin) concentration and C = total cortisol concentration (all units for concentration were converted to nmol/L).

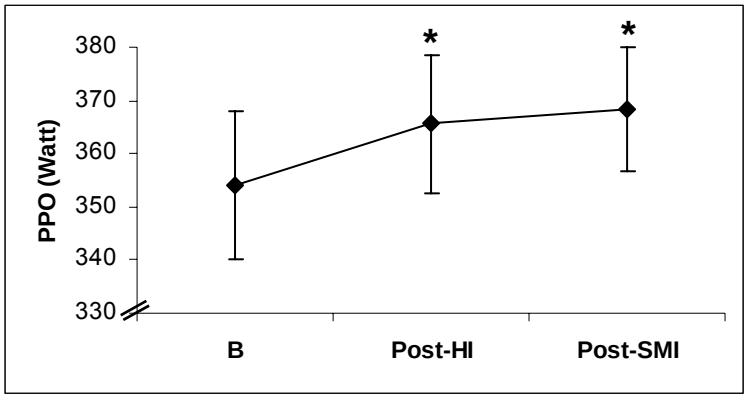
Data analysis: Data sets were compared using repeated measures analysis of variance (ANOVA) with Bonferroni multiple comparison *post hoc* tests, and Pearson's correlation coefficients. Performance and endocrine results are reported as means ± SD, and immune results as means ± SEM. The level of significance was set at $P < 0.05$.

4.3 Results

Performance: Repeated measures analysis of variance indicated a significant effect of time on PPO ($P < 0.01$), 5TT ($P < 0.01$) and 40TT ($P < 0.05$). Bonferroni *post hoc* analysis indicated that PPO, 5TT and 40TT performances improved significantly from B to post-HI (PPO: 354 ± 13 *vs*. 366 ± 12 Watt; $P < 0.05$; 5TT: 443 ± 15 *vs*. 427 ± 13 seconds $P < 0.05$; 40TT: 64.4 ± 3.0 *vs*. 61.7 ± 1.5 minutes; $P < 0.05$), and were maintained during SMI (Figure 4.1).

Figure 4.1 Improvement in (a) PPO, (b) 5TT and (c) 40TT performances in response to training intervention.

(a)



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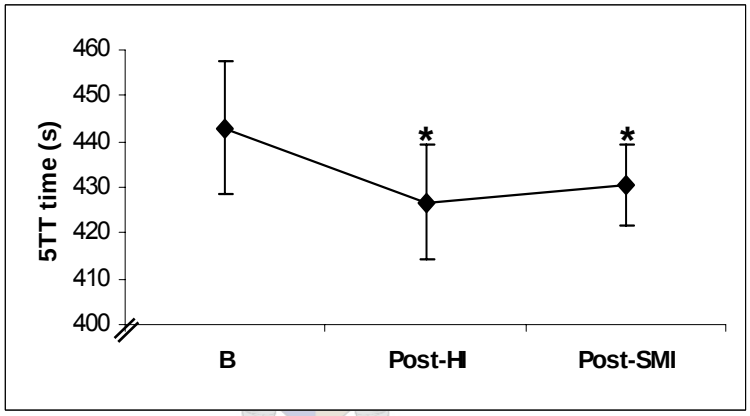

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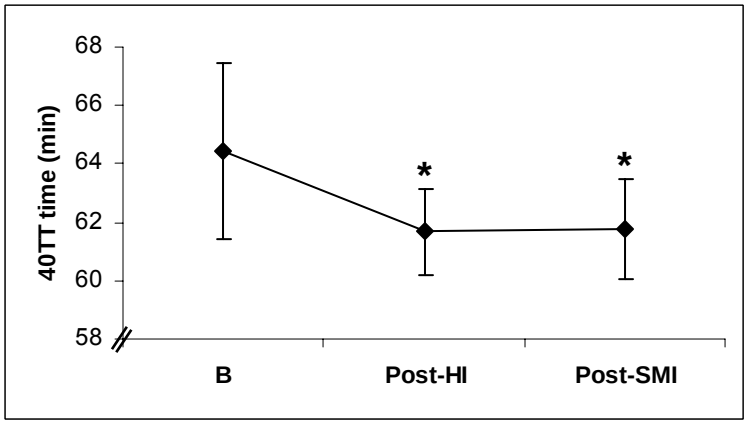


Error bars indicate SD

** Value significantly different from baseline (P < 0.05)*

T cell counts: Although the mean total WBC and means for all lymphocyte subpopulation counts tended to be lower post-HI compared to B, only that of the $CD8^+$ cells was

significantly decreased. No cell counts assessed were different from any other time point post-SMI (Table 4.1).

Table 4.1 Changes in lymphocyte subpopulation counts in response to the training intervention.

	Units	B	Post-HI	Post-SMI
WBC	cells/uL	6414 ± 1189	5414 ± 1068	5714 ± 855
CD3$^+$	cells/uL	2628 ± 711	2277 ± 559	2034 ± 1035
CD4$^+$	cells/uL	1598 ± 461	1152 ± 302	1240 ± 628
CD8$^+$	cells/uL	846 ± 199	**640 ± 147 ***	727 ± 132
CD4:CD8 ratio		1.82 ± 0.39	1.88 ± 0.60	1.72 ± 0.39
CD16$^+$/CD56$^+$	cells/uL	692 ± 194	491 ± 178	583 ± 169

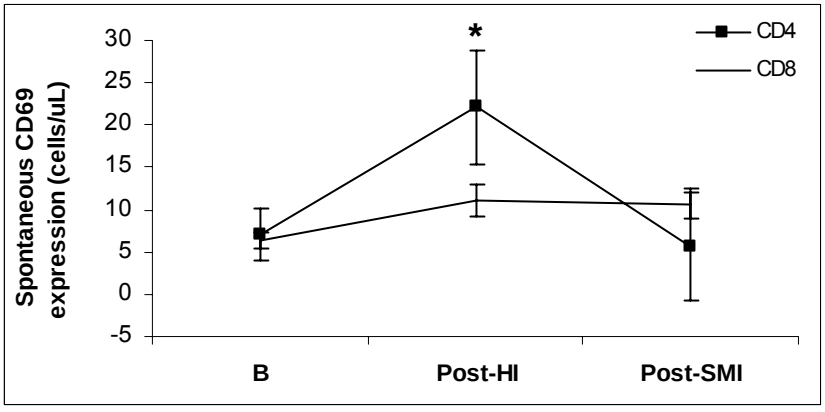
Values are means ± SEM.

** Value significantly different from baseline (P < 0.05).*

Spontaneous CD69 expression by CD4$^+$ cells, but not CD8$^+$ cells, was significantly increased post-HI ($P < 0.05$; Figure 4.2a), while the mitogen-induced CD69 expression was transiently decreased for both subpopulations at the same time point ($P < 0.05$; Figure 4.2b). Both spontaneous and mitogen-induced CD69 expression for both CD4$^+$ and CD8$^+$ cells had returned toward baseline levels post-SMI.

Figure 4.2 Changes in the (a) spontaneous and (b) mitogen-induced expression of CD69 by CD4$^+$ and CD8$^+$ cells as a result of changes in training volume and intensity.

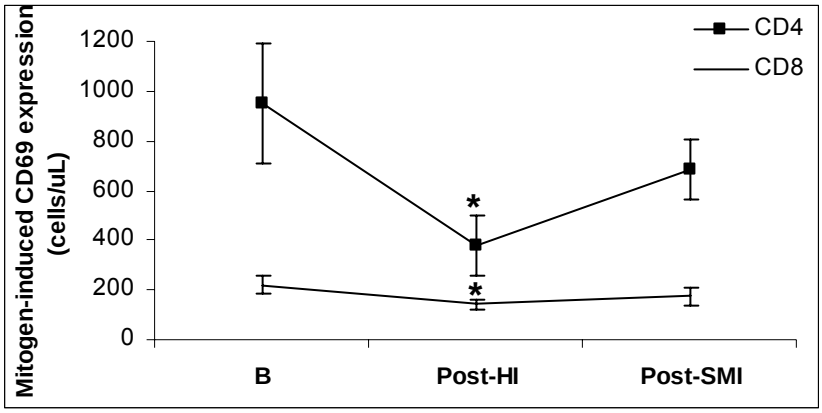
(a)



Error bars indicate SEM.

** Value significantly different from baseline (P < 0.05).*

(b)



Error bars indicate SEM.

**Value significantly different from baseline (P < 0.05).*

Endocrine measures: No effect of the training intervention was found on any of the endocrine parameters measured at rest (Table 4.2). However, a subsequent power analysis revealed that the low subject numbers in this study is not sufficient to exclude an effect of the intervention on these parameters. Unfortunately, since an estimated subject number in excess of 45 is required to yield an analytical power of 0.6 for the parameters assessed, testing sufficient numbers of athletes was not feasible. No correlations were found between any of the measured immune parameters and cortisol or DHEAs or CBG concentrations. However, several relationships existed between testosterone concentrations and immune parameters at B (Table 4.3), which were no longer evident after the training intervention. In addition, while total serum testosterone was positively correlated with the unbound fraction of testosterone throughout the protocol (R=0.90, 0.71, 0.96 and P< 0.01, 0.05, 0.001 respectively for B, post-HI and post-SMI), there was no correlation between the concentration of total and unbound cortisol at any time point.

Table 4.2 Serum concentrations of selected endocrine parameters and relationships between these parameters over time.

	B	post-HI	post-SMI
Cortisol (nmol/L)	608 ± 63	621 ± 75	599 ± 139
CBG (ug/mL)	51 ± 9	58 ± 12	52 ± 16
Unbound Cortisol (nmol/L)	390 ± 170	340 ± 150	380 ± 170
DHEAs (umol/L)	9.0 ± 2.3	7.9 ± 2.6	8.1 ± 2.6
Cortisol:DHEAs ratio	0.07 ± 0.01	0.09 ± 0.03	0.08 ± 0.03
Testosterone (nmol/L)	24 ± 6	20 ± 6	22 ± 6
SHBG (nmol/L)	28 ± 8	34 ± 17	29 ± 6
Free androgen index (%)	89 ± 11	68 ± 14	77 ± 8
Cortisol:Testosterone ratio	26.3 ± 4.5	35.4 ± 15.2	31.3 ± 15.4

Values are means ± SD

Level of significance: P < 0.05

Table 4.3 Associations between testosterone concentration and lymphocyte subpopulation counts and activation status.

Testosterone *vs.*:	**B**	**Post-HI**	**Post-SMI**
$CD3^+$	**0.85****	0.37	- 0.03
$CD4^+$	**0.83***	0.44	- 0.07
$CD8^+$	**0.86****	0.22	0.04
CD4:CD8 ratio	0.23	0.47	- 0.44
$CD16/CD56^+$	**0.84***	0.16	- 0.14
Spontaneous $CD4^+CD69^+$	**0.79***	- 0.01	- 0.21
Spontaneous $CD8^+CD69^+$	**0.84***	- 0.19	0.28
Mitogen-induced $CD4^+CD69^+$	**0.84***	0.39	0.03
Mitogen-induced $CD8^+CD69^+$	**0.90****	0.14	- 0.12

Values are R-values.

** P < 0.05; ** P < 0.01*

Change in individual training volume and intensity: The training diaries (summarised in Table 4.4) indicated that the participants in this study maintained their average weekly outdoor training volume (distance cycled) throughout the study, although there was a tendency to decrease individual training volume during SMI (P = 0.07). However, their training intensity outdoors (average speed) increased during HI and was maintained during SMI. Individual outdoor training volume correlated negatively with CD4:CD8 ratio at B and post-HI, but not post-SMI (Figure 4.3).

Table 4.4 Mean weekly outdoor training volume and intensity, as well as number of training sessions per week both outdoors and indoors.

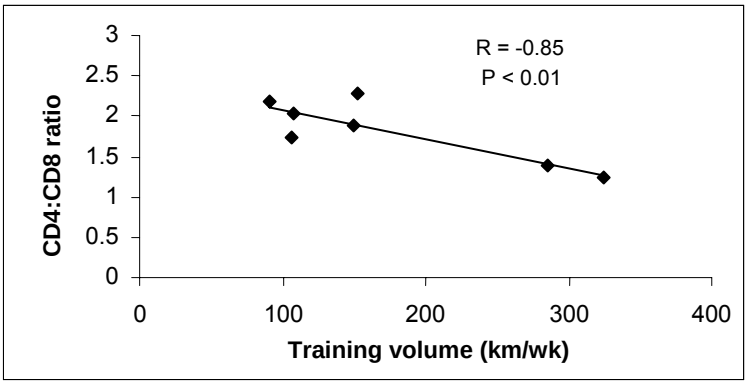
	Pre-B	**During HI**	**During SMI**
Volume (km cycled)	174 ± 93	177 ± 99	133 ± 60
Intensity (average speed, km/h)	26 ± 2	**29 ± 1***	**28 ± 2***
Training sessions per week	4.2 ± 0.6	**6.2 ± 1.3***	5.9 ± 0.9

Values are means ± SD.

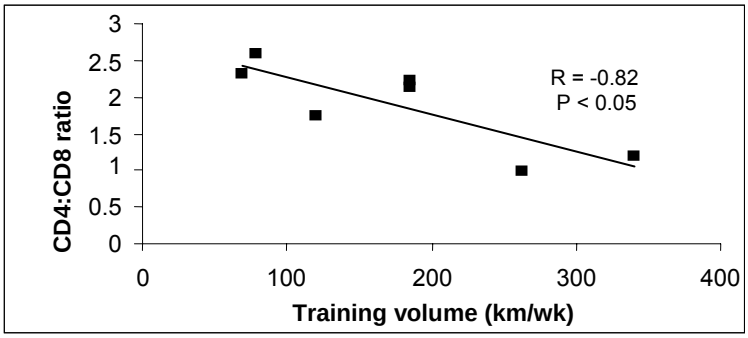
** Significantly higher than baseline value (P < 0.01).*

Figure 4.3 Relationships between training volume and the CD4:CD8 ratio at rest in recreationally competitive cyclists at (a) B, (b) post-HI training and (c) post-SMI training.

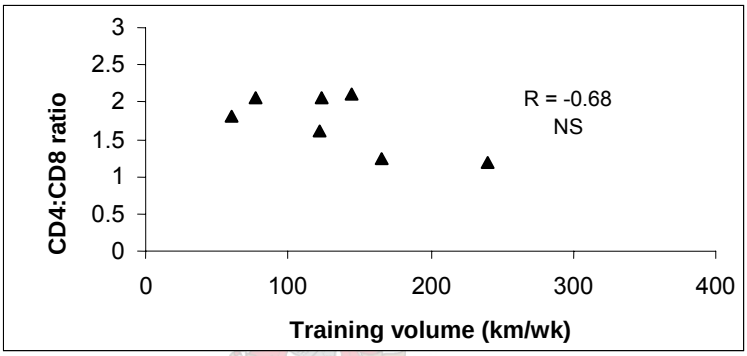
(a)



(b)



(c)



4.4 Discussion

The main findings of this study were that increased training intensity in the laboratory and outdoors with maintenance of individual outdoors training volume: a) increased performance significantly, b) was associated with decreased $CD8^+$ T cell counts at rest, c) increased spontaneous activation of $CD4^+$ and decreased mitogen-induced activation of $CD4^+$ and $CD8^+$ T cells at rest, but d) did not significantly affect the resting endocrine parameters assessed. Although the performance and endocrine measures remained unchanged after a subsequent period of supra-maximal intensity laboratory training, immune parameters returned toward baseline values, as did the number of training sessions per week.

Performance: The fact that all three performance measures improved after HI training was expected, since the HI training protocol used in this study was similar to those used in previous studies which reported both increased endurance performance and increased peak sustained power output post-training (Lindsay *et al.*, 1996; Westgarth-Taylor *et al.*, 1997).

The second finding, that SMI training did not further improve performance, has more than one possible explanation. Firstly, the supra-maximal training sessions (at 110 % maximum) may have been of too severe intensity for the recreationally competitive athletes to recover sufficiently within the one day allowed prior to performance assessments. This is in agreement with the earlier finding of no improvement in performance (running economy, treadmill VO_{2max}, time to fatigue and maximum distance, both run at minimal velocity associated with VO_{2max}) after 4 weeks of unaccustomed supra-maximal intensity interval training (Billat *et al.*, 1999). Alternatively, the specificity of the performance tests was more applicable to high intensity, but sub-maximal training than to training at maximal or supra-maximal intensities. This possibility is in agreement with an earlier training study, which reported increased 40 km time-trial performance after 3 weeks of interval training at 85 % PPO, but no significant change in this performance measure after 3 weeks of interval cycling training at 100 % PPO (Stepto *et al.*, 1999). Secondly, there was a tendency for subjects to reduce both the volume of their own individual training and training frequency during SMI, but to maintain their intensity of outdoor training while laboratory-based training increased in intensity (Table 4.4). The effect of 6 days of high-frequency taper (training daily) *vs*. moderate-frequency taper (training 2 – 3 times per week) on 800 m running performance was recently investigated in middle-distance runners (Mujika *et al.*, 2002). (Taper consisted of an 80 % progressive reduction in high-intensity training.) While high-frequency taper was associated with a significant increase in performance, moderate-frequency taper did not have an enhancing effect, possibly due to a detraining effect of training only 2 – 3 times per week. However, although slightly lower when compared to HI, the average number of weekly training sessions for our subjects during SMI (≈ 6) was still similar to the high-frequency regimen described by Mujika *et al.* (2002), so that changes in training frequency is unlikely to be the cause of our result of no performance improvement after SMI. Together, these results suggest that a decrease in high-intensity exercise with maintenance of training frequency is necessary to further enhance performance, while a high-frequency, high-intensity taper, while maintaining performance, does not result in further improvement.

Immune parameters: The significant decrease in resting $CD8^+$ lymphocyte count after HI training is in accordance with previous results of decreased T cell counts at rest after both short-term (5 days) and longer-term (12 weeks) submaximal training in previously sedentary

subjects (Hoffman-Goetz *et al.*, 1990; Shore *et al.*, 1999). This suggests that the decreased lymphocyte subpopulation counts we report in this study were as a result of either unaccustomed training or the intensity of the training. However, recently no change in resting lymphocyte subpopulation counts during or after intensified cycling training (4 weeks baseline training, 6 weeks increased volume training, 18 days maximal intensity interval training, 10 days taper) was reported (Dressendorfer *et al.*, 2002). The difference in results is possibly due to the fact that the athletes who participated in the study by Dressendorfer *et al.* (2002) were more experienced cyclists (on average had 4.4 ± 2.1 years of competition experience) than our recreationally competitive individuals. Also, while our study took place at the start of the racing season, that of Dressendorfer *et al.* (2002) took place at the end of the racing season. It is therefore likely that the training protocol followed by Dressendorfer *et al.* (2002) at the end of a competitive season did not represent an unaccustomed training load to subjects, while our high-intensity protocol at the start of a season was likely to represent an unaccustomed training load to our subjects, who have been doing maintenance training only during the off-season. The normalisation of lymphocyte subpopulation counts and activation status after SMI, when subjects had become more accustomed to a higher intensity of training, supports this contention.

Our data also clearly indicate decreased mitogen-induced CD69 expression by both $CD4^+$ and $CD8^+$ T cells *in vitro* post-HI. Although a different type of functional test, this is in accordance with previous reports of suppressed lymphocyte proliferative response after a few weeks of high intensity training (Verde *et al.*, 1992). Our results add to the literature by indicating that the changes in lymphocyte proliferation rate previously reported are not limited to a specific subpopulation of T cells, although the decreased in mitogen-induced CD69 expression was much greater for $CD4^+$ T cells than for $CD8^+$ T cells. Also, we show that training-induced decreases in resting lymphocyte function, previously reflected simply by decreased lymphocyte proliferation rate, are brought about by regulation of lymphocyte activation, rather than by a mechanism downstream from activation. Two recent studies assessed both $CD4^+$ and $CD8^+$ cell activation (CD69 expression) and T cell proliferative response on the same blood samples. In one study, an acute bout of exercise (60 minutes treadmill running at 90 % of ventilatory threshold) in well-trained endurance runners had no apparent effect on either of the two functional tests (Green *et al.*, 2003), while in the other

study (Vider *et al.*, 2001), exhaustive exercise resulted in a decrease in both CD69 expression and lymphocyte proliferation rate in response to mitogen stimulation, illustrating that changes in activation do result in similar changes downstream. Assessment of CD69 expression may indeed be a more time-economical, direct and informative test for lymphocyte responsiveness.

Apart from changes in mitogen-induced CD69 expression, which all groups investigating this parameter report, we assessed changes in spontaneous CD69 expression by T cells after a period of incubation without the presence of a mitogen. Although the proportion of cells expressing CD69 spontaneously is relatively small, these results may provide additional information on the chronic activation status of these cells in the absence of an additional stressor or immune challenge. Our results indicate an increased spontaneous reactivity of $CD4^+$ cells, but not $CD8^+$ cells, post-HI, which returned to baseline post-SMI (Figure 4.2a). Chronically increased spontaneously active cells may contribute to chronic inflammation after long-term high-intensity training, or may contribute to an inability to mount an appropriate acute response to exogenous stimuli. Although no significant correlation existed between spontaneous and mitogen-induced CD69 expression, the decreased average mitogen-induced CD69 expression post-HI compared to the capacity at baseline supports the latter possibility. The implication of this finding on immune function warrants further investigation to elucidate its clinical importance.

The relatively small percentage of cells spontaneously activated in comparison to the percentage of cells activated by mitogen stimulus (≈ 1.5 % vs. ≈ 49 %), implies that the regular stimulus provided by exercise training is mild. However, at the same time it was sufficient to result in general desensitisation of lymphocytes and a lower response to a mitogen challenge *in vitro*. A candidate mechanism for the desensitisation of lymphocytes is the increased cortisol released in response to unaccustomed exercise. Indeed, strenuous exercise was previously shown to result in decreased corticosteroid sensitivity of peripheral blood lymphocytes (decreased dexamethasone inhibition of LPS-induced IL-6 secretion in a whole blood culture) (DeRijk *et al.*, 1996). Although one or two repeated bouts of strenuous exercise is insufficient to result in altered $CD4^+$ and $CD8^+$ lymphocyte responsiveness

(Ronsen *et al.*, 2001c; Ronsen *et al.*, 2002), we show that unaccustomed training over several weeks has a suppressive effect on lymphocyte function.

The difference between our results, on blood samples taken at rest, and those of Ronsen *et al.* (Ronsen *et al.*, 2001b), who illustrated no effect of seasonal training on $CD4^+$ lymphocyte CD69 expression immediately post-exercise, illustrates the importance of investigating changes in immune measures at rest. In some instances the acute effect of exercise may mask chronic changes in the immune status of athletes. In addition, our results indicate the necessity of not only considering mitogen-induced CD69 expression, as previously done by other groups, but also spontaneous CD69 expression, for a more comprehensive interpretation of data.

Endocrine parameters: Our result of no significant change in any measured endocrine parameter assessed at rest (Table 4.1) has previously been established in various studies (Kraemer *et al.*, 1989; Urhausen & Kindermann, 1992; Urhausen *et al.*, 1987; Urhausen *et al.*, 1995). However, acute strenuous exercise was shown to decrease peripheral blood mononuclear cell (PBMC) sensitivity to exogenous glucocorticoid action, so that a higher concentration of dexamethasone was required to inhibit IL-6 secretion *in vitro* post-exercise compared to pre-exercise (DeRijk *et al.*, 1996). If this reported acute glucocorticoid insensitivity of immune cells after exercise becomes chronic with excessive training, an unchanged cortisol concentration would be insufficient to suppress inflammation. This may suggest, at least in part, a mechanism for the recent theory that chronic inflammation causes overtraining (Smith, 2000).

Although we report no significant change in any endocrine parameter assessed, testosterone concentration correlated positively with several lymphocyte subpopulation counts and their activation at baseline (Table 4.3). While some studies report a stimulatory effect of testosterone on both white and red blood cell counts in sedentary humans (Ellegala *et al.*, 2003; Palacios *et al.*, 1983), a study in rats reported a stimulatory effect of exogenous testosterone on $CD8^+$ cell count only, with no effect on other T cell subpopulation counts (Yao *et al.*, 2003). The resultant decrease in $CD4^+$:$CD8^+$ ratio suggested an immunosuppressive effect of testosterone. The results obtained at baseline in the current

study support a general, rather than subpopulation-specific, stimulatory effect of testosterone on white cell counts, with no effect on $CD4^+/CD8^+$ ratio, arguing against the results previously obtained in rats, but in agreement with the findings in human studies mentioned above. Furthermore, testosterone concentration at baseline correlated positively with the ability of both helper and suppressor T cells to become activated, but all these relationships were no longer apparent after training. Given the change in glucocorticoid sensitivity after exercise previously reported, one mechanism for the effect of testosterone may be a training-related change in T cell steroid receptor binding affinity for testosterone. Investigation into chronic alterations in the immune cell steroid receptor profiles of athletes after training is needed to further clarify this issue.

The finding that total testosterone concentration represents parallel changes in the fraction of unbound testosterone at all time points, is also supported by earlier reports (Kuoppasalmi, 1980), suggesting that measurement of SHBG is not necessary for interpretation. On the other hand, unbound cortisol concentration did not correlate with the concentration of either total cortisol or CBG at any time point. Our results therefore support earlier suggestions (Breuner & Orchinik, 2002; Dhillo *et al.*, 2002) that CBG concentration should be considered in conjunction with cortisol concentration for a more accurate interpretation of changes in the biological activity of cortisol. However, our data suggest that even this addition to the test battery may not be helpful for assessment of athletes. More complex assays could possibly be included, such as assessment of exercise-induced changes in glucocorticoid receptor number or binding affinity for cortisol on target cells such as skeletal muscle and lymphocytes.

4.5 Conclusion

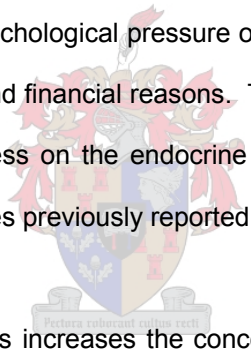
The results obtained in our study indicate that the decreased lymphocyte proliferative response seen after exercise training is the result of decreased responsiveness in both $CD4^+$ and $CD8^+$ T cells. Furthermore, our *in vitro* results illuminate the importance of assessing both spontaneous and mitogen-induced responsiveness of immune cells for a more comprehensive representation of immune status in athletes.

Chapter 5

A profile of selected endocrine and immune parameters in individuals exposed to chronic (occupational) psychological stress

5.1 Introduction

Acute exercise has been established as a stressor to both the endocrine and immune systems of athletes. In the previous chapter, we reported an increased immune activation status at rest after exposure to chronic exercise stress of unaccustomed volume and intensity, in athletes. However, the effects of chronic exercise stress are complicated by the inability to separate physical stress from psychological stress, particularly in competitive athletes. Psychological stress could include non-physical, occupation-related stress, life stressors and the constant psychological pressure on athletes to excel at their chosen sport, for both competition ranking and financial reasons. Therefore, it is necessary to evaluate the influence of psychological stress on the endocrine and immune systems, and to compare that to typical chronic responses previously reported in athletes.



Short-term psychological stress increases the concentration of circulating cortisol (Gerra *et al.*, 2001; Sapolsky *et al.*, 2000). Similar to results reported in response to acute exercise in athletes (Petrides *et al.*, 1997), the cortisol responses to acute experimentally-induced psychological stress allowed for division of subjects into either a high- or a low-responder group (Kunz-Ebrecht *et al.*, 2003). These results, obtained in models of acute stress, suggest similar effects of acute psychological and acute physiological stress on the activation of the HPA-axis, and thus an additive effect of psychological stress on the overall stress status in athletes is likely. However, since the endocrine effects of long-term psychological stress are commonly investigated in subjects suffering from stress-related illnesses, e.g. chronic fatigue syndrome (Scott *et al.*, 2000) and metabolic syndrome X (Pickup *et al.*, 1997), the chronic response to psychological stress in the absence of an underlying pathology is unclear. It is also unclear how the endocrine effects result in the pathology, although interactions with the immune system are likely.

The immune response to chronic psychological stress in relatively healthy, non-athlete populations has been investigated to a certain extent. Prolonged psychological stress resulted in dysregulation of immune function in previously healthy individuals, with the cytokine response shifting towards a T_H2 response (Matalka, 2003). Such a change in athletes, if prolonged, may have two undesirable outcomes: firstly, a reduced capacity for T_H1 function, which is part of the vital early defense system against infection, and secondly, a chronic up-regulation of the T_H2 response, which may predispose an athlete to overtraining. The former may contribute to the increased risk of athletes for URTI, as reported in epidemiological studies (Peters & Bateman, 1983; Tomasi *et al.*, 1982), whereas the latter may contribute to chronic inflammation, which is hypothesised to be implicated in the development of the overtraining syndrome (Smith, 2000).

Our recent studies on athletes indicate that direct, *in vitro* functional assessments of the immune system show changes in response to the stress of racing (Chapter 3) and increased training load (Chapter 4). Therefore, one of these may be suitable for monitoring changes in immune capacity in response to chronic psychological stress. Indeed, decreased T cell proliferative response has been reported in humans (Lacey *et al.*, 2000) and rats (Stefanski, 2000; Stefanski & Engler, 1999) after chronic psychological stress. However, this functional test is quite non-specific and the effects of chronic psychological stress on the $CD4^+$:$CD8^+$ T cell ratio and their activation status have not been established.

The interpretation of the physiological response to prolonged stress also depends on the extent of short-term biological variation. If the intra-individual day-to-day variation in a specific parameter is too high relative to inter-individual variation, it may be difficult to assess the physiological response to an acute intervention or to long-term accumulated effects (Terblanche *et al.*, 2004). Therefore, one aim of this study was to determine the extent of day-to-day variation in values obtained for selected immune and endocrine parameters in a population exposed to chronic stress. Other aims were to determine whether T cell reactivity (expression of CD69) was associated with any endocrine parameters in individuals exposed to occupation-related chronic psychological stress.

5.2 Methods

Subject recruitment and criteria: Subjects exposed to chronic occupation-related stress were recruited via advertisements on bulletin boards in office buildings and via word of mouth. 11 sedentary male subjects who were not on medication and who had no history of pathology (either physiological or psychological) and who had been in their current work positions for more than 2 years, were admitted to the study. All subjects were subjected to a clinical assessment by a medical doctor before participation in the study. Athletes and well-trained individuals were excluded from this group, to prevent the confounding effects of chronic exercise stress. Ethical approval for the study was gained from the University of Stellenbosch Research Sub-Committee B and all subjects gave informed, written consent prior to taking part in the study.

Sample collection: Early morning resting, fasted blood samples were collected by venepuncture from a forearm vein on two occasions separated by 7 days. At each time point, blood was collected in one EDTA-, one lithium heparin- and one SST Vacutainers (BD Systems, Plymouth, UK). Fresh EDTA blood were kept at room temperature and analysed for total and differential white cell count by automated analysis (STKS, Beckman/Coulter, Fullerton, CA, USA). Fresh heparinised blood samples were kept at room temperature and analysed within 4 hours for T lymphocyte subpopulation counts and *in vitro* spontaneous and mitogen-induced activation of helper- and suppressor T lymphocytes (CD69 expression) (for detailed description of assay procedure, refer to Appendix A). SST blood samples were left to coagulate at room temperature for 10 minutes, after which they were centrifuged at 3000 rpm for 10 minutes and frozen at –80 ºC until subsequent batch analysis of cortisol (Access B module 81600, Beckman/Coulter), dehydroepiandrosterone-sulphate (DHEAs) (Immulite I, Diagnostic Products Corporation, Los Angeles, CA, USA) and testosterone (Advia Centaur, Bayer Diagnostics, Leverkusen, Germany) concentrations.

Psychological assessments (in the form of various questionnaires) were used to measure stress perception at each time point, as a measure of the actual stress status of each individual. Subjects exhibited an “inverted iceberg” profile according to a profile of mood states (POMS) evaluation, i.e. the socre for vigour was decreased in relation to all other

parameters measured, which indicates a stressed condition (Morgan *et al.*, 1987; Wughalter & Gondola, 1991). This aspect of the study formed part of the baseline measurements of an M.Sc. thesis investigating the efficacy of a stress-relief intervention (Saunders, 2002) and will not be discussed here.

Statistical analysis: Data were analysed for differences between time points using repeated measures ANOVA and Bonferroni *post hoc* tests. Correlations between parameters, and correlations between time points, were assessed by determination of Pearson's product moment correlation coefficients. The relative influences of intra- and inter-individual variability on the repeatability of parameters were determined by calculation of repeatability (R) according to techniques described earlier (Krebs, 1999; Lessells & Boag, 1987), using the formulas: $R_b = s_b^2 / (s_b^2 + s_w^2)$ and $R_w = s_w^2 / (s_b^2 + s_w^2)$, where R_b and R_w are between-(inter-) individual and within-(intra-) individual repeatability respectively, and s^2 is the variance (mean square).

5.3 Results

Subject characteristics are summarised in Table 5.1.

Table 5.1 Characteristics for 11 sedentary subjects.

Age (yr)	33 ± 6
Height (m)	1.79 ± 0.05
Weight (kg)	82.7 ± 11.5
BMI	25.8 ± 4.0

Values are means ± SD

Means for all parameters assessed were within the normal range at both time points, with the exception of the average $CD4^+$ and $CD8^+$ cell counts, which were slightly lower than the reference ranges at time point 2, and the CD4:CD8 ratio which was slightly higher than the reference range at both time points (Table 5.2). Both cortisol and DHEAs concentrations were within the respective reference ranges at both time points (Table 5.2). Ratios calculated in this study (Table 5.2) were similar to ratios reported (or calculated from cortisol and

DHEAs data) for 7-9 am blood samples in the literature (0.08 – 0.10) (Hechter *et al.*, 1997; Straub *et al.*, 2002; Villette *et al.*, 1990). Repeated measures ANOVA showed no main effect of time (P = 0.59), and Bonferroni *post hoc* analysis showed no significant difference between the time points in any of the parameters measured. Pearson intra-class correlations indicated good correlation coefficients for cortisol, testosterone and DHEAs concentrations and the $CD4^+$:$CD8^+$ ratio (R > 0.80; Table 5.2). Variability for all parameters were high. For parameters pertaining to the endocrine system inter-individual variability was much higher than intra-individual variability, while for immune parameters inter- and intra-individual variability was quite similar, except when considering the $CD4^+$:$CD8^+$ ratio (Table 5.2).

Table 5.2 Immune and endocrine parameters measured at two time points one week apart.

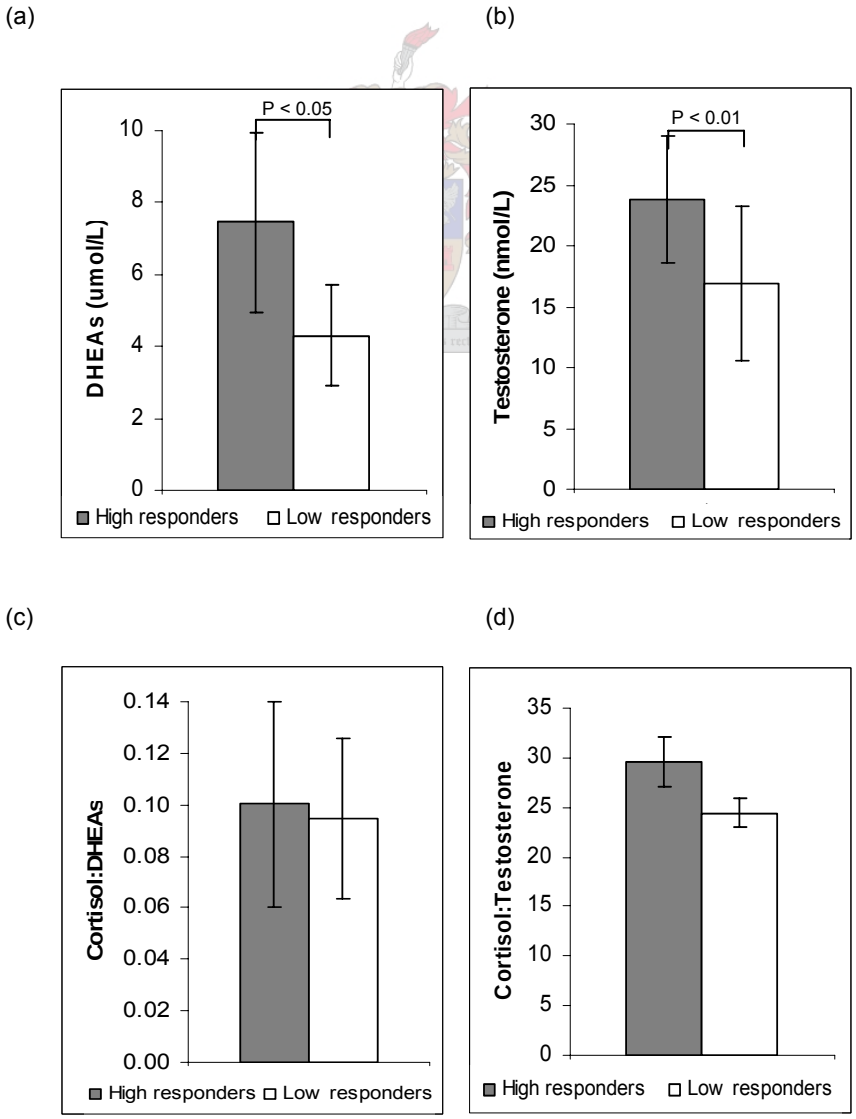
	Reference ranges	Time point 1	Time point 2	Pearson's R-value	Inter-individual R	Intra-individual R
Cortisol	140 – 650 nmol/L	523 ± 194	559 ± 142	0.84	0.83	0.17
DHEAs	2.2 – 15.2 umol/L	6.0 ± 2.8	6.1± 2.4	0.94	0.94	0.06
Cortisol:DHEAs	†	0.09 ± 0.03	0.10 ± 0.04	0.76	0.80	0.20
Testosterone	9.5 - 35 nmol/L	21.9 ± 7.4	19.5 ± 6.2	0.82	0.81	0.19
Cortisol: Testosterone	†	25.1 ± 9.6	30.2 ± 7.9	0.75	0.71	0.29
Total WBC	4000 – 11000 cells/uL	5590 ± 1140	5680 ± 1020	0.62	0.74	0.26
Total lymphocytes	1000 – 4000 cells/uL	2130 ± 460	1770 ± 420	-0.07	0.46	0.54
CD3	1100 – 700 cells/uL	1473 ± 273	1238± 349	-0.05	0.48	0.52
CD4	700 – 1100 cells/uL	832 ± 143	692 ± 169	0.25	0.57	0.43
CD8	500 – 900 cells/uL	548 ± 154	476 ± 189	0.23	0.54	0.46
CD4:CD8 ratio	1.0 – 1.5	1.6 ± 0.5	1.6 ± 0.6	0.85	0.87	0.13
NK	200 – 400 cells/uL	371 ± 179	303 ± 143	0.62	0.72	0.28
Spontaneous CD4CD69	† cells/uL	14 ± 7	8 ± 4	0.36	0.45	0.55
Spontaneous CD8CD69	† cells/uL	40 ± 24	24 ± 25	-0.04	0.47	0.53
Induced CD4CD69	† cells/uL	738 ± 229	621 ± 214	-0.20	0.47	0.53
Induced CD8CD69	† cells/uL	386 ± 192	258 ± 136	-0.03	0.47	0.53

Values are means ± SD. † No reference range available

Statistical analysis: Repeated measures ANOVA, with Bonferroni post hoc tests. Not significant for all parameters.

Individual values for the two time points were averaged and used for further analysis. The subjects were divided into either a low- or a high-responder group according to their average resting cortisol concentrations (low: below 500 nmol/L, n = 5; high: above 500 nmol/L, n = 6), so that the average cortisol concentrations were significantly different between the two groups (high-responders: 668 ± 55 *vs*. low-responders: 388 ± 86 nmol/L, P < 0.0001). Although the cut-off value of 500nmol/L was arbitrary, there was a clear natural division of subjects above and below this concentration. Although average resting DHEAs and testosterone concentrations were also significantly higher in high- vs. low-responders, cortisol:DHEAs and cortisol:testosterone ratios were not different between the two groups (Figure 5.1).

Figure 5.1 Resting (a) DHEAs and (b) testosterone concentrations, as well as the ratios between cortisol concentration and (c) DHEAs and (d) testosterone concentrations for high- *vs*. low-responder groups.



Values are means ± SD.Statistical analysis: One-way ANOVA with Bonferroni post hoc tests.

A comparison of the immune parameters between time point-averaged values for the cortisol high-responder *vs*. low-responder groups is given in Table 5.3. One-way ANOVA and Bonferroni post hoc analysis indicated a significantly lower spontaneous activation of $CD8^+$ cells and a tendency for the same effect in $CD4^+$ T cells in the low-responder group compared to the high-responder group.

Table 5.3 Immune and endocrine parameters in the cortisol high-responder group compared to that of the cortisol low-responder group.

	Reference ranges	**High-responders**	**Low-responders**
Total WBC	4 000 – 11 000 cells/uL	5967 ± 611	5240 ± 1243
Total lymphocyte count	1 000 – 4 000 cells/uL	2080 ± 270	1800 ± 280
CD3	1 100 – 1 700 cells/uL	1198 ± 105	1038 ± 282
CD4	700 – 1 100 cells/uL	700 ± 139	597 ± 243
CD8	500 – 900 cells/uL	432 ± 71	386 ± 103
CD4:CD8 ratio	1.0 – 1.5	1.7 ± 0.6	1.6 ± 0.5
NK	200 – 400 cells/uL	182 ± 50	209 ± 178
Spontaneous CD4CD69	† cells/uL	**14 ± 9***	5 ± 3
Spontaneous CD8CD69	† cells/uL	**35 ± 13****	17 ± 10
Induced CD4CD69	† cells/uL	583 ± 56	575 ± 267
Induced CD8CD69	† cells/uL	290 ± 67	243 ± 62

Values are means for two time points ± SD

Statistical analysis: One-way ANOVA with Bonferroni post hoc tests.

** P = 0.06; ** P < 0.05, values different between groups*

† no reference range available

5.4 Discussion

This study assessed various endocrine and immune parameters in sedentary individuals experiencing stress, at two time points separated by 7 days. No significant change over time could indicate a chronic but stable stress situation. However, high standard deviations were reported at both time points for most parameters assessed (Table 5.2), with magnitudes similar to those reported in previous studies by other groups (Green *et al.*, 2003; Ronsen *et al.*, 2001c; Vider *et al.*, 2001). All these studies indicated great variation in these parameters,

which may have been a factor confounding conclusions. However, even if intra-individual coefficient of variation are high, but inter-individual variability is much higher, it is possible to draw conclusions on the adaptability of a parameter (Terblanche *et al.*, 2004).

To assess the relative difference between inter-individual and intra-individual variability, repeatability (R) was calculated and revealed that for the endocrine parameters, relative inter-individual variability was sufficiently low. However, for immune parameters only the $CD4^+$:$CD8^+$ ratio seems to be a robust, repeatable indicator of immune status over time in a specific individual. This result suggests that measurement of lymphocyte subsets and activation status may not be repeatable enough to draw firm conclusions, at least not in individuals who are subjected to stressors similar to that of the subjects in our study.

Although average cortisol, DHEAs and testosterone concentrations were within the normal range at both time points, subjects in our group were clearly not all similar. Thus, considering only the average for the whole group may not reveal the full picture. Dividing subjects into cortisol high- and low-responders (Figure 5.1) confirms a dual response of cortisol to psychological stress similar to that reported previously in the literature in chronically stressed individuals at rest (Rosmond *et al.*, 1998), and in response to exercise (Deuster *et al.*, 1999; Petrides *et al.*, 1997). This dual response was previously suggested to be a result of stress exposure during a critical time of development, which led to altered responses of the nervous, immune and endocrine systems to stressors (Hurwitz & Morgenstern, 2001). This is in turn likely to be a result of changes on the level of glucogorticoid receptors, e.g. changes in the balance between GR type I and type II (Peters *et al.*, 2004). However, since we did not assess GR concentration or binding affinity, we cannot draw conclusions in this regard. Our results also indicate the same dual response for both DHEAs and testosterone, which is a novel finding in chronically stressed individuals, although it has been reported in athletes in response to exercise (Petrides *et al.*, 1997).

Previously, decreased cortisol:DHEAs ratio (as low as 0.05) was reported in chronic fatigue syndrome patients (Scott *et al.*, 2000), in athletes suffering from the overtraining syndrome (Lehmann *et al.*, 1998), and in individuals with AIDS (Villette *et al.*, 1990). The range of our results indicate that none of the subjects in the stress group (lowest cortisol:DHEAs ratio

measured was 0.07) suffered from chronic stress to the same extent as the patients with pathological conditions. Also, cortisol:DHEAs ratios in the two groups in our study were not different. Therefore, measurement of the cortisol:DHEAs ratio may be a useful tool to monitor extreme stress, but possibly not mild stress. In a study in elite athletes, average cortisol:testosterone ratio was ≈ 32 prior to increased training load, but decreased to ≈ 26 after the 12-month protocol (Hakkinen *et al.*, 1987). Although there was no significant inter-group difference in the cortisol:testosterone ratios, the average cortisol:testosterone ratio for the high-responders in our study was similar to the ratios obtained before training, and that of the low-responders was similar to the average ratio reported after training in the study by Hakkinen *et al*. (1987). This may suggest that monitoring the decline in cortisol:testosterone ratio over time may provide an indication of changes in stress status, which is valid for both physical and psychological stress.

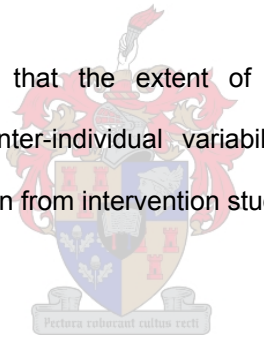
Not many studies have investigated the expression of CD69 on T cells, and most of these were performed on athletic populations (Green *et al.*, 2003; Ronsen *et al.*, 2001c; Ronsen *et al.*, 2002; Vider *et al.*, 2001), or individuals suffering a pathology (Schmid-Ott *et al.*, 2001). However, some studies using mononuclear cell cultures of normal healthy people showed spontaneous CD69 expression in less than 10% of cells (Mardiney, III *et al.*, 1996; Werfel *et al.*, 1997), similar to our results in $CD4^+$ and $CD8^+$ cells of the low-responder group. The significantly higher spontaneous activation status indicated in these immune cell subpopulations in the high-responder group, suggests that the divergent stress response influences immune function without necessarily influencing cell counts. It is difficult to draw a conclusion about the clinical significance of this finding, since it did not appear to influence the ability of the immune cells to become activated in response to an exogenous challenge. Due to inter-laboratory differences in technique, e.g. the type of mitogen used to stimulate cells *in vitro,* it is not possible to compare the absolute values of our results to those reported in the literature. Further studies are required to investigate the possibility of other adverse effects of a chronically increased spontaneous T cell activation status, e.g. whether this may result in a chronic low-grade inflammation. Also, repeatability of this variable needs to be assessed in a healthy, unstressed population, as well as their response to an acute psychological stressor.

5.5 Conclusion

We conclude that in a population whose lifestyle subjects them to psychological stress, cortisol concentration alone is not sufficient to indicate the stress status of an individual. Rather, DHEAs and testosterone concentrations should be measured in conjunction. However, the ratios between concentrations of these parameters and cortisol may be more relevant to assess in more extreme situations of stress.

Furthermore, comparing our results to the literature on athletes elucidates various similarities in the endocrine and immune system adaptations to chronic psychological and chronic exercise stress. Therefore, results obtained and conclusions drawn from studies investigating the physiological response to psychological stress may also be applicable to exercise stress.

Finally, it became apparent that the extent of intra-individual variability in immune parameters relative to the inter-individual variability places severe limitations on the conclusions that could be drawn from intervention studies.



Chapter 6

The effect of acute immobilisation stress on the concentrations of corticosterone, testosterone and selected inflammatory cytokines in male Wistar rats

6.1 Introduction

Human studies have investigated the response to psychological stress in both free-living situations and in more controlled laboratory-settings. These studies, recently reviewed by Matalka (2003), which investigated the response of the endocrine stress-axis and the cytokine system to acute psychological stress, report variable responses even to a similar stressor (Matalka, 2003).

Acute psychological stress in response to an academic examination was reported to increase circulating concentrations of cortisol, IL-6 and TNF-α (Maes *et al.*, 1998). However, under similar conditions in another study, IL-1β concentrations were not increased (Lacey *et al.*, 2000), although cortisol concentrations were increased. These results may seem contradictory, but IL-6 is known to down-regulate the concentration of IL-1β in the circulation, so that IL-1β is only present early in the inflammatory response to stress. Apart from differences in results due to timing of blood samples, multiple other factors may have an influence, such as the severity and duration of the stressor (Lacey *et al.*, 2000) and inter-individual differences in stress perception and adaptation to a stressor (Gerra *et al.*, 2001), most of which cannot be eliminated in humans. For example in humans, repeated participation in a similar psychosocial stress test in a controlled environment, separated by one week, resulted in habituation to the stressor in 12 participants, so that they had a reduced ACTH and cortisol response to the second test, while 8 other participants displayed a similarly increased ACTH and cortisol response after both tests with no apparent adaptation (Gerra *et al.*, 2001).

Since confounding factors cannot be accurately controlled for or standardised between individuals (although a questionnaire may give some indication of perceived stress), human studies are often difficult to interpret. However, a more controlled, standardised stress protocol that would be ethically possible in animal studies, should enable clearer interpretations of the relationships between cortisol and cytokines in the response to psychological stress. Also, experimental animal populations are relatively homogeneous and should therefore exhibit smaller inter-individual variability.

Laboratory rat models of stress include immobilisation, prone restraint, forced swimming and inescapable tail shock (Arancibia *et al.*, 2000; Armario *et al.*, 1995; Deak *et al.*, 1999; Fleshner *et al.*, 1995b; Garcia *et al.*, 2000; Marti *et al.*, 2001). Immobilisation stress is one of the less extreme and thus most applicable models to relatively healthy humans undergoing a stressor for a defined time period per day (e.g. the stress response to exercise training may be similar to that after short-duration immobilisation stress in rats). Although studies in rats subjected to immobilisation stress consistently report increased corticosterone concentration (Armario *et al.*, 1995; Duclos *et al.*, 2001; Garcia *et al.*, 2000; Heiman *et al.*, 1997; Marti *et al.*, 2001), and some also report increased pro-inflammatory cytokine release after exposure to the stressor (Zhou *et al.*, 1993), much inter-study variation exists, in part due to variations in the severity and duration of the stress episode (Garcia *et al.*, 2000; Pitman *et al.*, 1988), but also due to variations in sensitivity to stress in different strains of rat (Armario *et al.*, 1995), and an additive effect of previous chronic stress (Almeida *et al.*, 2000). Therefore, animal models of stress should be validated when first used by a new research group, to ensure that the protocol is effective, repeatable and sensitive enough for the purposes of the intended investigations, in their hands.

Therefore, in order to establish a relevant animal model for stress in our laboratory, we investigated the effect of one 2-hour period of immobilisation stress on the corticosterone and pro-inflammatory cytokine response in male Wistar rats. Particular aims were to investigate whether we could prevent a stress effect of the sacrifice protocol in the control group, and to determine if the stress was indeed mild and relatively transient, but still sufficient to produce a robust stress response in the experimental group.

6.2 Methods

Experimental animals: 23 male Wistar rats were used in this study. All rats were housed in standard rat cages and fed rat chow and tap water *ad libitum*. Temperature in the housing facility was controlled at 21 ºC and lights set to a 12-hour light/dark cycle (lights on at 7 am). Rats were bought from a breeding facility and allowed 3 weeks to acclimatise to their new conditions before initiation of the study protocol. Acclimatisation included purposeful handling by one researcher only, weighing of the animals once per day, as well as transport between experimental rooms in the animal house to accustom the rats to all actions necessary for the study protocol, bar the actual stress intervention.

Stress intervention: Rats were divided into three weight-matched groups: a control group (C, n = 7), an acute stress group (S, n = 8) and a stress recovery group (R, n = 8). C underwent no intervention, while both S and R were subjected to one 2-hr period of immobilisation in a Perspex cage (dimensions 7 cm x 8 cm x 15 cm) designed for this purpose. S rats were sacrificed immediately after the immobilisation period. R rats were allowed to recover for 24 hours after immobilsation before sacrifice. All rats were sacrificed between 10h00 and 11h00. Ethical approval for the study was obtained from the Stellenbosch University Sub-Committee C prior to the start of the protocol.

Sample collection: Rats were decapitated and whole blood collected via a heparinised funnel into 5 ml SST Vacutainers (BD Vacutainer Systems, Plymouth, UK). Blood was kept on ice, and centrifuged within 20 minutes after collection at 3000 rpm for 10 minutes at 0 ºC. Serum was aliquoted and frozen at –80 ºC for subsequent batch analysis of IL-1β, TNF-α, IL-6 and corticosterone concentrations.

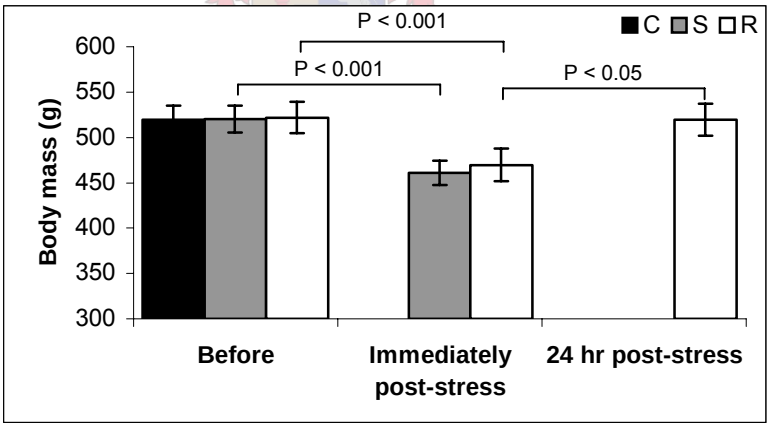
Analysis: Commercially available rat-specific ELISA kits were used for determination of IL-1β (Biotrak RPN 2743, AEC Amersham Biosciences, UK), TNF-α (865.000.096, Diaclone, France), IL-6 (Biotrak RPN 2742, AEC Amersham Biosciences, UK), corticosterone (AC-14F1, Octeia, Immunodiagnostic Systems, USA) and testosterone (EIA-1559, DRG Instruments, Germany) concentrations.

Data analysis: Differences in corticosterone and cytokine concentrations between groups were assessed using one-way ANOVA and Bonferroni *post hoc* tests (Statistica 6, StatSoft Inc, OK, USA). Level of significance was set at $P < 0.05$.

6.3 Results

Rats in the different groups were matched for body mass at the start of the protocol, but immobilisation stress resulted in significant changes in body mass (Figure 6.1). Mean corticosterone concentrations at the time of sacrifice were significantly increased in S *vs*. C and R, but C and R mean corticosterone concentrations did not differ (Figure 6.2a). Mean testosterone concentration was similar in S vs. C, but significantly increased in R in comparison to C and S (Figure 6.2b). The mean corticosterone:testosterone ratio followed a similar pattern to that of the corticosterone concentration (Figure 6.2c).

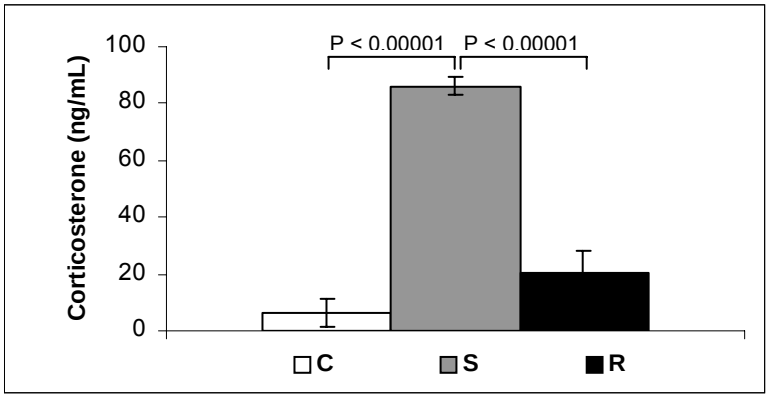
Figure 6.1 Comparison of mean body mass in control rats and in rats subjected to acute, short-term immobilisation stress with or without subsequent recovery.



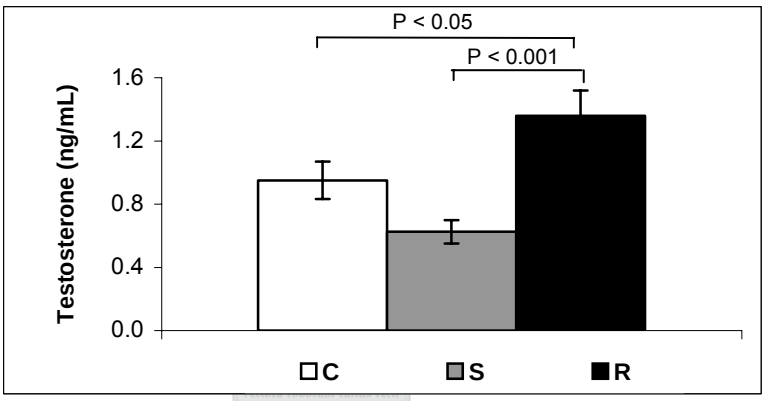
Error bars indicate SEM.
Abbreviations: C = Control Non-stressed; S = Acutely Stressed; R = 24 hours Recovery after acute stress

Figure 6.2 Effect of acute short-term immobilisation stress and recovery from stress on mean a) serum corticosterone concentration, b) serum testosterone concentration and c) the corticosterone:testosterone ratio.

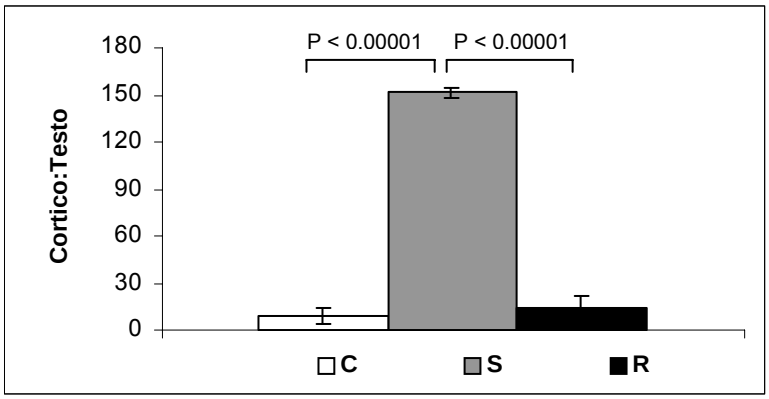
(a)



(b)



(c)



Error bars indicate SEM.

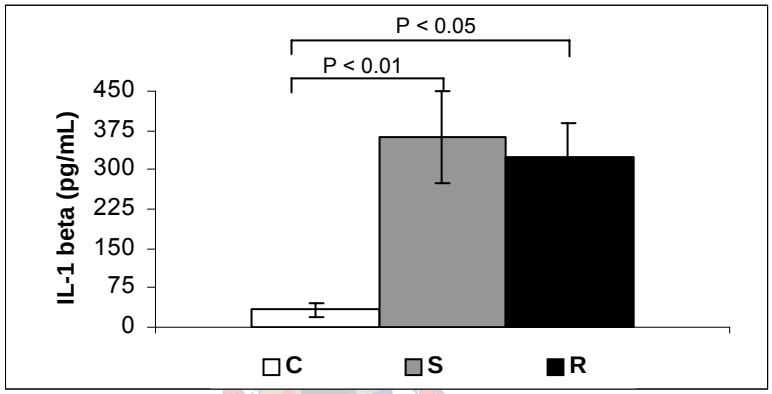
Abbreviations: Cortico, Corticosterone; Testo, Testosterone

IL-6 concentration was below detectable levels in all rats (i.e. less than 15 pg/mL). Also, in C and S, less than 30 % of rats had detectable TNF-α concentrations (i.e. more than 20 pg/mL) and in R, more than 60 % of rats had detectable TNF-α concentrations (range: 22 – 54

pg/mL). For illustrative purposes, the samples with concentrations below detectable levels were assigned an arbitrary concentration value of 10 pg/mL (Figure 6.3b). Due to the high percentages of non-detectable TNF-α concentrations, statistical analysis was not performed for this parameter. One-way ANOVA indicated a group effect for IL-1β concentration ($P < 0.00001$), and *post hoc* analysis indicated that IL-1β concentration was significantly higher in both S and R compared to C (Figure 6.3a).

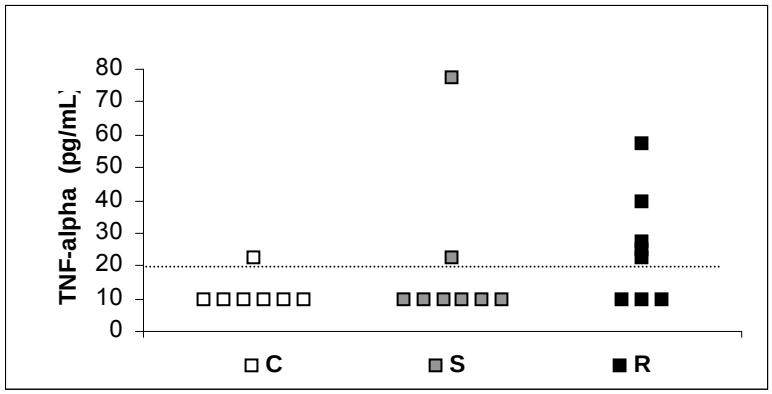
Figure 6.3 The effect acute immobilisation stress with or without 24 hr recovery on a) IL-1β and b) TNF-α concentrations.

(a)



Error bars indicate SEM.

(b)



Dotted line indicates detectable concentration limit (20 pg/mL).

6.4 Discussion

The main findings of this study are a) that we were able to induce stress only in the experimentally stressed animals and b) that, although corticosterone concentrations returned

to normal, the complex downstream response to stress was not abolished after 24 hours of recovery.

Although rats seemed to suffer significant weight loss as an acute result of immobilisation (Figure 6.1), the recovering group's result indicates that the acute effect is quickly reversed, since they regained this weight within 24 hours. This weight loss was most probably the result of dehydration during exposure to stress from excessive panting, urination and diarrhoea. Cumulative effects of a more chronic protocol of exposure to this relatively mild stressor may indeed result in long-term weight loss, since a previous study reported rapid increase in somatostatin release from the hypothalamus after only 15 minutes of prone (taped-down) restraint stress in Sprague-Dawley rats (Arancibia *et al.*, 2000). However, the latter is a more severe stressor than that used in this study.

The finding of significantly increased corticosterone levels after acute immobilisation stress is in agreement with previous studies investigating effects of both chronic (Almeida *et al.*, 1998; Almeida *et al.*, 2000; Pitman *et al.*, 1988) and acute stress (Pitman *et al.*, 1988; Strausbaugh *et al.*, 1999; Tinnikov, 1999) in rats. Although the corticosterone concentrations in C rats and R rats were similar, acute stress was previously shown to result in greater numbers of corticosteroid receptors for more than 24 hours after cessation of the stress, as well as a decrease in CBG levels after acute stress (Deak *et al.*, 1999; Fleshner *et al.*, 1995b). Therefore, although corticosterone concentrations had themselves returned to baseline after 24 hours, we cannot exclude the possibility of maintenance of enhanced bioactivity at cellular level for longer than 24 hours after stress exposure.

Our result of similar testosterone concentrations in control rats and immediately after stress exposure, is in accordance with reports of unchanged testosterone concentrations during acute exercise stress exposure in human subjects, with decreased concentrations only occurring during the recovery period (Kuoppasalmi *et al.*, 1980). An earlier study in rats reported a decrease from baseline in testosterone concentration in response to 3 hours of acute immobilisation (tube restraint) stress (Akinbami *et al.*, 1999), while another reported increased testosterone concentration after acute (5 minutes) immobilisation stress (Almeida *et al.*, 2000). Since the stress protocol used in the first study was more severe than our

protocol, our result and that of Almeida *et al*. (2000) suggest that a moderate acute stressor is not severe enough to negatively influence the testosterone synthetic pathway and may even result in up-regulation of testosterone production, while a severe acute stressor may result in decreased testosterone production, possibly via a shift away from androgen production in favour of corticosteroid production. Our finding of significantly higher testosterone concentration after 24 hours of recovery from 2 hours of immobilisation stress (group R) compared to C and S, supports this contention. Furthermore, a study using a rat model of chronic social stress (7 days), reported that testosterone concentrations were only decreased in bitten loser rats, but either unchanged or increased in winner rats (Stefanski, 2000), providing further proof that the testosterone response to stress (both acute and chronic) is dependent on the intensity of the stressor.

Despite the fact that mean testosterone concentration was higher in R compared to C and S, the corticosterone:testosterone ratio followed the same pattern as corticosterone concentration. This suggests that the increased testosterone concentration after recovery from acute mild psychological stress may not be sufficient to counter the effect of the highly increased corticosterone concentration seen immediately after stress exposure. This may suggest a cumulative negative effect in the long-term. A model of chronic mild stress could possibly elucidate this further.

IL-1β, IL-6 and TNF-α are released in response to stress as a result of neural stress perception and cytokine interaction. (These cytokines perform many similar and overlapping functions in response to stress, such as macrophage activation, increased NK cytotoxic activity, activation of T and B lymphocytes and release of acute phase reactants (Mackinnon, 1992), and activation of the HPA-axis (Sapolsky *et al.*, 2000; Watkins, 1994)). One might therefore expect increased levels of all three cytokines after exposure to stress. Indeed, IL-1β concentrations were increased after acute stress. This was not unexpected, since IL-1β was previously shown to be responsible for enhancing the secretion of CRH, ACTH and corticosterone in stressed rats (Berkenbosch *et al.*, 1987; Sapolsky *et al.*, 2000; Watkins, 1994). IL-1β is also known to result in up-regulation of IL-6 secretion (Mackinnon, 1992), but in this study IL-6 concentrations were below the detectable range in all samples. Since our model is one of mild stress only, it is possible that either the intensity or the duration of

the stress episode, or both, was not sufficient to result in IL-6 secretion. However, the substantial increase in IL-1β argues against this. Rather, it would seem that the peak in IL-6 concentration was not detected with the timing of sample collection used in this protocol, since previous rat studies reported increased IL-6 excretion in urine after only 30 minutes (Boucher *et al.*, 2002), and increased plasma IL-6 already after one hour of immobilisation (Ishikawa *et al.*, 2001) with the peak reported at 90 minutes during a 2-hour immobilsation protocol (Takaki *et al.*, 1994), suggesting an early response and relatively quickly clearance compared to the other cytokines assessed in this study.

Previous reports in humans indicate that TNF-α concentration may increase within one hour after exercise stress exposure (Espersen *et al.*, 1990; Madrigal *et al.*, 2002), but that the magnitude of change is often too small to be detected by ELISA (Ullum *et al.*, 1994). The fact that most rats in C and S had TNF-α levels below the detectable range confirms this and suggests that circulating TNF-α is not a useful marker of acute psychological stress in rats.

The high IL-1β levels 24 hours after cessation of stress may suggest incomplete recovery from the acute stressor. However, although chronic exercise stress in humans is associated with chronically elevated IL-1β concentration at rest (Evans *et al.*, 1986), an acute bout of exercise resulted in either no significant change in IL-1β concentration (Evans *et al.*, 1986), or return to baseline levels within 9 hours of recovery (Cannon *et al.*, 1986). However, the assays used in these relatively old studies, was much less specific compared to modern assay methods. Since our protocol involved one acute episode of stress only, it is unlikely to result in a chronically elevated IL-1β concentration. Also, most rats in R had comparatively higher TNF-α concentrations. Therefore, it is more likely that the rats associated their weighing prior to sacrifice with the weighing prior to subjection to the stress protocol the previous day, despite the fact that they were handled and weighed prior to starting the protocol as well. This neural stress perception may have resulted in an acute stress response and therefore increased TNF-α concentrations just prior to decapitation in this group. However, since rats were sacrificed within one minute of removal from their cages, only the cytokines released very early in response to stress would be elevated, explaining why downstream parameters such as IL-6 and corticosterone concentrations were not yet elevated. This result however questions the usefulness of using rats in studies investigating

recovery from acute stress exposure, because unlike human subjects, they cannot be informed that no further stress challenge will take place. Nevertheless, the slightly delayed nature of the corticosterone response would allow for this more robust marker of stress to be used in laboratories that have validated their expertise in using such a model.

6.5 Conclusion

The protocol used to induce psychological stress indeed resulted in increased secretion of upstream pro-inflammatory cytokines (IL-1β) as well as the major anti-inflammatory hormone (corticosterone) immediately following a two-hour stress exposure. Also the control rats were truly habituated and unstressed by human handling. The mild nature of the stressor is indicated by the return of corticosterone concentrations to baseline after 24 hours, and the fact that testosterone concentration was not depressed. However, if the aim of a future study is to investigate the response of downstream cytokines, e.g. IL-6, blood samples should possibly be taken via an indwelling catheter, to allow multiple blood sampling time points.

6.6 Limitations

The use of an assay kit using a monoclonal antibody directed against IL-6 is a possible confounder in this study, since lower concentrations of IL-6 could not be detected. Use of a polyclonal antibody or a high-sensitivity assay kit would possibly allow for more accurate data pertaining to IL-6 concentration, especially when the concentration is very low. However, neither a polyclonal antibody, nor a high-sensitivity kit specific for rat IL-6 was available at the time of the study. Although more sensitive kits are available (e.g. from Biosource), these kits are only suitable for use in a semi-automated analysis using specialised instrumentation (so-called “bead-systems” such as Luminex^{TM} and Multiplex^{TM}) which were not available to us.

Chapter 7

The efficacy of *Sutherlandia frutescens* supplementation to reduce stress levels in rats subjected to chronic intermittent immobilisation stress

7.1 Introduction

Two of the most severe and common chronic diseases, cancer and HIV/AIDS, result in heightened activation of the hypothalamic-pituitary-adrenal (HPA) –axis and therefore chronically increased blood cortisol concentration (Christeff *et al.*, 2000; Clerici *et al.*, 1997; Costelli *et al.*, 1993). The long-term effects of raised cortisol include decreased capacity of the cellular immune response (Hässig *et al.*, 1996), chronic inflammation due to glucocorticoid insensitivity (Miller *et al.*, 2002), and increased muscle catabolism (Gore *et al.*, 1993; Woolf, 1992). All these effects result in chronically decreased standard of living. Considering the high cost of most commercial (synthetic) medicines prescribed for symptomatic treatment, it is of extreme importance to find a less expensive treatment for these debilitating symptoms. A natural remedy, *Sutherlandia frutescens*, has been used in traditional medicine in South Africa for many decades, for the treatment of stress-related illnesses (Van Wyk *et al.*, 1997; Van Wyk & Gericke, 2000).

Some of the components of the *Sutherlandia frutescens* plant, identified through chemical analyses (Brümmerhoff, 1969; Levy *et al.*, 1999; Moshe, 1999; Viljoen, 1969), include L-canavanine, gamma-aminobutyric acid (GABA) and pinitol. The individual effects of these compounds have been illustrated in previous studies on rats: L-canavanine had anti-viral (Green, 1988) and anti-cancer action (Crooks & Rosenthal, 19940; Swaffar *et al.*, 1995), and was proved to be a selective inhibitor of inducible nitric oxide synthase (Anfossi *et al.*, 1999), which has been used effectively to treat endotoxic shock in rats (Levy *et al.*, 1999). GABA is an inhibitory neurotransmitter and is known for having mood elevating properties. Pinitol has anti-inflammatory properties (Singh *et al.*, 2001). Although the individual actions of these substances are known, the combined palliative effects of these substances in one compound have not been investigated before. Nevertheless, a toxicology study in vervet monkeys

(Seier *et al.*, 2002; Seier, 2002) has shown that *Sutherlandia* leaves, even at nine times the recommended dose, had no toxic or any other side effects with regard to haematological and biochemical parameters measured.

The native words for this herb, “motlepelo” (Sotho for “bringing back the heart”), “*insiswa*” (ancient Zulu word meaning “the one which dispels darkness”) and “*unwele*” (Zulu for “hair” – alluding to the fact that the plant stops people from “pulling out their hair” with distress) are indicative of the main effect claimed – to relieve symptoms such as irritability, anxiety and depression. Although much anecdotal evidence exists to validate this herb as having medicinal properties (Rood, 1994; Van Wyk *et al.*, 1997; Van Wyk & Gericke, 2000), to our knowledge, no scientific proof exists of its action.

Therefore the aim of this study was to determine the effects of daily supplementation with an extract of *Sutherlandia frutescens subs. microphylla* on selected serum hormone (both anabolic and catabolic) and cytokine levels, in a rat model of chronic immobilisation stress.

7.2 Methods

Experimental groups: Forty adult male Wistar rats (average body mass of 376 ± 40 g at start of intervention protocol) were used in this study. All rats were housed in groups of 4 in standard plastic-bottomed wire mesh cages with a 12-hour light-dark cycle. Rats were fed rat chow and tap water *ad libitum*. All rats were allowed 4 weeks to acclimatise after transport to the animal house, before commencing with the stress intervention and supplementation protocols.

Rats were divided into four mass-matched groups (n = 10 for each group). Two groups represented the control groups. One of these received supplementation with *Sutherlandia* extract (CS), while the other received supplementation with placebo (isotonic saline) (CP) for the same period of time. The third and fourth groups were subjected to chronic immobilisation stress in addition to receiving either *Sutherlandia* extract (IS) or placebo (IP).

Interventions: All rats were weighed and handled once a day for 3 weeks before the start of the immobilisation protocol to accustom them to humans, preventing stress caused by (unaccustomed) handling from being a confounding factor in this study. Immobilisation was achieved by placing rats individually into small Perspex cages (dimensions 7 cm x 8 cm x 15 cm) designed for this purpose, which do not allow free movement. Duration of immobilisation sessions was 2 hours, once daily, for 28 consecutive days.

Commercially available *Sutherlandia* is sold as 700 mg tablets, each containing 300 mg *Sutherlandia* leaf powder. Manufacturers of these tablets (Phyto Nova (Pty) Ltd, Noordhoek, Cape Town) recommend a dose of one tablet twice daily. This recommended dose of commercially available *Sutherlandia* leaf powder equals 9 mg/kg body mass for humans. This corresponds to a daily dose of about 3.4 mg/rat/day, which was rounded up to 4 mg/rat/day, to allow for weight gain during the study protocol. Specimens of *Sutherlandia* were harvested in the vicinity of Murraysburg, Western Cape Province, South Africa, and identified as *Sutherlandia frutescens* subspecies *microphylla* by Professor Ben-Erik van Wyk, of the Botany Department of Rand Afrikaans University (Voucher specimen from Mrs Grobler: C. Albrecht s.n sub B-E van Wyk 4126 (JRAU)). Since the traditional way of administration is in the form of an herbal infusion, a warm water extract was prepared: boiling water was added to dried *Sutherlandia* leaves (8 mg/mL) and left to infuse overnight at room temperature. The infusion was not filtered, as this might have resulted in removal of an active component, but poured through a sieve to remove any bigger leaf particles. Hereafter, the extract was diluted 1:2 with 1.7 % saline to result in an extract of 4 mg/mL in isotonic saline. Placebo consisted of a solution of sterile 0.85 % saline. Since the *Sutherlandia* extract has a bitter taste, rats will not voluntarily ingest it, and forced oral administration would represent an uncontrolled additional stressor. Therefore, all rats were subjected to intraperitoneal injection twice per day, of either 0.5 ml of 0.85 % saline (CP and IP) or 0.5 ml of *Sutherlandia* extract (CS and IS). The dosage of *Sutherlandia* used in this study was not adjusted to compensate for the higher metabolic rate of rats compared to humans. However, treatment was administered just prior to stress exposure, which was intermittent rather than constant as in humans, so that any preventative effect of *Sutherlandia* on the stress response would still occur despite the relatively faster metabolism of the compound.

Sample collection: At the end of the protocol, all rats were sacrificed between 11h00 and 13h00 by decapitation. All rats were sacrificed in a rested state, i.e. 24 hours after the last stress or treatment intervention. Rats were taken from the housing cage one at a time, placed into a weighing basket and carried to another room, where it was weighed and then decapitated. (The sacrifice process, from removal from the cage to decapitation took less than 1 minute per rat.) Whole blood collected by exsanguination from the aorta, into SST blood collection tubes (BD Vacutainer Systems, Preanalytical Solutions, Plymouth, UK). Blood was allowed to clot at room temperature for 10 minutes, after which it was centrifuged at 3000 rpm for 10 minutes at 4 °C, and the serum aliquoted and frozen at –80 °C until subsequent analysis. Between subsequent sacrifices, all traces of blood and animal waste was removed from the room, and the working area disinfected with the disinfectant routinely used to clean housing cages, to prevent acute stress in rats just prior to sacrifice.

Analysis: Serum samples were analysed for corticosterone concentration by radio-immunoassay (Biotrak RPA 548, Amersham), testosterone concentration by immunoassay (Advia Centaur, Bayer Diagnostics) and IL-6 and TNF-α concentrations by ELISA (Biotrak RPN 2742 and RPN 2734, Amersham). Data were analysed using 2-way ANOVA with Fisher *post hoc* tests to assess differences between subgroups. Relationships between variables were assessed using Pearson correlations.

7.3 Results

Effects of *Sutherlandia* treatment, stress and interaction of supplementation and stress on blood parameters are illustrated in Table 7.1 and Figure 7.1. 2-way ANOVA indicated no significant main effect of *Sutherlandia* treatment alone. There was however a significant main effect of stress on both corticosterone ($P < 0.05$) and testosterone ($P < 0.05$) concentrations, associated with a significant change in corticosterone:testosterone ratio ($P < 0.01$), as well as a significant effect of interaction of stress and *Sutherlandia* treatment on serum corticosterone concentration ($P < 0.005$) (Table 7.1).

Table 7.1 Effects of stress, *Sutherlandia* treatment and interaction on concentrations of parameters measured.

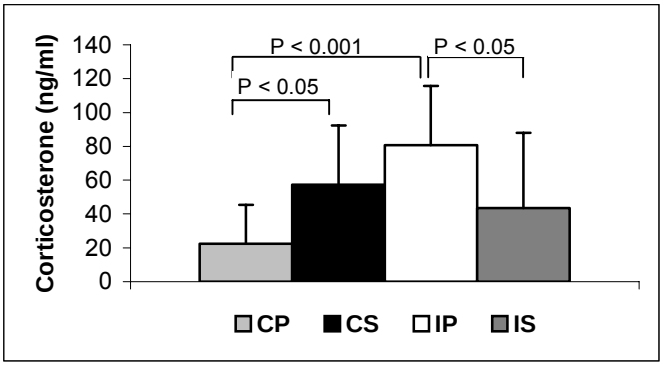
	Corticosterone (ng/mL)	Testosterone (nmol/mL)	C:T ratio	IL-6 (pg/mL)
Treatment	NS	NS	NS	NS
Stress	P < 0.05	P < 0.05	P < 0.01	NS
Stress x Treatment	P < 0.005	NS	NS	NS

Statistical analysis: Values are P- values obtained by 2-way ANOVA analysis.
Abbreviations: C:T = Corticosterone:Testosterone

Fisher's *post hoc* analysis indicated significantly higher corticosterone concentration in IP *vs*. CP (P < 0.001), and significantly lower corticosterone concentration in IS *vs.* IP (P < 0.05; Figure 7.1a). Despite the (conservative) ANOVA result of no effect of Sutherlandia treatment alone on corticosterone concentration, which were most probably as result of large inter-individual variations in all four groups, *post hoc* analysis indicated that corticosterone concentration was significantly higher in CS *vs*. CP (P < 0.05; Figure 7.1a). Both CP and CS had significantly higher testosterone concentrations compared to IS (P < 0.01 and P < 0.05; Figure 7.1b), while the corticosterone:testosterone ratio were significantly higher in IP *vs*. CP (P < 0.05; Figure 7.1c).

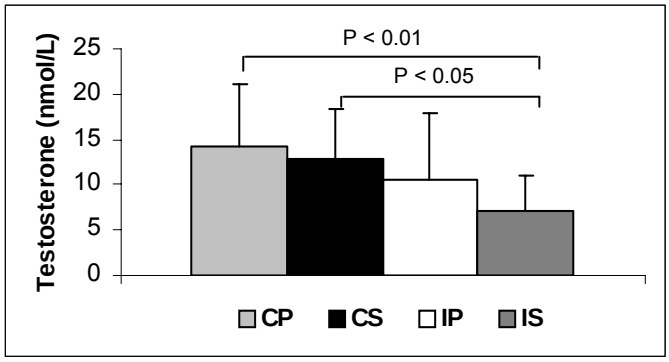
Figure 7.1 Serum concentrations of (a) corticosterone, (b) testosterone and (c) the corticosterone:testosterone ratio.

(a)

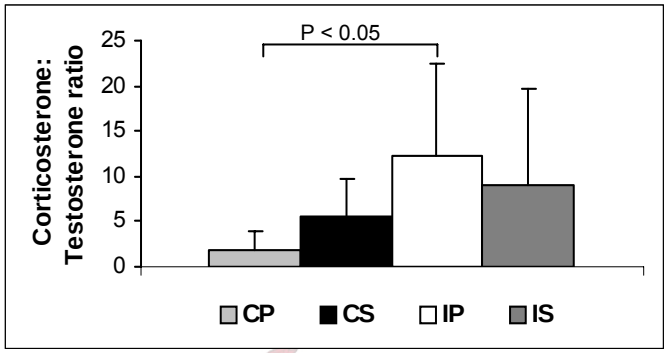


Error bars indicate SD.
Statistical analysis: P-values indicate significant differences obtained by Fisher post hoc analysis that followed 2-way ANOVA.

(b)



(c)

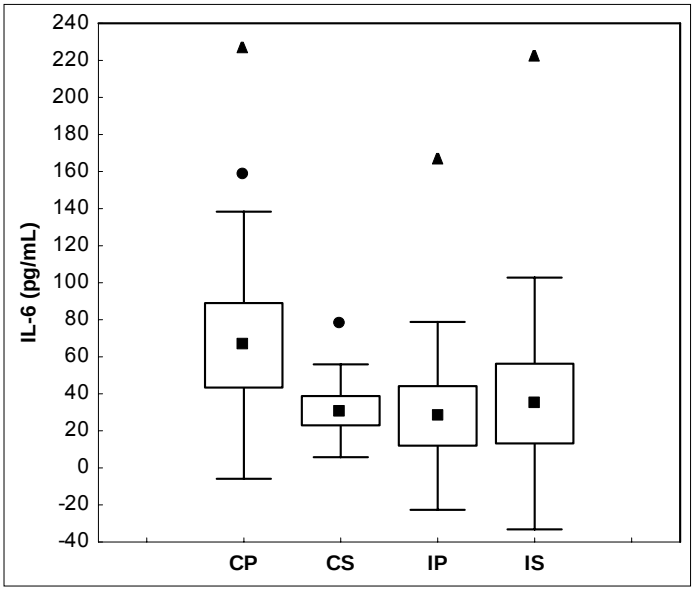


Error bars indicate SD.

Statistical analysis: P-values indicate significant differences obtained by Fisher post hoc analysis that followed 2-way ANOVA.

IL-6 concentration showed a high level of inter-individual variation and was not significantly different from the others in any group (Figure 7.2). TNF-α concentration was below detectable levels (< 10 pg/mL) in all samples.

Figure 7.2 Box-plot analysis of serum IL-6 concentrations.



Boxes indicate mean ± SEM, error bars indicate SD, circles indicate outliers and triangles indicate extreme outliers.

7.4 Discussion

We reported increased cortiscosterone concentrations after a single exposure to immobilisation stress in rats in Chapter 6. Also, intermittent immobilisation of rats is frequently used as a model for inducing mild but physiologically significant stress (Almeida *et al.*, 1998; Hu *et al.*, 2000; Pellegrini *et al.*, 1998; Pitman *et al.*, 1988). Therefore, the finding in the current study that stress significantly increased basal serum corticosterone concentration ($P < 0.05$; Table 7.1) was expected. Although a single exposure to mild psychological stress only transiently decreased testosterone concentrations in a previous study by our group (Chapter 6), the finding that chronic stress resulted in a decreased basal serum testosterone concentration ($P < 0.05$; Table 7.1), is in accordance with results from previous studies (Akinbami *et al.*, 1999; Kostic *et al.*, 2000; Pellegrini *et al.*, 1998).

In addition, we present two main novel findings: Firstly, that *Sutherlandia* treatment decreased the corticosterone response to chronic intermittent immobilisation stress, and secondly, that *Sutherlandia* treatment in control rats increased basal corticosterone concentrations in these rats when compared to control placebo-supplemented rats. The first main finding, of an attenuated corticosterone response to stress, supports the indigenous

knowledge that *Sutherlandia* has stress-relieving properties. However, further investigation is necessary to establish the exact mechanism by which this is achieved. Possible target tissues for an anti-stress function of the herb include the hypocampus (down-regulated stress perception), the hypothalamus or pituitary gland (down-regulation of HPA-axis activation) and the adrenal gland (down-regulation of corticosterone production). Since the herb has not been fully characterised and all the active ingredients are not yet known, it is too early to speculate further on possible mechanisms.

The second main finding has more than one possible explanation. Firstly, the herb was prepared in the traditional way, after which it was filtered through a coarse sieve (pore size 0.5mm), but not sterilised, since sterilisation at high temperature held the possibility that an as yet unknown active substance may have been inactivated. Similarly, filtration could possibly remove a larger molecule from the extract. The *Sutherlandia* infusion was then administered by intraperitoneal injection, and not via the upper digestive tract, as it would traditionally be ingested (reasons discussed in methods section). The possibility therefore exists that the herb may have elicited an inflammatory response, resulting in increased basal corticosterone concentrations, compared to placebo-controls, which received sterile saline. However, TNF-α concentrations was below detectable levels in all samples, and IL-6 concentrations in the treated control rats were not significantly different from the other groups, arguing against a chronic inflammatory state at the time of sacrifice. A second, more likely explanation, is that the *Sutherlandia* herb acts as an adaptogen (a substance increasing the body's ability to adapt and increase its resistance to stress and disease, changing the course of an illness into a favourable outcome by normalising body functions), bringing about a more functional basal corticosterone concentration through allostasis. Allostasis is the term used to describe the process of maintaining homeostasis by means of multiple interacting adaptive processes (Goldstein & McEwen, 2002), which may be achieved by combinations of mediators, produced by the immune system, autonomic nervous system and the HPA-axis (McEwen, 2002; McEwen & Seeman, 1999).The fact that the average basal corticosterone and IL-6 concentrations in the two *Sutherlandia* groups are similar, despite the fact that one group was also subjected to immobilisation stress, supports this idea. However, the mechanism(s) by which this result is achieved is not yet known, and is unlikely to be elucidated while using the "intact" extract administered as treatment in this

study: *Sutherlandia* is a compound with several active substances which may be role-players in the corticosterone response, as discussed in the introduction. Furthermore, some as yet unidentified role players may also come into play. Therefore, future studies should focus on investigating these substances individually after extraction and isolation (if possible), to more clearly determine the possible interactive pathways that resulted in the results reported here.

While acute stress is known to increase both IL-6 (Maes *et al.*, 1998; Nukina *et al.*, 1998) and corticosterone levels (Armario *et al.*, 1995; Nukina *et al.*, 1998), these increases in IL-6 levels are inversely related to the increases in glucocorticoid levels (Kunz-Ebrecht *et al.*, 2003) and short-lived, due to the anti-inflammatory action of the increased circulating corticosterone produced during stress (Li *et al.*, 2000). However, chronic stress is known to result in glucocorticoid insensitivity of monocytes, resulting in chronically increased IL-6 concentration (Miller *et al.*, 2002). Our result of no significant difference in IL-6 concentration between experimental groups seems to differ from the available literature. However, taking into account the high inter-individual variation reported for IL-6 in this study (Figure 7.2) and in the literature (Lenczowski *et al.*, 1997; Tsirpanlis *et al.*, 2004), the relative small sample number used in the current study may have masked effects of *Sutherlandia* treatment and chronic intermittent stress on secretion of IL-6. Further studies on the acute cytokine responses to *Sutherlandia* treatment, possibly using larger sample numbers, are required to investigate this possibility. A more sensitive assay method should also be used (a high-sensitivity kit for rat blood is currently not available). Furthermore, the phasic release of TNF-α secretion, as well as its down-regulation by both IL-6 (Nukina *et al.*, 1998; Xing *et al.*, 1998) and glucocorticoids (Fantuzzi *et al.*, 1995), may account for the non-detectable levels of TNF-α reported here.

The suppressive effect of stress alone on testosterone levels ($P < 0.05$; Table 7.1) and the resultant effect of increasing the corticosterone:testosterone ratio ($P < 0.01$; Table 7.1) may be due to inhibition of the nocturnal rise in testosterone levels by glucocorticoid action (Doerr & Pirke, 1976). Although *Sutherlandia* treatment did not appear to have any direct effect on testosterone levels when compared to control rats (Table 7.1 & Figure 7.1b), small sample size and large inter-individual variation may mask such an effect in the current study. This issue warrants further investigation, since it is of particular importance in chronic illness to be

able to maintain testosterone levels, since it is vital for tissue growth and recovery (Bhasin *et al.*, 1996; Bhasin *et al.*, 2001).

7.5 Conclusion

Our data for the first time confirms scientifically the indigenous knowledge that the *Sutherlandia frutescens* herb has stress-relieving properties. In addition, our results indicate the necessity for further investigations of the effects of this herb, since it appears to have a complex mechanism of action and may therefore prove to be appropriate for other illnesses as well.

7.6 Acknowledgements

We would like to thank Dr. Carl Albrecht for donation of the dried *Sutherlandia* leaf specimen used in preparation of the experimental extract.

Chapter 8

Effect of *in vivo* administration of an anti-IL-6 antibody on the response of selected endocrine and immune parameters to short-term intermittent immobilisation stress in rats

8.1 Introduction

The effect of psychological stress on the immune system is complex and incompletely understood. Acute social stress in rats was previously shown to depress T cell count after 2 hours, and to a lesser extent after 48 hours of constant stress exposure. The *in vitro* mitogen-induced T cell proliferative response followed the same pattern and was depressed after 2 hours, but not after 48 hours of stress (Stefanski & Engler, 1998). However, impaired T cell-dependent antibody production was recently reported after 6 weeks of chronic psychological stress of greater severity, in a murine model of chronic mild depression (Silberman *et al.*, 2004). This change was not related to the circulating total glucocorticoid concentration, which remained unchanged. This suggests either an increase in T cell sensitivity to stress hormones, or an alternative indirect mechanism for the effect of glucocorticoids, possibly via cytokine action, on T cell function after chronic stress.

Acute psychological stress is known to result in increased release of inflammatory cytokines in humans (Maes *et al.*, 1998) and in rats (Mastronardi *et al.*, 2001). Apart from triggering the release of cortisol (Vander *et al.*, 1998a), IL-6 has also been shown to inhibit the synthesis of cortisol binding globulin (CBG) in culture (Bartalena *et al.*, 1993), which suggests an additional role for IL-6 in enhancing bioavailability of cortisol. These results suggest a role for cytokines, and in particular IL-6, in regulating both the secretion and downstream effects of cortisol in response to stress. However, these relationships are complex and may be complicated by additional regulatory factors, e.g. IL-1β, which has a role in the stimulation of cortisol production and is in turn down-regulated by cortisol. Contrary to the role of IL-6 as a stimulator of cortisol release, increases in IL-6 concentration was shown to be inversely associated with that of cortisol concentration in response to

chronic mild psychological stress in humans (Kunz-Ebrecht *et al.*, 2003). We suggest that this may have been due to negative feedback.

Considering the known negative effects of cortisol on inflammation and immune cell function (Genuth, 1983a; Vander *et al.*, 1998a), it is important to establish more clearly the mechanism of this hormone's effects on the immune system, including both cytokines and immune cells. However, very few *in vivo* studies have investigated the effect of stress on cytokines in conjunction with both endocrine and immune parameters. Recently, the possibility of blocking the effects of one or another cytokine in the cascade of the inflammatory response has become possible by either using knockout models, e.g. an IL-6 knockout mouse model (Xing *et al.*, 1998), or administration of antiserum or antibody, e.g. antibodies directed against IL-6 (Arruda *et al.*, 2000; Marby *et al.*, 2001). Such studies, although not close to *in vivo* physiological models of imposed stress, can illuminate our understanding of the complex responses to stress or illness.

In a rat model of meningitis, treatment with anti-IL-6 antibodies resulted in a decreased immune response to the disease (fewer WBC in cerebrospinal fluid) (Marby *et al.*, 2001), indicating a direct role for IL-6 in up-regulation of WBC proliferation. In IL-6 knockout-mice, TNF-α concentrations were higher after experimental endotoxemia (LPS injections) compared to those in IL-6 intact mice (Xing *et al.*, 1998). Since IL-1 is the primary stimulus for TNF- α production and is also known to be upstream of IL-6 secretion (Roitt, 1994), this led to the conclusion that IL-6 normally limits the up-regulation of TNF-α and possibly other pro-inflammatory cytokines. However, in the absence of other circulating immune and stress-endocrine measurements, the significance of this result is difficult to interpret. Although it is clear from the above literature that cytokines are an important link in the disease-induced immune response, the exact mechanisms and role-players, and also their roles in response to milder psychological stressors, are still very unclear.

Therefore, the aim of this study was to induce a stress response in rats, while blocking the function of IL-6, to determine a) the role of this cytokine in the inflammatory stress response, b) its effect on immune cell function, and c) the responses of other cytokines, e.g. IL-1, and their downstream functions in the absence of IL-6.

8.2 Methods

Experimental animals: 40 male Wistar rats were used in this study. Rats were housed in standard rat cages (6x7x18cm) and fed rat chow and tap water ad libitum. Temperature in the housing facility was controlled at 21 ºC and lights set to a 12-hour light/dark cycle (lights on at 7am). Rats were bought in from a breeding facility and allowed 3 weeks to acclimate before initiation of the study protocol, during which time they were handled and weighed once per day (7 times per week) to accustom them to this procedure.

Preparation of anti-IL-6 antibody treatments: An antibody raised in goats immunised with purified *E.coli*-derived recombinant rat IL-6 (AF506, R&D Systems, Germany) was used as intervention treatment in this study. 100 ug lyophilised antibody was reconstituted with 1 mL sterile PBS, according to manufacturer's instructions, to yield an antibody concentration of 0.1 mg/mL. This stock solution was further diluted in sterile PBS to yield a final concentration of 2 ug/mL, so that an injection of 0.5 mL per day would result in a dose of 1 ug/rat/day of anti-IL-6 antibody, in accordance with the manufacturer's indicated neutralisation dosage, as well as with previous studies (Arruda *et al.*, 2000; Hogan *et al.*, 2003). This solution was kept at 4 ºC for the duration of the protocol (stable for one month at this temperature, according to manufacturer).

Intervention: Rats were divided into four experimental groups of 10 rats each: control placebo (CP), control antibody (CA), immobilisation placebo (IP) and immobilisation antibody (IA). Placebo rats (CP and IP) were injected intraperitoneally with 0.5 mL of sterile isotonic saline once per day, and antibody rats (CA and IA) were injected intraperitoneally with 0.5 mL of anti-IL-6 antibody, prepared as described above. In addition, immobilisation rat groups were subjected to immobilisation stress once per day, using the protocol described in Chapter 6. Both the injection treatment and immobilisation stress protocols lasted 4 days, after which rats were sacrificed by decapitation.

Sample collection: Whole blood was collected by exsanguination, via a heparinised funnel, into one lithium heparin and one SST Vacutainer tube (BD Systems, Plymouth, UK). The heparinised blood was analysed for total and differential white blood cell counts and

mitogen-induced PBMC IL-6 secretion *in vitro*. SST blood was centrifuged at 3000 rpm for 10 minutes at 0 ºC, before being aliquoted and frozen at –80 ºC for subsequent batch analysis of IL-1β, TNF-α, IL-6 and corticosterone concentrations.

Sample analysis: All analyses on fresh blood were performed within 3 hours of collection. 500 uL of heparinsed blood was decanted immediately into a 1.5 mL reaction vial (Eppendorff, Sigma) for determination of a total and differential white blood cell count, performed by automated analysis in a professional veterinary pathology laboratory (Pathcare Vetcare, Parow). The rest of the blood was used to determine the ability of PBMCs in culture to secrete IL-6 both spontaneously (no added stimulation) and in response to mitogen (*E. coli* LPS) stimulation, using an adaptation of an earlier method used for investigations in human blood (Pool *et al.*, 1998; Pool, 1999) (refer to appendix C for details of the assay procedure). Values obtained for spontaneous stimulation are termed "spontaneous IL-6 release", while those termed "mitogen-induced IL-6 release" reflects the concentration of IL-6 released when stimulated with LPS, after subtraction of the spontaneously released concentration, so that it represents only the extra amount of IL-6 secreted as result of the mitogen stimulus.

Commercially available ELISA kits were used for determination of serum IL-1β (Biotrak RPN 2743, AEC Amersham Biosciences, UK), TNF-α (865.000.096, Diaclone, France), IL-6 (Biotrak RPN 2742, AEC Amersham Biosciences, UK) and corticosterone (AC-14F1, Octeia, Immunodiagnostic Systems, USA) concentrations.

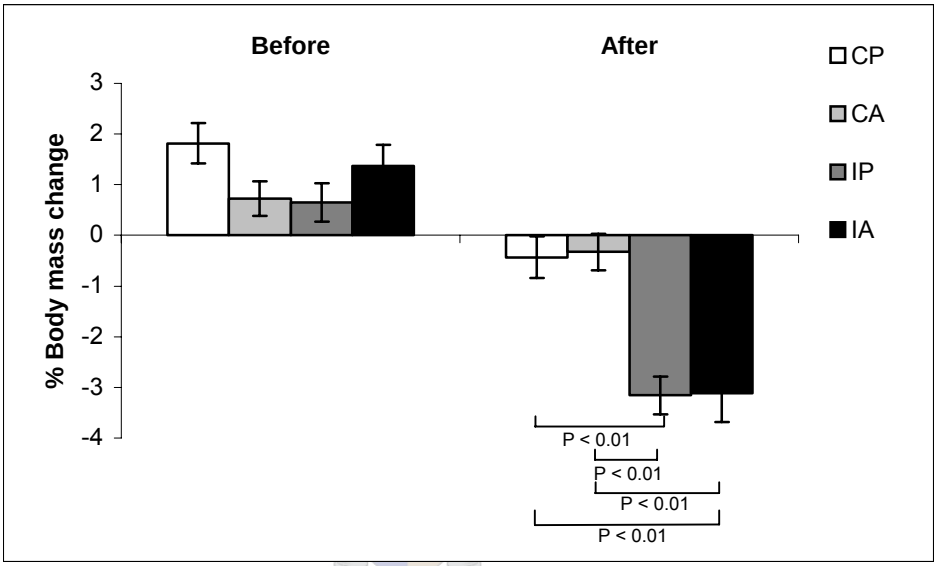
Statistical analysis: Effects of interventions were assessed by ANOVA and differences between experimental groups determined with Bonferroni and Tukey-Kramer *post hoc* tests. Correlations between parameters measured were determined by Pearson's correlations.

8.3 Results

Average change in body mass was calculated for days 1-4 before the start of the intervention protocol, as well as for the 4 days of the intervention protocol. Average change in body mass was similar in all groups during the 4 days before entry into the study, and groups had similar

average body masses on day 1 of the intervention protocol (means ± SD: CP: 519 ± 57, CA: 509 ± 33, IP: 517 ± 47 and IA: 498 ± 67 g). Both groups subjected to immobilisation stress lost a significantly greater percentage body mass during the intervention protocol compared to the non-stressed groups (Figure 8.1). The non-significant decrease in body mass in control rats may be ascribed to a very mild stress effect of the placebo injections administered (also refer to discussion).

Figure 8.1 Percentage change in body mass for 4 days before the start of the intervention protocol *vs*. during the 4 days of the intervention protocol.

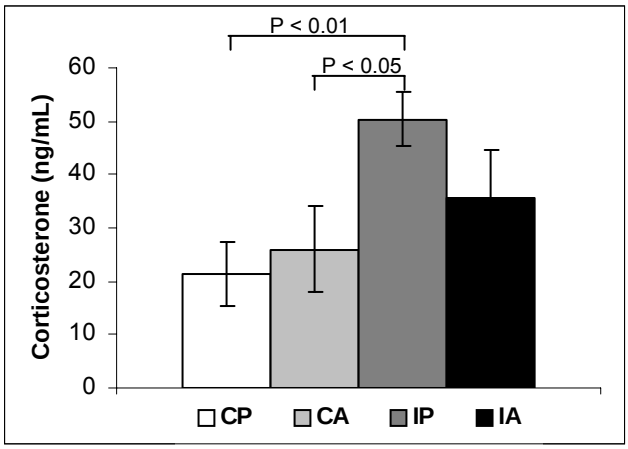


Values are means ± SEM.

Statistical analysis: P-values were obtained with Bonferroni post hoc analysis. Abbreviations: CP = Control, Placebo treated; CA = Control, Anti-IL-6 antibody treated; IP = Immobilised, Placebo treated; IA = Immobilised, Anti-IL-6 treated

Mean corticosterone concentration was significantly higher in IP vs. both CP and CA (Figure 8.2). Although this increase tended to be ameliorated in IA, mean corticosterone concentration of IA was not different from any other group.

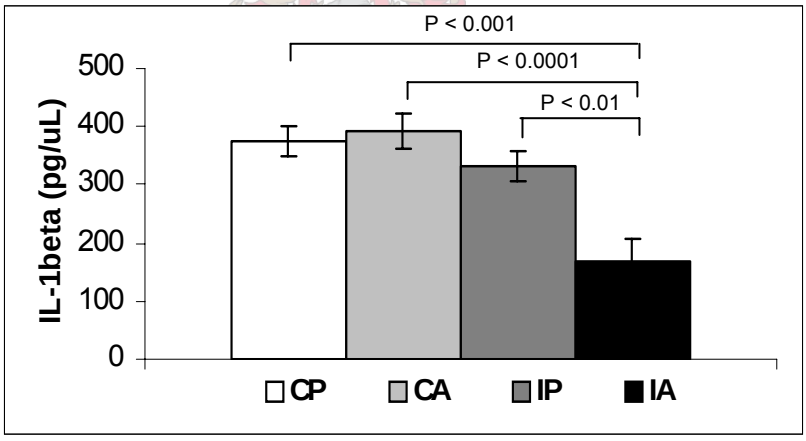
Figure 8.2 Differences in corticosterone concentration between experimental groups.



Values are means ± SEM. Statistical analysis and abbreviations as for Figure 8.1.

IL-6 concentration was below the detectable level in all but four samples, and TNF-α was detectable in only two (different) samples. However, IL-1β concentration was significantly lower in IA when compared to all other groups (Figure 8.3).

Figure 8.3 Differences in serum IL-1β concentrations between experimental groups.



Values are means ± SEM. Statistical analysis and abbreviations as for Figure 8.1.

Total and differential WBC counts were similar for all experimental groups (Table 8.1).

Table 8.1 Total and differential WBC counts at sacrifice.

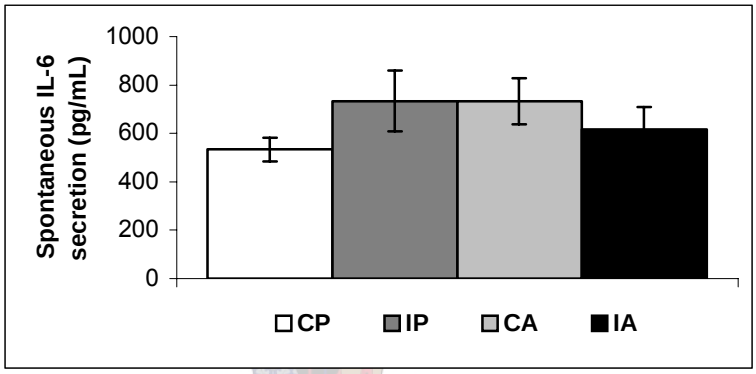
	CP	CA	IP	IA
WBC ($x10^9$/L)	8.4 ± 0.8	9.8 ± 0.4	8.4 ± 0.6	7.8 ± 0.4
Neutrophils ($x10^9$/L)	0.8 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	1.0 ± 0.3
Lymphocytes ($x10^9$/L)	7.2 ± 0.7	8.6 ± 0.5	7.3 ± 0.6	6.6 ± 0.5
Monocytes ($x10^9$/L)	0.15 ± 0.07	0.15 ± 0.05	0.15 ± 0.06	0.10 ± 0.05

Values are means ± SEM. Statistical analysis and abbreviations as for Figure 8.1.

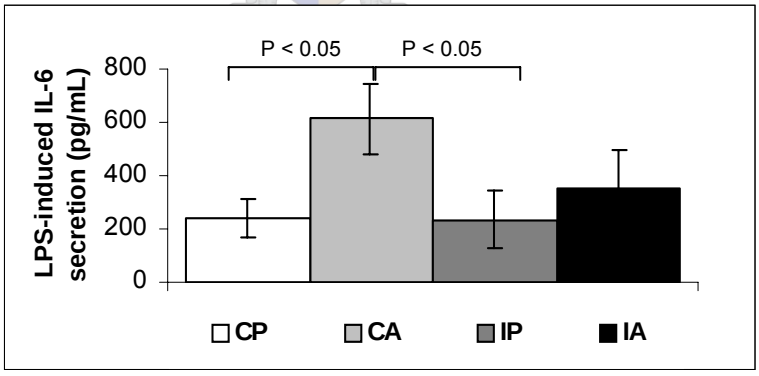
Although spontaneous *in vitro* IL-6 release by PBMCs was not influenced by the study protocol (Figure 8.4a), treatment with anti-IL-6 antibody was associated with significantly increased IL-6 release from PBMCs after 24 hours of incubation ($P < 0.05$). Although the treatment effect was significant, *post hoc* analysis indicated a more robust difference from non-treated groups in CA than IA, for both absolute IL-6 release (Figure 8.4b), as well as IL-6 release expressed relative to PMBC count (CP: 31.0 ± 8.1, CA: 72.4 ± 16.1, IP: 31.8 ± 16.9, IA: 47.6 ± 15.8 pg/cell x 10^6).

Figure 8.4 Effect of *in vivo* anti-IL-6 treatment on (a) spontaneous and (b) mitogen-induced secretion of IL-6 by PBMCs in plasma-replaced blood culture, after 24 hours of incubation.

(a)



(b)



Values are means ± SEM

Statistical analysis and abbreviations as for Figure 8.1.

LPS-induced IL-6 secretion *in vitro* was positively correlated with serum IL-1β concentration in both antibody-treated groups (Table 8.2). While serum corticosterone concentration was positively correlated with LPS-induced secretion of IL-6 in CP, this relationship was lost after intervention with either antibody, or immobilisation, or both (Table 8.2).

Table 8.2 Correlations between LPS-induced IL-6 secretion by PBMCs in culture and serum IL-1β and corticosterone concentration.

	CP	CA	IP	IA
Serum IL-1β concentration (pg/uL) *vs*. LPS-induced IL-6 (pg/mL)	-0.18	**0.70***	-0.07	**0.78***
Serum corticosterone concentration (ng/mL) *vs*. LPS-induced IL-6 (pg/mL)	**0.66***	-0.49	-0.12	0.14

Values are R-values.

** P < 0.05*

8.4 Discussion

The main findings we report here are a) significant loss of body mass in response to short-term intermittent mild psychological stress, b) immobilisation-induced increases in corticosterone secretion that were at least partially dependent on the presence of IL-6, c) decreased basal serum IL-1β concentration after stress exposure combined with blocking of IL-6, that was not apparent at all without stress, and d) a positive correlation between basal serum IL-1β concentration and LPS-induced PBMC function *in vitro* after blocking of IL-6, a finding that was independent of stress.

Similar to earlier results by our group (refer to Chapters 6 & 7) as well as other research groups (Dronjak *et al.*, 2004; Pitman *et al.*, 1988), immobilisation stress resulted in significantly increased serum corticosterone concentrations when compared to control groups. However, a novel finding is that in the IL-6-antibody-treated stressed group, this increase was attenuated (Figure 8.2). These results are not in agreement with a previous study investigating the acute stress response in anti-IL-6-treated mice, that showed no difference in the corticosterone response to immobilisation between experimental groups treated with antibody and those treated with placebo (Nukina *et al.*, 1998). However, there may have been a few confounding factors in the latter study. Firstly, the mean corticosterone concentration reported for the control (non-stressed) group was very high (108 ± 35 ng/mL compared to our result of 20 ± 6 ng/mL in CP and even only 50 ± 5 ng/mL in IP), which

suggests moderate stress in the controls. This is supported by another study in mice, where reported corticosterone concentrations in control mice were < 20 ng/mL, and 30 minutes of restraint stress resulted in values of ≈ 50 ng/mL (Lund *et al.*, 2004). Excess corticosterone may therefore have been present before the start of the stress-inducing intervention protocol in all groups. The fact that IL-1β, which is down-regulated by corticosterone, was below detectable levels in all groups in the study by Nukina *et al.* (1998), supports this possibility. Secondly, highly variable results within the stressed groups (279 ± 110 and 262 ± 80 pg/mL), combined with small sample size (n=5 in some groups), may account for not finding any differences between groups. Our result therefore does not support the conclusion made by this group, that restraint stress activates the HPA-axis independently of IL-6. Rather, our results suggest that the full-blown corticosterone response is dependent, in part, on IL-6.

Increased serum IL-6 concentration has been reported to result in a decreased TNF-α production in a rodent model of pathogen-induced weight loss (Matthys *et al.*, 1995). In a different study, treatment with anti-IL-6 resulted in an increase in TNF-α concentration immediately after and 60 minutes after restraint stress (Nukina *et al.*, 1998). However, IL-6 levels have also been reported to return to baseline levels within 12 - 24 hours after exposure to acute moderate stress, albeit in other models (Northoff *et al.*, 1994; Xing *et al.*, 1998). Therefore, our results of non-detectable levels of both IL-6 and TNF-α at rest 24 hours after short-term intermittent exposure to a mild stressor are not inexplicable. Furthermore, since the duration of chronic stress exposure that resulted in up-regulation of basal IL-6 concentration in the circulation of human subjects was in excess of 6 years (Kiecolt-Glaser *et al.*, 2003), our results suggest that four days of intermittent exposure to a mild stressor in rodents, is not analogous to a model of chronically elevated circulating IL-6, but is clearly a model of early phase adaptation to mild stress. Nonetheless, the phenotype of weight loss in response to stress was apparent. Changes in the concentration of IL-6 mRNA levels in immune cells may possibly elucidate the early adaptive response to mild stress further. However, since we did not assess this parameter, we cannot make any conclusions in this regard.

Chronic stress is commonly associated with weight loss in both human (Anker *et al.*, 1997; Kotler, 1994; Zinna & Yarasheski, 2003) and animal models (Stefanski, 1998). Although the

mechanisms are not fully elucidated, TNF-α and cortisol may be possible mediators of this process. It is possible that the immobilisation stress protocol used in our study, although relatively mild, was severe enough to result in a shift in the protein balance in favour of catabolism, particularly of skeletal muscle. Other studies have found evidence of severe muscle atrophy in rats subjected to more severe immobilisation protocols of longer duration (Pattison *et al.*, 2003; Sakakima *et al.*, 2004). While 10 days of hind limb immobilisation resulted in a 27 – 37 % decrease in soleus muscle mass (Pattison *et al.*, 2003), 14 days of ankle immobilisation resulted in a decrease in muscle fibre cross-sectional area of 62 – 66% in the soleus muscle and a reduction of 68 – 78 % in the gastrocnemius muscle (Sakakima *et al.*, 2004). Although the results of these studies are of greater magnitude than our result, these models of disuse immobilisation are far more severe than ours and invoke several mechanisms for atrophy. We suggest that our model, although yielding only a mild phenotype, is a more physiological model for psychological stress than the latter two studies, which models were specifically intended for investigating muscle unloading in the absence of mechanical stresses, which induces local atrophy-inducing factors. Further studies on muscle tissue are currently underway, as part of a M.Sc. thesis, to describe further the characteristics of stress-induced muscle atrophy present in our model, in which immobilisation is kept to a minimum and circulating atrophy-inducing factors are likely to predominate.

Normally, IL-1β production results in an up-regulation of IL-6 secretion (Ishikawa *et al.*, 2001; Roitt, 1994), with a resultant increase in circulating corticosterone, which provides negative feedback to attenuate IL-1β secretion. This negative feedback appeared to be sufficient in IP rats for their IL-1β concentrations to be similar to their controls (CP) despite stress. A novel finding in our study was that blocking of IL-6 is associated with a decrease in IL-1β only in the presence of stress (Figure 8.3). However, IL-1β also activates the release of CRH and ACTH directly, again with negative feedback provided by cortisol (Sapolsky *et al.*, 2000; Watkins, 1994). In our study it appeared as if the effect of down-regulation of IL-1β secretion by cortisol was obscured in the stressed groups, with significantly lower IL-1β levels in IA than in IP. This suggests a positive feedback by IL-6 on IL-1β secretion that can partially negate the effect of cortisol on IL-1β in mild stress. However, since the same effect was not observed in CA, it is unlikely to be a direct effect, but rather associated with some aspect of

the complex stress response. This finding and our interpretation is novel. That it has not been seen before could be related to the fact that many stress protocols are more extreme than ours, resulting in a greater corticosterone response which would down-regulate IL-1β more clearly. Also, anti-IL-6 antibodies have not previously been used in a model of mild stress.

The interventions used in this study did not significantly affect total or differential white blood cell counts (Table 8.1). Absolute total WBC and lymphocyte counts were slightly higher, and the neutrophil count lower, than those reported earlier in Long-Evans rats subjected to either no stress, or 2 or 48 hours of social stress (Stefanski & Engler, 1998). However, since these immune cell counts did not respond to stress in either study, the difference in cell counts between the two studies are most likely due to inter-strain differences. However, considering the reported decrease in peripheral node lymphocyte function after acute inescapable tail shock (Fleshner *et al.*, 1995a), an unchanged total WBC or lymphocyte cell count does not necessarily imply unchanged cell function. Therefore, functional test results should also be considered in a comprehensive evaluation of the immune response to stress.

PBMCs in culture exhibited a similar ability to spontaneously secrete IL-6 during incubation in all groups. This suggests that neither 4 days of immobilisation stress, nor short-term blocking of IL-6, is sufficiently severe to result in a change in excitation of mononuclear cells in the absence of a specific stimulant. However, a main effect of anti-IL-6 antibody treatment was a significant increase in mitogen-induced IL-6 secretion by the combined antibody-treated group compared to the combined placebo group ($P < 0.05$), with no independent or interactive effect of immobilisation. The result of no effect of immobilisation is in accordance with previous reports of unchanged mitogen-induced lymphocyte proliferation rate in rats subjected to social stress (Stefanski & Engler, 1998). If the replacement of blood plasma by culture medium in the assay procedure did not successfully remove all the antibody from the cell culture, it is possible that the antibody present initially bound to the IL-6 secreted in response to LPS. This would result in an increased cytokine secretion due to lack of early negative feedback by IL-6 on cell function. Alternatively, if anti-IL-6 antibody became membrane-bound and was not washed out by plasma replacement, it may have prevented

negative feedback throughout the assay time period. However, given the magnitude of increase in IL-6 secretion in the antibody-treated rats, the concentration of residual antibody is unlikely to have such a substantial effect. More likely, blocking of IL-6 may have caused adaptation of PBMCs to render them more sensitive to mitogen stimulation, so that stimulation resulted in excess cytokine secretion by these cells. The fact that mitogen-induced IL-6 release was positively correlated with serum IL-1β concentrations in the antibody-treated groups only, suggests that the proposed effect on PBMCs may be mediated by IL-1β. It also argues against the finding being an artefact of residual antibody in the assay procedure, since the correlation was with serum IL-1β, and not with *in vitro* mitogen-induced PBMC-secreted IL-1β.

8.5 Conclusion

Our results illustrate three important points regarding IL-6: a) that psychological stress activates the HPA-axis in a manner augmented by IL-6, b) that IL-6 plays a role in maintenance of the IL-1β concentration under conditions of mild stress, thus allowing a continued elevation of corticosterone release, and c) that IL-1β plays a role in priming PBMCs for enhanced function, a function normally obscured by IL-6.

Chapter 9

Synthesis

9.1 Introduction

Acute exercise stress results in physiological changes in the immune system and endocrine stress-axis that are usually only transient. Repeated exercise bouts in quick succession may however prevent the body from fully recovering between bouts. In training regimens followed by elite athletes, insufficient recovery time from exercise is frequent, rather than the exception. These athletes are also under constant psychological stress (both sport and every-day life stresses), which further contributes to their overall stress status. Unless an athlete is able to adapt and cope with the increased stress, the body may become unable to react effectively to additional stressors, resulting in increased risk of acute illness, inability to train and decreased performance in competitive events. Alternatively, the body may be rendered unable to down-regulate the stress response, which may lead to chronic disease. Both these possible outcomes are equally detrimental to a career in sports.

From the reviewed literature it is clear that chronic exercise-related stress affects both the immune system and endocrine stress-axis, but there are still many uncertainties (2.7). The contributions of this thesis to filling some of the gaps will be highlighted.

9.2 Impact of results

Even though the *in vitro* PBMC assay has been used before by exercise physiologists, the spontaneous secretion of IL-6 after incubation was not reported as a finding. This may be because it was assumed to be a response to the assay conditions, and thus merely used in calculation of the mitogen-induced response. However, one of the most important results in Chapter 3 was that there was an inverse relationship between the spontaneous and mitogen-induced IL-6 release by PBMCs at rest. Higher spontaneous release was associated with lower mitogen-induced release of IL-6. In a previous study of which I was a

co-author (Pool *et al.*, 2002), we showed that spontaneous IL-6 release in sedentary subjects was very low (≈ 25-fold lower) compared to that in endurance athletes at rest. Together, these results indicate that chronic exercise stress results in a chronic low-grade activation of pro-inflammatory activity, which renders PBMCs less capable of mounting a response to an additional stressor. This first study posed some questions:

a) Which other immune cells are chronically activated?

b) Is this up-regulation the result of an increased upstream activation, or a lack of negative feedback? For example, a lower level of $CD8^+$ suppressor T cell activation could lead to an overactivation of $CD4^+$ cells. Similarly, chronic exposure to cortisol may decrease the sensitivity of its inhibitory action on T lymphocyte cytokine release.

c) Is reduced mitogen-induced IL-6 release at rest after chronic stress the result of a direct feedback of circulatory IL-6?

In the next study (Chapter 4) we purposefully subjected recreationally competitive cyclists to a training regimen more similar to that of elite athletes. A main finding in the training study was the decreased absolute $CD8^+$ cell count at rest after training, which could lead to a lack of suppressor cell capacity to inhibit $CD4^+$ cell activation. However, the average $CD4^+$:$CD8^+$ ratio for the group was not affected by the intervention with unaccustomed training. Rather, the $CD4^+$:$CD8^+$ ratio at rest was associated with individual habitual training volume, with lower ratios associated with higher training volumes.

Another important finding was that spontaneous activation of $CD4^+$ lymphocytes was increased at rest after unaccustomed high-intensity interval training. However, this was not true for the $CD8^+$ cells, indicating that $CD4^+$ cell activation may be more sensitive to exercise stress. Similar to the finding in the *in vitro* assay in Chapter 3, the mitogen-induced response in the $CD4^+$ cells was lower, suggesting that a negative effect of chronic spontaneous cell activation on the immune response to a subsequent mitogenic challenge is generally applicable.

Cortisol high-responders to chronic psychological stress (Chapter 5) also showed significantly higher spontaneous $CD4^+$ (as in Chapter 4) as well as $CD8^+$ (not shown in Chapter 4) cell activation when compared to cortisol low-responders in the same study,

indicating that chronic exposure to cortisol may reduce the effectiveness of its normal inhibitory role in spontaneous T cell reactivity. This led us to develop an animal model in which stress exposure could be controlled, to further investigate this possibility.

In the first animal study (Chapter 6) we confirmed that the model of mild physiological stress worked in our hands. This was confirmed when a known anxiety-reducing supplement abolished the stress response (Chapter 7). Another interesting finding in the first study (Chapter 6) was that IL-1β concentration was elevated immediately after acute stress exposure, as well as 24 hours later, whereas IL-6 was not elevated at either time point. This result, as well as no detectable IL-6 at rest at any time point during the training study (Chapter 4, result not reported), led us to question the significance of IL-6 as essential to a) the chronic stress response and b) immune cell reactivity.

Our final study was designed to investigate the influence of stress on the immune and endocrine systems in the absence of IL-6 (Chapter 8). We successfully demonstrated that IL-6 is required to induce a proper corticosterone response to immobilisation stress (Figure 8.2). Since a marker for CD69 expression in rat T cells is not available, we reverted to the less specific PBMC assay. Unfortunately, spontaneous IL-6 release was not affected by the 4 days of exposure to mild stress. Neither did the stress protocol affect mitogen-induced IL-6 secretion. These results imply that the 4-day animal stress model is not similar to chronic exercise stress and that if IL-6 does play a role in immune cell reactivity, it will either be more acute, i.e. not evident in a sample taken at rest, or only develop with longer-term exposure.

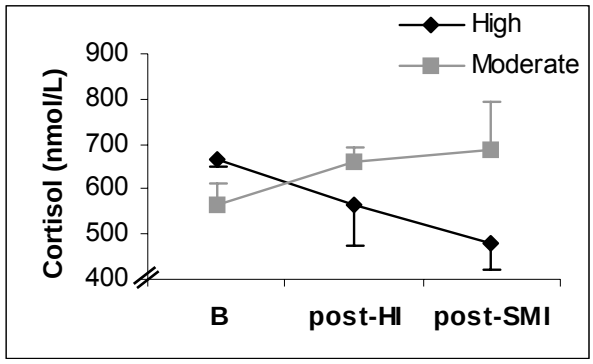
Nevertheless, influences of both corticosterone and IL-6 on the mitogen-induced IL-6 response were evident. Blocking of IL-6 in control rats resulted in an increased mitogen-induced IL-6 response, suggesting moderate self-regulation of IL-6 release under normal conditions. This is not dissimilar to the conclusion that can be drawn from the relationship reported between spontaneous and mitogen-induced IL-6 release in the first study (Figure 3.3). However, as in the first study, this relationship was not evident in the presence of stress. This implies that another factor associated with stress can inhibit IL-6 secretion. The complex interactions between the pro-inflammatory and anti-inflammatory markers we measured are very clearly indicated in Table 8.2. Under normal conditions (no stress),

within the low normal range of corticosterone, its concentration correlated positively with mitogen-induced IL-6 release, but when one or more conditions were manipulated (either stress exposure, or blocking of IL-6, or both), this relationship was not evident. In the presence of normal low corticosterone and anti-IL-6 antibody, corticosterone concentration no longer affected mitogen-induced IL-6 release directly. Instead, IL-1β correlated with the enhanced response. In the presence of stress, despite the presence of sufficient IL-1β, corticosterone down-regulated IL-6 secretion. This also occurred in the presence of the antibody, although IL-1β was able to partially rescue the mitogen-induced secretion of IL-6.

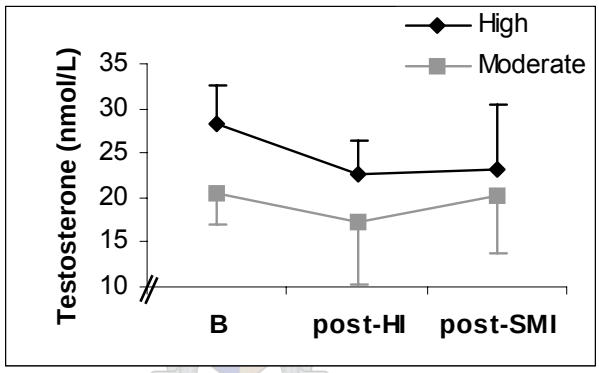
Given the complexities in the interpretation of resting immune cell counts and functions (which may be related in part also to their intra-individual variability) reverting back to the endocrine system markers of stress status (which have more acceptable intra-individual variability; Table 5.2) should be considered. In the well-controlled animal model of chronic, intermittent immobilisation stress lasting 28 days, corticosterone concentration, as well as the corticosterone:testosterone ratio, was increased (Figure 7.1). However, in the training study (Chapter 4), no training effect was evident on either the mean absolute concentrations of endocrine parameters, or the relationships between them (Table 4.2). Nonetheless, after re-analysing results from Chapter 4, dividing these subjects into two groups according to their cortisol concentration at baseline, there was a clear difference in the cortisol responses to training between the two groups (Figure 9.1a). While the group with higher initial cortisol concentrations (n = 3) showed a decrease in resting cortisol concentration during training, the opposite effect was evident for the group who started with a moderate cortisol concentration (n = 4) at baseline. (No subject exhibited resting cortisol concentrations as low as those in the low-responder group in Chapter 5, and this group was therefore termed “moderate” responders.) Although the testosterone responses appeared similar for the two groups (Figure 9.1b), the cortisol:testosterone ratio in the group with moderate baseline cortisol concentration was more responsive (albeit with high variability) to the exercise training intervention when compared to the group with high baseline cortisol concentration (Figure 9.1c).

Figure 9.1 Differences in the effect of training on the responses of a) cortisol, b) testosterone and c) the cortisol:testosterone ratio at rest in subjects with either high or moderate serum cortisol concentration at baseline (data from Chapter 4, re-analysed).

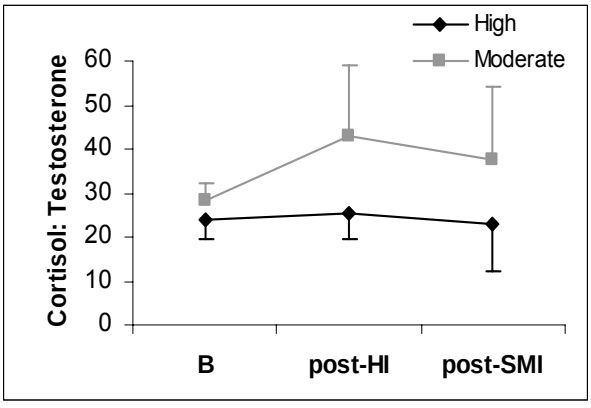
(a)



(b)



c)



Values are means ± SD.

These results indicate that consideration of both cortisol and the cortisol:testosterone ratio may be useful for the assessment of changes in stress status. However, careful classification of the subject group at baseline is necessary for comprehensive interpretation of results,

since a lack of this precaution may mask such changes. This approach requires higher subject numbers so that division into groups is more feasible than in the current study.

9.3 Conclusions and recommendations for future studies

Although the interactions between role-players in the systemic stress response cannot be accurately simulated in *in vitro* studies, the latter techniques allow for the controlled manipulation of potential stress-response mechanisms. I conclude that the effects of stress on specific role-players, or interactions between them, may never be elucidated unless the *in vitro* systems are artificially manipulated. For example, our results suggest a positive feedback loop between IL-1β and IL-6 in the presence of a stressor, rather than a one-way up-regulation of secretion of IL-6 by IL-1β only. Follow-up studies in appropriate subject populations are needed to illuminate the mechanism by which this may be achieved. Possible mechanisms include a) cytokine-mediated alterations to the setpoint (balance) between different types of glucocorticoid receptors present on immune cells, as recently suggested (Peters *et al.*, 2004; Strickland *et al.*, 2001), or b) increases in cytokine gene transcription rates, which may be a result of decreased glucocorticoid-mediated transrepression (transcriptional interference) of key regulatory factors in gene expression, such as nuclear factor-kappaB and activator protein I, or an increase in cytokine response element-mediated transactivation (gene transcription).

Our results of the chronic up-regulation of spontaneous activation status of both PBMCs and T lymphocytes in athletes in response to exercise stress are novel. However, the PBMC assay that we used, does not distinguish between monocytes and lymphocytes, while the assessment of CD69 expression by T cells cannot distinguish between the activation of T_H1 and T_H2 lymphocytes. Given the different roles or these cells in the immune response, namely activation of the cell-mediated *vs*. the antibody-mediated immune responses, it is of importance to determine the nature of this up-regulated spontaneous activation in response to stress, in a cell type specific manner. This could be done by culturing isolated cell populations, instead of whole blood. Furthermore, given the divergent cortisol response to chronic stress (illustrated in both sedentary and athlete populations), and its implication for

immune reactivity (illustrated in sedentary subjects after psychological stress), we recommend that future studies should investigate possible differences in the regulation of spontaneous T_H1 and T_H2 cell activation in response to chronic exercise stress in cortisol high- *vs.* low-responder athletes.

Finally, we report that our result of unchanged cortisol concentration at rest in response to chronic stress, is the net result of a divergent response to stress, rather than a reflection of no change. Serum cortisol concentration at rest, and potentially the cortisol:testosterone ratio, may in fact be useful indicators of stress status, with the precondition of subject classification at baseline. Further studies, using larger subject numbers and a more varied population, are required to further substantiate our finding in studies with relatively low subject numbers.

Appendix A

Determination of T lymphocyte subpopulation distribution and responsiveness using flow cytometry

Assay principle

Flow cytometry uses either structural features of cells, or components on the cell, such as fluorescent antibodies directed against the cells, to quantify these cells by optical means. The cells may be alive or fixed at the time of measurement, but must be in monodisperse (single cell) suspension. They are passed single-file through a laser beam by continuous flow of a fine stream of the suspension. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The flow cytometer measures several parameters simultaneously for each cell:

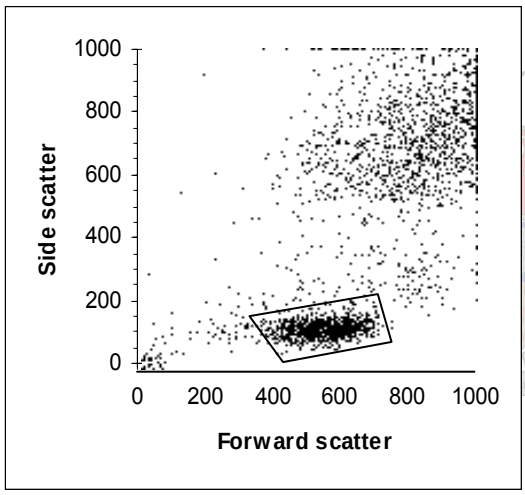
1) forward scatter intensity, which is approximately proportional to cell diameter
2) 90º side scatter intensity, which is approximately proportional to the quantity of granular structures within the cell, and
3) fluorescence intensities at several wavelengths, so that if particles have been stained with one or more fluorescent dyes, the light source excites these dyes to provide additional biological information about each particle, such as presence of specific surface and intracellular markers (Lewis & Rickman, 1992; Shapiro, 1995; Steen, 1990).

These fluorescent pulses and scattered light are converted into digital pulses and sent to a computer for analysis. Light scatter alone is sufficient to distinguish lymphocytes from monocytes from granulocytes in blood leukocyte samples. Fluorescent antibodies are often used to report the densities of specific surface receptors, and thus to distinguish subpopulations of differentiated cell types (Hannet *et al.*, 1992; Lewis & Rickman, 1992; Maino *et al.*, 1995; Shapiro, 1995; Steen, 1990).

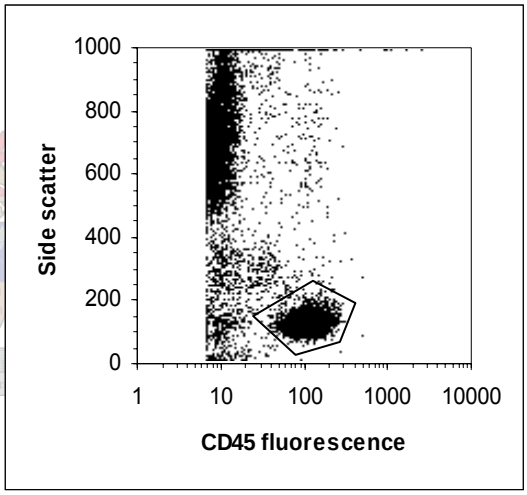
Interpretation of results

There are two ways to identify lymphocytes in a whole blood sample. One way is to label the lymphocytes with a fluorescence-labeled antibody directed against CD45, a general lymphocyte surface marker. A scatter plot is then generated according to CD45 fluorescence (X-axis) and side scatter (Y-axis). The other way is to generate a scatter plot without using a cellular marker antibody, according to forward scatter (X-axis) and side scatter (Y-axis). (The latter is typically used for analysing non-human samples.) Forward scatter increases with increased cell size, while increased side scatter indicates higher cell granularity. Typical scatter plots obtained in these ways are illustrated below:

a) Using scatter characteristics only

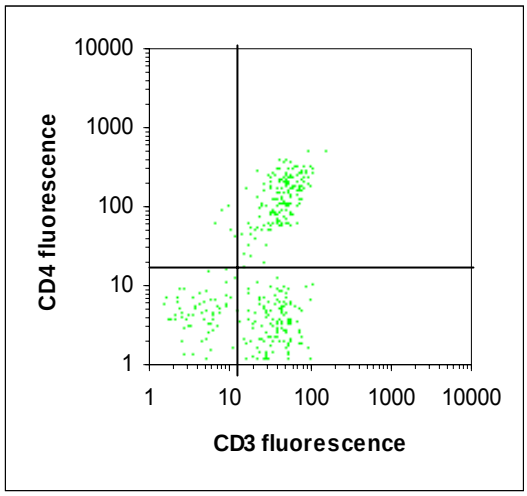


b) Using fluorescence labeled CD45 antibody

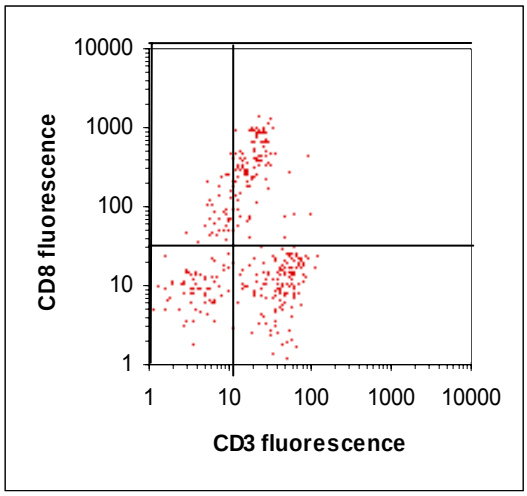


Lymphocytes may be distinguished on the two graphs by the characteristics (a) "FSC^{high} SSC^{low}", which means that lymphocytes exhibit high forward and low side scatter, or (b) "$CD45^{bright}$ SSC^{low}", which means that they are positive for CD45, and exhibits low side scatter on a flow cytometry scatter plot, (Lewis & Rickman, 1992). These lymphocytes are then selected, or "gated" manually (indicated on the plots above). These cells only are then plotted again on a similar scatter plot, but according to fluorescence of CD3 (for T cells) on the X-axis *vs*. fluorescence of CD4 or CD8 on the Y-axis. Representative scatter plots achieved this way are illustrated below:

a) CD3 *vs*. CD4 fluorescence

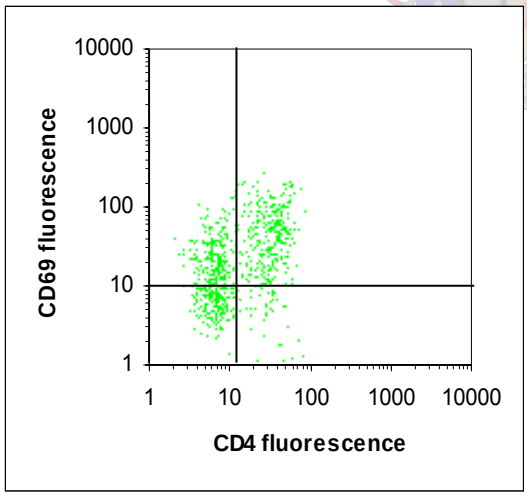


b) CD3 *vs*. CD8 fluorescence

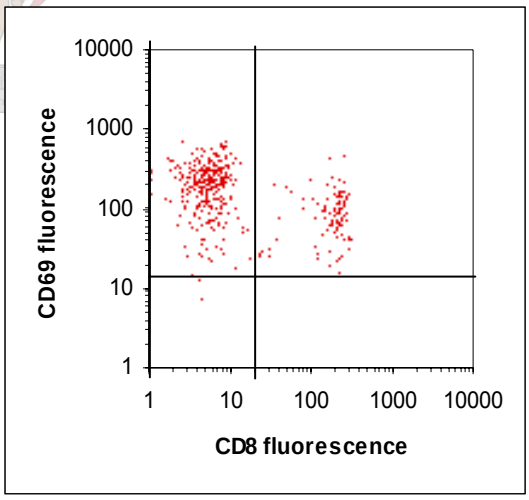


From this it is clear that the $CD4^+$ and $CD8^+$ T cells are in the right hand upper quadrant of each graph respectively. These groups of cells can then each be gated again and another scatter plot generated according to CD4 or CD8 vs. CD69 fluorescence. Typical plots are illustrated below:

a) CD4 vs. CD69



b) CD8 vs. CD69



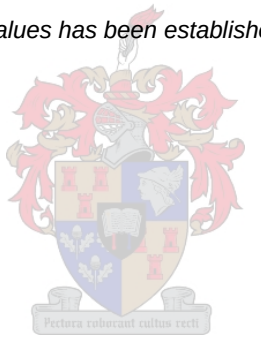
Again, cells expressing both CD69 and CD4, or both CD69 and CD8, will appear in the right hand upper quadrant of the scatter plot. For all graphs, all gated cells are automatically expressed as a percentage of total lymphocytes counted. Using the differential white cell count, these percentages can be converted into absolute cell counts.

Expected normal values for human lymphocyte subpopulations*

(Lewis & Rickman, 1992; Maino *et al.*, 1995)

Subpopulation	**Range (18 – 70 years)**
$CD3^+$	1 100 – 1 700 cells/uL
$CD4^+$	700 – 1 100 cells/uL
$CD8^+$	500 – 900 cells/uL
$CD4^+$:$CD8^+$ ratio	1.0 – 1.5 : 1
$CD19^+$ (B cells)	200 – 400 cells/uL
$CD16/56^+$ (NK cells)	200 – 400 cells/uL
$CD4^+CD69^+$	No reference range established yet
$CD8^+CD69^+$	No reference range established yet

** No normal values has been established for rat blood samples*



Analytical procedure

1. Lymphocyte phenotyping

Reagents and equipment

Multitest CD3FITC/CD4APC/CD8PE/CD45PerCP antibody

Multitest CD3FITC/CD16+56PE/CD19APC/CD45PerCP antibody

Fluorescent Activated Cell Sorter (FACS) Lysing Solution

FACS tubes

3-colour flow cytometer (FACS Calibur)

All reagents and equipment are available from Becton Dickinson, New Jersey, USA

Method

1. Label two tubes per sample.
2. Prepare a 1:3 dilution of each Multitest antibody reagent in phosphate buffered saline (PBS).
3. Add 20ul of antibody dilutions to two separate tubes.
4. Add 50ul of heparin/EDTA whole blood to each tube.
5. Mix gently and incubate at room temperature for 15 minutes in the dark.
6. Mix again and add 450ul FACS Lysing Solution (diluted 1:10 in distilled water).
7. Mix and incubate for 10 minutes at room temperature in the dark.
8. Analyse on flow cytometer.

2. Determination of CD69 expression

Reagents and equipment

12-O-tetradecanoylphorbol-13-acetate (TPA)

Ionomycin

Multitest CD3PerCP/CD4FITC/CD69PE antibody

Multitest CD3PerCP/CD8FITC/CD69PE antibody

Fluorescent Activated Cell Sorter (FACS) Lysing Solution

FACS Cell Fixative

FACS tubes

3-colour flow cytometer (FACS Calibur)

All reagents and equipment are available from Becton Dickinson, New Jersey, USA.

Method

1. Preparation of TPA:
    a) Prepare a 1mg/ml stock solution in DMSO medium (aliquots of 20ul stored at –20 °C).
    b) Dilute 20ul in 980ul culture medium (1:100).
2. Preparation of Ionomycin:
    a) Make up a stock solution of 1mg/ml in ethanol (store in aliquots of 50ul at –20 °C).
    b) Dilute 50ul in 450ml culture medium (1:10)
3. Label 4 tubes per sample: two for unstimulated cells and two for stimulated cells.
4. To unstimulated-labelled tubes, add 100ul whole blood (heparin/EDTA).
5. To stimulated-labelled tubes, add 100ul whole blood, 4ul ionomycin and 5ul TPA.
6. Incubate for 4 hours at 37 °C at 5 % CO_2 and 98 % humidity.
7. Add 10ul of CD4/CD69 and 10ul CD8/CD69 to one ‘unstim.’ and one ‘stim.’ tube each.
8. Incubate for 20 minutes at room temperature in the dark.
9. Add 500ul FACS Lysing Solution to all tubes.
10. Incubate for 10 minutes at room temperature in the dark.

11. Analyse on flow cytometer within 60 minutes.
12. If not possible, centrifuge samples at 1800 rpm for 5 minutes. Decant supernatant. Add 2ml PBS and centrifuge again. Decant. Add 500ul fixative to the cells and store at 2 – 8 °C until analysis.

Appendix B

ELISA for IL-6 secretion in whole blood culture supernatant (human)

Reagents

Rabbit anti-IL-6 (Sigma)

Rabbit serum (Highveld Biologicals)

Trition X100 wash solution (Merck Chemicals)

Recombinant human IL-6 (Sigma)

Biotinylated rabbit anti-IL-6 (Sigma IL-6 antibody biotinylated using Boehringer Mannheim protein biotinylation kit)

Avidin-peroxidase complex (Amdex, Denmark)

TBM substrate (Boehringer Mannheim, Germany)

0.2 M H_2SO_4

96-well plates

Method

A) Plate preparation:

1. Dilute rabbit anti-IL-6 antibody 1:1000 with saline.
2. Coat plates with rabbit anti-IL-6 by adding 100uL/well and incubating overnight at room temperature.
3. Aspirate and block wells by adding 200uL/well of 1% rabbit serum in saline, incubating 30 minutes at room temperature.
4. Wash plates once with saline containing 0.1 % Triton X100 (wash solution).

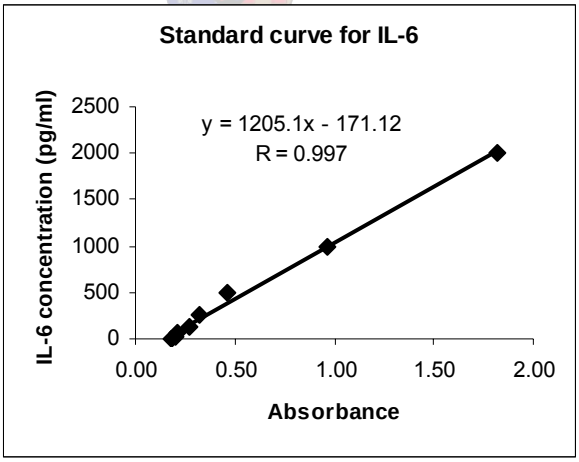
B) IL-6 assay:

1. Prepare standard solutions for IL-6 using human recombinant IL-6.
2. Add 100uL/well of samples or standards were added (100 uL/well) and incubated at 37 °C for 90 minutes.

3. Wash plate three times in wash solution.
4. Dilute biotinylated rabbit anti-IL-6 1:200 in 1 % rabbit serum, add 100 uL/well and incubate at 37 °C for 90 minutes.
5. Wash plate three times in wash solution.
6. Dilute avidin-peroxidase complex 1:1000 in 1 % rabbit serum, add 100 uL/well and incubate at room temperature for 20 minutes.
7. Wash plate four times in wash solution.
8. Add 100 uL/well of TBM substrate and incubate at room temperature in the dark for 20 minutes. Stop the chromogenic reaction by addition of 100 uL/well of 0.2 M H_2SO_4.
9. Read optical density on a plate reader at 450 nm.

C) Standard curve:

1. Use standard solutions to generate a linear standard curve, from which IL-6 concentrations in samples can be extrapolated.
2. A representative typical standard curve is illustrated below:



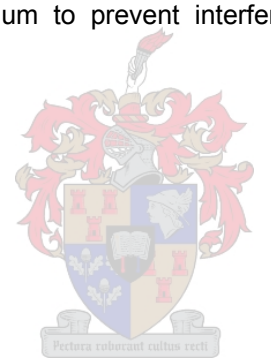
3. NOTE: The linear detection range was previously established at 10 – 1240 pg/mL. Coefficients of variation between samples analysed in duplicate, and between assays, are typically less than 7 %.

Appendix C

Determination of *in vitro* mitogen-induced IL-6 secretion after *in vivo* administration of anti-IL-6 antibody (rat)

Assay principle

This assay is similar to the one described earlier, used in humans participating in an ultra-marathon (refer to Chapter 3). However, since this was the first time the assay was used to analyse rat blood, it had to be optimised first by doing a dose-time response determination. Also, since the whole blood would contain the administered anti-IL-6 antibody, the plasma of all samples was removed (taking care not to remove white cells as well) and replaced by an equal volume of RPMI medium to prevent interference of the antibody with the assay procedure.



Reagents

E.coli LPS (Sigma)

RPMI 1640 medium, containing 5% glutamine (Highveld Biologicals)

Rat IL-6 kit (Biotrak 2742, AEC Amersham)

Sterile test tubes

Sterile pipette tips

A) Dose-time response assay (performed on pooled rat whole blood)

Method

1. Make up LPS concentrations of 0.0 ug/mL, 0.5 ug/mL, 1.0 ug/mL and 1.5ug/mL (dosage most frequently used in literature, is 1ug/mL (Li *et al.*, 2002; Sautebin *et al.*, 1999)) in RPMI 1640 medium.
2. Label 4 tubes each for 4, 8, 12, 16, 18, 20 and 24 hours (incubation times reported for rat and murine cell cultures are 12 – 24 hours (Li *et al.*, 2002; Sautebin *et al.*, 1999), and for human whole blood 4 – 18 hours (Pool, 1999; Pool *et al.*, 2002; Tantak *et al.*, 1991).
3. Into all tubes, pipette 500 uL whole blood.
4. For each time point, pipette into 4 test tubes 2000 uL of 0.0, 0.5, 1.0 or 1.5 ug/L LPS-RPMI solution.
5. Incubate at 37 °C, in the presence of 98 % humidity and 5 % CO_2.
6. Centrifuge a set of 4 tubes at incubation time points 4, 8, 12, 18 and 24 hours and collect supernatant.
7. Freeze at –80 °C until batch analysis of IL-6.
8. Determine IL-6 concentration using assay kit.
9. Draw curve of time and IL-6 concentration for each LPS concentration.

Results

Figure 1 Standard curve obtained for IL-6

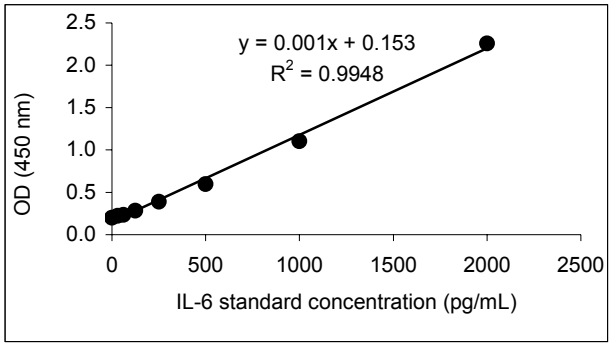
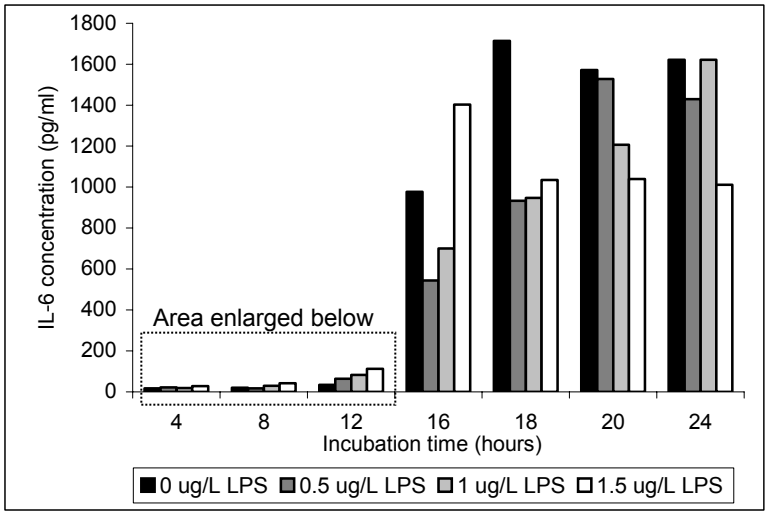
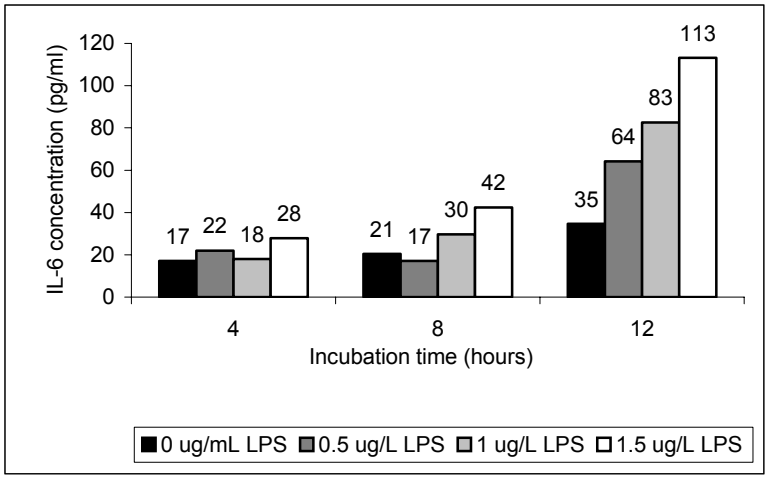


Figure 2 IL-6 secretion measured for each incubation time and LPS dosage





Discussion

Cells showed the best reaction to 1.5 ug/L LPS after 12 hours of incubation. However, the values obtained after 16, 18, 20 and 24 hours suggested that cells responded in absence of LPS, indicating stress, while addition of LPS as well resulted in a decreased response. This may indicate that the cells were experiencing oxidative stress in the closed reaction tubes, so that cells died off after 16 hours, resulting in a less that optimal response. In addition, the large volume of blood necessitated the use of pooled rat blood, so that this pilot assay does not give an indication of the inter-individual variation that can be expected in the actual study assay. Therefore, we decided to incubate cells with 1.5ug/L LPS for both 12 and 24 hours for our study protocol. In addition, the reaction tubes were not closed airtight, to prevent oxygen starvation.

B) Culture assay for determination of LPS-induced IL-6 secretion in rat whole blood

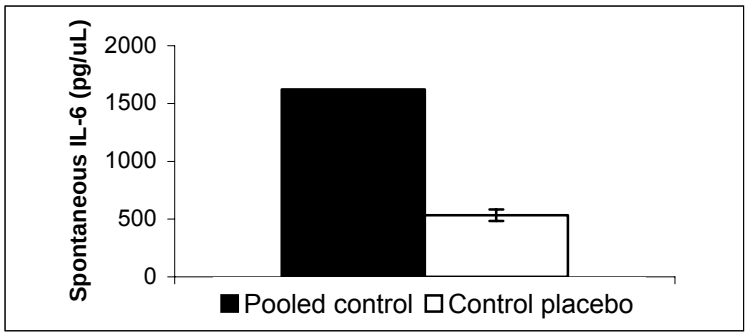
Method

1. Prepare of LPS-RPMI medium with a final concentration of 1.5 ug/mL LPS.
2. Prepare whole blood: To reduce the concentration of anti-IL-6 antibody in the whole blood, centrifuge blood for 10 minutes at 3000 rpm at room temperature. Then remove plasma and replace with an equal volume of RPMI medium. Mix gently and use for blood culture.
3. Prepare cell culture:
    a. Prepare 4 tubes for each rat: 1x stim. 12 hours, 1x stim. 24 hours, 1x unstim. 12 hours, 1x unstim. 24 hours
    b. To stim tubes add 1ml RPMI-LPS medium and to unstim tubes 1ml RPMI medium.
    c. To all tubes add 250ul blood, prepared in (2).
    d. Incubate for 12 and 24 hours at 37 °C, in the presence of 98 % humidity and 5 % CO_2.
    e. Centrifuge at 3000 rpm for 10 minutes and draw off supernatant into 2 eppies.
    f. Freeze at –80 °C until batch analysis using IL-6 kits, as used in the dose-time response assays.
4. Determine IL-6 concentrations in all supernatants.
5. To calculate the amount of IL-6 secreted in response to mitogen stimulation, subtract the IL-6 concentration measured in the “stim” tube from that of the “unstim” tube.

Results

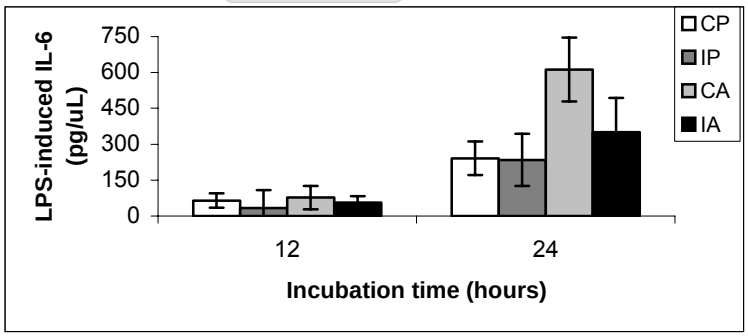
Results obtained indicated that the cells in the time-dose response assay may indeed have been oxygen starved, since values for spontaneous release of IL-6 was much lower in the control group (CP) in the latter assay (Figure 3).

Figure 3 Comparison between spontaneous IL-6 release by PBMCs in culture (i.e. in absence of LPS) in dose-time response assay (pooled control) vs. the actual study protocol (control placebo) to illustrate the effect of oxygen starvation on cell activation.



Furthermore, due to high inter-individual variation (Figure 4), incubation for 24 hours proved more optimal than 12 hours for our purposes. Therefore, only the results of the 24-hour culture are reported in Chapter 9.

Figure 4 Comparison of the mitogen-induced release of IL-6 *in vitro* after 12 and 24 hours of incubation.



References

1. Akinbami, M. A., Philip, G. H., Sridaran, R., Mahesh, V. B., & Mann, D. R. (1999). Expression of mRNA and proteins for testicular steroidogenic enzymes and brain and pituitary mRNA for glutamate receptors in rats exposed to immobilization stress. *J.Steroid Biochem.Mol.Biol.* **70**, 143-149.

2. Alarrayed, F., Hartman, A. D., & Porter, J. R. (1992). Is there a role for the adrenals in the development of hypercholesterolemia in Zucker fatty rats? *Am J Physiol* **263**, E287-E295.

3. Almeida, S. A., Anselmo-Franci, J. A., Rosa e Silva AA, & Carvalho, T. L. (1998). Chronic intermittent immobilization of male rats throughout sexual development: a stress protocol. *Exp.Physiol* **83**, 701-704.

4. Almeida, S. A., Petenusci, S. O., Franci, J. A., Rosa e Silva AA, & Carvalho, T. L. (2000). Chronic immobilization-induced stress increases plasma testosterone and delays testicular maturation in pubertal rats. *Andrologia* **32**, 7-11.

5. Alvarez, B., Quinn, L. S., Busquets, S., Quiles, M. T., Lopez-Soriano, F. J., & Argiles, J. M. (2002). Tumor necrosis factor-alpha exerts interleukin-6-dependent and - independent effects on cultured skeletal muscle cells. *Biochim.Biophys.Acta* **1542**, 66-72.

6. Anfossi, G., Massucco, P., Mattiello, L., Cavalot, F., Perna, P., Giori, A., Tassone, F., & Trovati, M. (1999). Modulation of human platelet function by L-canavanine: differential effects of low and high concentrations. *Gen.Pharmacol.* **32**, 321-328.

7. Angeli, A., Dovio, A., Sartori, M. L., Masera, R. G., Ceoloni, B., Prolo, P., Racca, S., & Chiappelli, F. (2002). Interactions between glucocorticoids and cytokines in the bone microenvironment. *Ann.N.Y.Acad.Sci.* **966**, 97-107.

8. Anker, S. D., Ponikowski, P., Varney, S., Chua, T. P., Clark, A. L., Webb-Peploe, K. M., Harrington, D., Kox, W. J., Poole-Wilson, P. A., & Coats, A. J. (1997). Wasting as independent risk factor for mortality in chronic heart failure. *Lancet* **349**, 1050-1053.

9. Anker, S. D. & Sharma, R. (2002). The syndrome of cardiac cachexia. *Int J Cardiol.* **85**, 51-66.

10. Arancibia, S., Rage, F., Grauges, P., Gomez, F., Tapia-Arancibia, L., & Armario, A. (2000). Rapid modifications of somatostatin neuron activity in the periventricular nucleus after acute stress. *Exp.Brain Res.* **134**, 261-267.

11. Armario, A., Gavalda, A., & Marti, J. (1995). Comparison of the behavioural and endocrine response to forced swimming stress in five inbred strains of rats. *Psychoneuroendocrinology* **20**, 879-890.

12. Arruda, J. L., Sweitzer, S., Rutkowski, M. D., & DeLeo, J. A. (2000). Intrathecal anti-IL-6 antibody and IgG attenuates peripheral nerve injury-induced mechanical allodynia in the rat: possible immune modulation in neuropathic pain. *Brain Res.* **879**, 216-225.

13. Baj, Z., Kantorski, J., Majewska, E., Zeman, K., Pokoca, L., Fornalczyk, E., Tchorzewski, H., Sulowska, Z., & Lewicki, R. (1994). Immunological status of competitive cyclists before and after the training season. *Int.J.Sports Med.* **15**, 319-324.

14. Barron, J. L., Noakes, T. D., Levy, W., Smith, C., & Millar, R. P. (1985). Hypothalamic dysfunction in overtrained athletes. *J Clin Endocrinol Metab* **60**, 803-806.

15. Bartalena, L., Hammond, G. L., Farsetti, A., Flink, I. L., & Robbins, J. (1993). Interleukin-6 inhibits corticosteroid-binding globulin synthesis by human hepatoblastoma-derived (Hep G2) cells. *Endocrinology* **133**, 291-296.

16. Baum, M., Muller-Steinhardt, M., Liesen, H., & Kirchner, H. (1997). Moderate and exhaustive endurance exercise influences the interferon- gamma levels in whole-blood culture supernatants. *Eur.J Appl.Physiol Occup.Physiol* **76**, 165-169.

17. Baumann, H., Jahreis, G. P., Sauder, D. N., & Koj, A. (1984). Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells from man, rat, and mouse. *J Biol Chem.* **259**, 7331-7342.

18. Benten, W. P., Lieberherr, M., Giese, G., Wrehlke, C., Stamm, O., Sekeris, C. E., Mossmann, H., & Wunderlich, F. (1999a). Functional testosterone receptors in plasma membranes of T cells. *FASEB J.* **13**, 123-133.

19. Benten, W. P., Lieberherr, M., Stamm, O., Wrehlke, C., Guo, Z., & Wunderlich, F. (1999b). Testosterone signaling through internalizable surface receptors in androgen receptor-free macrophages. *Mol.Biol.Cell* **10**, 3113-3123.

20. Berglund, B. & Hemmingsson, P. (1990). Infectious disease in elite cross-country skiers: a one-year incidence study. *Clinical Sports Medicine* **2**, 19-23.

21. Berk, L. S., Nieman, D. C., Youngberg, W. S., Arabatzis, K., Simpson-Westerberg, M., Lee, J. W., Tan, S. A., & Eby, W. C. (1990). The effect of long endurance running on natural killer cells in marathoners. *Med.Sci.Sports Exerc.* **22**, 207-212.

22. Berkenbosch, F., van Oers, J., del Rey, A., Tilders, F., & Besedovsky, H. (1987). Corticotropin-releasing factor-producing neurons in the rat activated by interleukin-1. *Science* **238**, 524-526.

23. Bester, C. C. (1991). Morphology. In *Fundementals of Medical Microbiology*, ed. Bester, C. C., pp. 13-42. Lindi Enterprises Publications, Cape Town.

24. Bhasin, S., Storer, T. W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., Bunnell, T. J., Tricker, R., Shirazi, A., & Casaburi, R. (1996). The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *N.Engl.J Med* **335**, 1-7.

25. Bhasin, S., Taylor, W. E., Singh, R., Artaza, J., Sinha-Hikim, I., Jasuja, R., Choi, H., & Gonzalez-Cadavid, N. F. (2003). The mechanisms of androgen effects on body composition: mesenchymal pluripotent cell as the target of androgen action. *J.Gerontol.A Biol.Sci.Med.Sci.* **58**, M1103-M1110.

26. Bhasin, S., Woodhouse, L., & Storer, T. W. (2001). Proof of the effect of testosterone on skeletal muscle. *J Endocrinol* **170**, 27-38.

27. Bierman, H. R., Kelly, K. H., Cordes, F. L., Petrakis, N. L., Kass, H., & Shpil, E. L. (1952). The influence of respiratory movements upon circulating leukocytes. *Blood* **7**, 533-534.

28. Billat, V. L., Flechet, B., Petit, B., Muriaux, G., & Koralsztein, J. P. (1999). Interval training at VO2max: effects on aerobic performance and overtraining markers. *Med.Sci.Sports Exerc.* **31**, 156-163.

29. Blannin, A. K., Chatwin, L. J., Cave, R., & Gleeson, M. (1996). Effects of submaximal cycling and long-term endurance training on neutrophil phagocytic activity in middle aged men. *Br.J.Sports Med.* **30**, 125-129.

30. Borrego, F., Robertson, M., Ritz, J., Peña, J., & Solana, R. (1999a). CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor. *Immunology* **97**, 159-165.

31. Borrego, F., Alonso, M. C., Galiani, M. D., Carracedo, J., Ramirez, R., Ostos, B., Pena, J., & Solana, R. (1999b). NK phenotypic markers and IL2 response in NK cells from elderly people. *Exp.Gerontol.* **34**, 253-265.

32. Bosenberg, A. T., Brock-Utne, J. G., Gaffin, S. L., Wells, M. T., & Blake, G. T. (1988). Strenuous exercise causes systemic endotoxemia. *J Appl.Physiol* **65**, 106-108.

33. Boucher, W. S., Letourneau, R., Huang, M., Kempuraj, D., Green, M., Sant, G. R., & Theoharides, T. C. (2002). Intravesical sodium hyaluronate inhibits the rat urinary mast cell mediator increase triggered by acute immobilization stress. *J.Urol.* **167**, 380-384.

34. Breuner, C. W. & Orchinik, M. (2002). Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J Endocrinol* **175**, 99-112.

35. Breytenbach, U., Clark, A., Lamprecht, J., & Bouic, P. (2001). Flow cytometric analysis of the Th1-Th2 balance in healthy individuals and patients infected with the human immunodeficiency virus (HIV) receiving a plant sterol/sterolin mixture. *Cell Biol.Int.* **25**, 43-49.

36. Brien, T. G. (1981). Human corticosteroid binding globulin. *Clin Endocrinol (Oxf)* **14**, 193-212.

37. Brown, R. L., Frederick, E., Falsetti, H., Burke ER, & Ryan, A. (1983). Overtraining of athletes: a round table. *The Physician and Sportsmedicine* **11**, 93-110.

38. Brümmerhoff, S. Some content substances of Sutherlandia microphylla Burch. 1969. University of the Free State, Bloemfontein, South Africa.
Ref Type: Thesis/Dissertation

39. Bruunsgaard, H., Galbo, H., Halkjaer-Kristensen, J., Johansen, T. L., MacLean, D. A., & Pedersen, B. K. (1997). Exercise-induced increase in serum interleukin-6 in humans is related to muscle damage. *J.Physiol* **499 (Pt 3)**, 833-841.

40. Buckley, C. D., Pilling, D., Lord, J. M., Akbar, A. N., Scheel-Toellner, D., & Salmon, M. (2001). Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol.* **22**, 199-204.

41. Butcher, E. C., Williams, M., Youngman, K., Rott, L., & Briskin, M. (1999). Lymphocyte trafficking and regional immunity. *Adv.Immunol.* **72**, 209-253.

42. Cannon, J. G., Evans, W. J., Hughes, V. A., Meredith, C. N., & Dinarello, C. A. (1986). Physiological mechanisms contributing to increased interleukin-1 secretion. *J.Appl.Physiol* **61**, 1869-1874.

43. Christeff, N., Nunez, E. A., & Gougeon, M. L. (2000). Changes in cortisol/DHEA ratio in HIV-infected men are related to immunological and metabolic perturbations leading to malnutrition and lipodystrophy. *Ann.N.Y.Acad Sci* **917**, 962-970.

44. Clerici, M., Sarin, A., Coffman, R. L., Wynn, T. A., Blatt, S. P., Hendrix, C. W., Wolf, S. F., Shearer, G. M., & Henkart, P. A. (1994). Type 1/type 2 cytokine modulation of T-cell programmed cell death as a model for human immunodeficiency virus pathogenesis. *Proc Natl Acad Sci U S A* **91**, 11811-11815.

45. Clerici, M. & Shearer, G. M. (1993). A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol.Today* **14**, 107-111.

46. Clerici, M. & Shearer, G. M. (1994). The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol.Today* **15**, 575-581.

47. Clerici, M., Trabattoni, D., Piconi, S., Fusi, M. L., Ruzzante, S., Clerici, C., & Villa, M. L. (1997). A possible role for the cortisol/anticortisols imbalance in the progression of human immunodeficiency virus. *Psychoneuroendocrinology* **22 Suppl 1**, S27-S31.

48. Coolens, J. L., Van Baelen, H., & Heyns, W. (1987). Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. *J.Steroid Biochem.* **26**, 197-202.

49. Costelli, P., Carbo, N., Tessitore, L., Bagby, G. J., Lopez-Soriano, F. J., Argiles, J. M., & Baccino, F. M. (1993). Tumor necrosis factor-alpha mediates changes in tissue protein turnover in a rat cancer cachexia model. *J Clin Invest* **92**, 2783-2789.

50. Crooks, P. & Rosenthal, G. Use of L-canavanine as a chemotherapeutic agent for the treatment of pancreatic cancer. [5,552,440]. 19940. United States.
Ref Type: Patent

51. Daynes, R. A. & Araneo, B. A. (1989). Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4. *Eur.J Immunol.* **19**, 2319-2325.

52. Daynes, R. A., Araneo, B. A., Dowell, T. A., Huang, K., & Dudley, D. (1990). Regulation of murine lymphokine production in vivo. III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function. *J Exp.Med* **171**, 979-996.

53. de Metz, J., Sprangers, F., Endert, E., Ackermans, M. T., ten Berge, I. J., Sauerwein, H. P., & Romijn, J. A. (1999). Interferon-gamma has immunomodulatory effects with minor endocrine and metabolic effects in humans. *J.Appl.Physiol* **86**, 517-522.

54. Deak, T., Nguyen, K. T., Cotter, C. S., Fleshner, M., Watkins, L. R., Maier, S. F., & Spencer, R. L. (1999). Long-term changes in mineralocorticoid and glucocorticoid receptor occupancy following exposure to an acute stressor. *Brain Res.* **847**, 211-220.

55. Delany, A. M., Gabbitas, B. Y., & Canalis, E. (1995). Cortisol downregulates osteoblast alpha 1 (I) procollagen mRNA by transcriptional and posttranscriptional mechanisms. *J.Cell Biochem.* **57**, 488-494.

56. Dellwo, M. & Beauchene, R. E. (1990). The effect of exercise, diet restriction, and aging on the pituitary-- adrenal axis in the rat. *Exp.Gerontol.* **25**, 553-562.

57. DeRijk, R. H., Petrides, J., Deuster, P., Gold, P. W., & Sternberg, E. M. (1996). Changes in corticosteroid sensitivity of peripheral blood lymphocytes after strenuous exercise in humans. *Journal of Clinical Endocrinology and Metabolism* **81(1)**, 228-235.

58. Deuster, P. A., Zelazowska, E. B., Singh, A., & Sternberg, E. M. (1999). Expression of lymphocyte subsets after exercise and dexamethasone in high and low stress responders. *Med Sci.Sports Exerc* **31**, 1799-1806.

59. Devenport, L., Doughty, D., Heiberger, B., Burton, D., Brown, R., & Stith, R. (1993). Exercise endurance in rats: roles of type I and II corticosteroid receptors. *Physiol Behav.* **53**, 1171-1175.

60. Devenport, L., Knehans, A., Thomas, T., & Sundstrom, A. (1991). Macronutrient intake and utilization by rats: interactions with type I adrenocorticoid receptor stimulation. *Am J Physiol* **260**, R73-R81.

61. Dhillo, W. S., Kong, W. M., Le Roux, C. W., Alaghband-Zadeh, J., Jones, J., Carter, G., Mendoza, N., Meeran, K., & O'Shea, D. (2002). Cortisol-binding globulin is important in the interpretation of dynamic tests of the hypothalamic--pituitary--adrenal axis. *Eur.J Endocrinol* **146**, 231-235.

62. Di Somma, C., Pivonello, R., Loche, S., Faggiano, A., Klain, M., Salvatore, M., Lombardi, G., & Colao, A. (2003). Effect of 2 years of cortisol normalization on the impaired bone mass and turnover in adolescent and adult patients with Cushing's disease: a prospective study. *Clin.Endocrinol.(Oxf)* **58**, 302-308.

63. Doerr, P. & Pirke, K. M. (1976). Cortisol-induced suppression of plasma testosterone in normal adult males. *J Clin Endocrinol Metab* **43**, 622-629.

64. Dressendorfer, R. H., Petersen, S. R., Moss Lovshin, S. E., Hannon, J. L., Lee, S. F., & Bell, G. J. (2002). Performance enhancement with maintenance of resting immune status after intensified cycle training. *Clin.J.Sport Med.* **12**, 301-307.

65. Dronjak, S., Gavrilovic, L., Filipovic, D., & Radojcic, M. B. (2004). Immobilization and cold stress affect sympatho-adrenomedullary system and pituitary-adrenocortical axis of rats exposed to long-term isolation and crowding. *Physiol Behav.* **81**, 409-415.

66. Duclos, M., Martin, C., Malgat, M., Mazat, J. P., Chaouloff, F., Mormede, P., & Letellier, T. (2001). Relationships between muscle mitochondrial metabolism and stress- induced corticosterone variations in rats. *Pflugers Arch.* **443**, 218-226.

67. Duclos, M., Minkhar, M., Sarrieau, A., Bonnemaison, D., Manier, G., & Mormede, P. (1999). Reversibility of endurance training-induced changes on glucocorticoid sensitivity of monocytes by an acute exercise. *Clin Endocrinol (Oxf)* **51**, 749-756.

68. Eichner, E. R. (1995). Overtraining: consequences and prevention. *J.Sports Sci.* **13 Spec No**, S41-S48.

69. Ellegala, D. B., Alden, T. D., Couture, D. E., Vance, M. L., Maartens, N. F., & Laws, E. R., Jr. (2003). Anemia, testosterone, and pituitary adenoma in men. *J.Neurosurg.* **98**, 974-977.

70. Espersen, G. T., Elbaek, A., Ernst, E., Toft, E., Kaalund, S., Jersild, C., & Grunnet, N. (1990). Effect of physical exercise on cytokines and lymphocyte subpopulations in human peripheral blood. *APMIS* **98**, 395-400.

71. Evans, W. J., Meredith, C. N., Cannon, J. G., Dinarello, C. A., Frontera, W. R., Hughes, V. A., Jones, B. H., & Knuttgen, H. G. (1986). Metabolic changes following eccentric exercise in trained and untrained men. *J.Appl.Physiol* **61**, 1864-1868.

72. Fantuzzi, G., Di Santo, E., Sacco, S., Benigni, F., & Ghezzi, P. (1995). Role of the hypothalamus-pituitary-adrenal axis in the regulation of TNF production in mice. Effect of stress and inhibition of endogenous glucocorticoids. *J Immunol.* **155**, 3552-3555.

73. Feldman, D., Mondon, C. E., Horner, J. A., & Weiser, J. N. (1979). Glucocorticoid and estrogen regulation of corticosteroid-binding globulin production by rat liver. *Am.J.Physiol* **237**, E493-E499.

74. Ferrando, A. A., Sheffield-Moore, M., Wolf, S. E., Herndon, D. N., & Wolfe, R. R. (2001). Testosterone administration in severe burns ameliorates muscle catabolism. *Crit Care Med.* **29**, 1936-1942.

75. Filaire, E., Duche, P., & Lac, G. (1998). Effects of amount of training on the saliva concentrations of cortisol, dehydroepiandrosterone and on the dehydroepiandrosterone: cortisol concentration ratio in women over 16 weeks of training. *Eur.J Appl.Physiol Occup.Physiol* **78**, 466-471.

76. Filaire, E. & Lac, G. (2000). Dehydroepiandrosterone (DHEA) rather than testosterone shows saliva androgen responses to exercise in elite female handball players. *Int.J.Sports Med.* **21**, 17-20.

77. Fleshner, M., Bellgrau, D., Watkins, L. R., Laudenslager, M. L., & Maier, S. F. (1995a). Stress-induced reduction in the rat mixed lymphocyte reaction is due to macrophages and not to changes in T cell phenotypes. *J.Neuroimmunol.* **56**, 45-52.

78. Fleshner, M., Deak, T., Spencer, R. L., Laudenslager, M. L., Watkins, L. R., & Maier, S. F. (1995b). A long-term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. *Endocrinology* **136**, 5336-5342.

79. Flynn, M. G., Pizza, F. X., & Brolinson, P. G. (1997). Hormonal responses to excessive training: influence of cross training. *Int.J.Sports Med.* **18**, 191-196.

80. Foster, N. K., Martyn, J. B., Rangno, R. E., Hogg, J. C., & Pardy, R. L. (1986). Leukocytosis of exercise: role of cardiac output and catecholamines. *J Appl.Physiol* **61**, 2218-2223.

81. Fry, A. C., Kraemer, W. J., & Ramsey, L. T. (1998). Pituitary-adrenal-gonadal responses to high-intensity resistance exercise overtraining. *J Appl.Physiol* **85**, 2352-2359.

82. Fry, R. W., Morton, A. R., Garcia-Webb, P., & Keast, D. (1991a). Monitoring exercise stress by changes in metabolic and hormonal responses over a 24-h period. *Eur.J.Appl.Physiol Occup.Physiol* **63**, 228-234.

83. Fry, R. W., Morton, A. R., & Keast, D. (1991b). Overtraining in athletes. An update. *Sports Med* **12**, 32-65.

84. Fry, R. W., Morton, A. R., & Keast, D. (1992). Acute intensive interval training and T-lymphocyte function. *Med.Sci.Sports Exerc.* **24**, 339-345.

85. Fujisawa, H., Wang, B., Sauder, D. N., & Kondo, S. (1997). Effects of interferons on the production of interleukin-6 and interleukin-8 in human keratinocytes. *J Interferon Cytokine Res.* **17**, 347-353.

86. Fukaura, H., Kent, S. C., Pietrusewicz, M. J., Khoury, S. J., Weiner, H. L., & Hafler, D. A. (1996). Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J.Clin.Invest* **98**, 70-77.

87. Gabriel, H., Schmitt, B., Urhausen, A., & Kindermann, W. (1993). Increased CD45RA+CD45RO+ cells indicate activated T cells after endurance exercise. *Med Sci Sports Exerc* **25**, 1352-1357.

88. Gabriel, H. H., Urhausen, A., Valet, G., Heidelbach, U., & Kindermann, W. (1998). Overtraining and immune system: a prospective longitudinal study in endurance athletes. *Med.Sci.Sports Exerc.* **30**, 1151-1157.

89. Gallagher, P. & Young, A. (2002). Cortisol/DHEA ratios in depression. *Neuropsychopharmacology* **26**, 410.

90. Garcia, A., Marti, O., Valles, A., Dal Zotto, S., & Armario, A. (2000). Recovery of the hypothalamic-pituitary-adrenal response to stress. Effect of stress intensity, stress duration and previous stress exposure. *Neuroendocrinology* **72**, 114-125.

91. Genuth, S. M. (1983a). The adrenal glands. In *Physiology*, ed. Farrell, R., pp. 949-979. Mosby-Year Book Inc., St. Louis, Missouri.

92. Genuth, S. M. (1983b). The hypothalamus and pituitary gland. In *Phyisology*, ed. Farrell, R., pp. 897-931. Mosby-Year Book, Inc., St. Louis, Missouri.

93. Gerra, G., Zaimovic, A., Mascetti, G. G., Gardini, S., Zambelli, U., Timpano, M., Raggi, M. A., & Brambilla, F. (2001). Neuroendocrine responses to experimentally-induced psychological stress in healthy humans. *Psychoneuroendocrinology* **26**, 91-107.

94. Gleeson, M. (2000). Mucosal immune responses and risk of respiratory illness in elite athletes. *Exerc Immunol.Rev.* **6**, 5-42.

95. Gleeson, M., Blannin, A. K., Sewell, D. A., & Cave, R. (1995). Short-term changes in the blood leucocyte and platelet count following different durations of high-intensity treadmill running. *J.Sports Sci.* **13**, 115-123.

96. Gleeson, M., McDonald, W. A., Pyne, D. B., Clancy, R. L., Cripps, A. W., Francis, J. L., & Fricker, P. A. (2000). Immune status and respiratory illness for elite swimmers during a 12-week training cycle. *Int.J.Sports Med.* **21**, 302-307.

97. Gleeson, M., Walsh, N. P., Blannin, A. K., Robson, P. J., Cook, L., Donnelly, A. E., & Day, S. H. (1998). The effect of severe eccentric exercise-induced muscle damage on plasma elastase, glutamine and zinc concentrations. *Eur.J.Appl.Physiol Occup.Physiol* **77**, 543-546.

98. Goldstein, D. S. & McEwen, B. (2002). Allostasis, homeostats, and the nature of stress. *Stress.* **5**, 55-58.

99. Gore, D. C., Jahoor, F., Wolfe, R. R., & Herndon, D. N. (1993). Acute response of human muscle protein to catabolic hormones. *Ann.Surg.* **218**, 679-684.

100. Grasso, G., Lodi, L., Lupo, C., & Muscettola, M. (1997). Glucocorticoid receptors in human peripheral blood mononuclear cells in relation to age and to sport activity. *Life Sci* **61**, 301-308.

101. Green, K. J., Rowbottom, D. G., & Mackinnon, L. T. (2003). Acute Exercise and T-Lymphocyte Expression of the Early Activation Marker CD69. *Med.Sci.Sports Exerc.* **35**, 582-588.

102. Green, M. H. Method of treating viral infections with amino acid analogs. [5,110,600]. 1988. United States Patent.
Ref Type: Patent

103. Grinspoon, S., Corcoran, C., Stanley, T., Rabe, J., & Wilkie, S. (2001). Mechanisms of androgen deficiency in human immunodeficiency virus- infected women with the wasting syndrome. *J Clin Endocrinol Metab* **86**, 4120-4126.

104. Hackney, A. C. (1996). The male reproductive system and endurance exercise. *Med Sci Sports Exerc* **28**, 180-189.

105. Hakkinen, K., Pakarinen, A., Alen, M., Kauhanen, H., & Komi, P. V. (1987). Relationships between training volume, physical performance capacity, and serum hormone concentrations during prolonged training in elite weight lifters. *Int J Sports Med* **8 Suppl 1**, 61-65.

106. Halson, S. L., Bridge, M. W., Meeusen, R., Busschaert, B., Gleeson, M., Jones, D. A., & Jeukendrup, A. E. (2002). Time course of performance changes and fatigue markers during intensified training in trained cyclists. *J.Appl.Physiol* **93**, 947-956.

107. Halson, S. L., Lancaster, G. I., Jeukendrup, A. E., & Gleeson, M. (2003). Immunological responses to overreaching in cyclists. *Med.Sci.Sports Exerc.* **35**, 854-861.

108. Hammarqvist, F., Ejesson, B., & Wernerman, J. (2001). Stress hormones initiate prolonged changes in the muscle amino acid pattern. *Clin Physiol* **21**, 44-50.

109. Hammond, G. L., Smith, C. L., Goping, I. S., Underhill, D. A., Harley, M. J., Reventos, J., Musto, N. A., Gunsalus, G. L., & Bardin, C. W. (1987). Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. *Proc.Natl.Acad.Sci.U.S.A* **84**, 5153-5157.

110. Hammond, G. L., Smith, C. L., Paterson, N. A., & Sibbald, W. J. (1990). A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. *J.Clin.Endocrinol.Metab* **71**, 34-39.

111. Hannet, I., Erkeller-Yuksel, F., Lydyard, P., Deneys, V., & DeBruyere, M. (1992). Developmental and maturational changes in human blood lymphocyte subpopulations. *Immunol.Today* **13**, 215, 218.

112. Hartmann, U. & Mester, J. (2000). Training and overtraining markers in selected sport events. *Med.Sci.Sports Exerc.* **32**, 209-215.

113. Hässig, A., Wen-Xi, L., & Stampfli, K. (1996). Stress-induced suppression of the cellular immune reactions: on the neuroendocrine control of the immune system. *Medical Hypothesis* **46**, 551-555.

114. Heath, G. W., Macera, C. A., & Nieman, D. C. (1992). Exercise and upper respiratory tract infections. Is there a relationship? *Sports Med.* **14**, 353-365.

115. Hechter, O., Grossman, A., & Chatterton, R. T., Jr. (1997). Relationship of dehydroepiandrosterone and cortisol in disease. *Med Hypotheses* **49**, 85-91.

116. Heiman, M. L., Ahima, R. S., Craft, L. S., Schoner, B., Stephens, T. W., & Flier, J. S. (1997). Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology* **138**, 3859-3863.

117. Hennebold, J. D. & Daynes, R. A. (1994). Regulation of macrophage dehydroepiandrosterone sulfate metabolism by inflammatory cytokines. *Endocrinology* **135**, 67-75.

118. Hoffbrand, A. F. & Pettit, J. E. (1994a). The white cells 1: Granulocytes, monocytes and their benign disorders. In *Essential Haematology* pp. 141-160. Blackwell Scientific Publications, Cambridge, MA.

119. Hoffbrand, A. F. & Pettit, J. E. (1994b). The white cells 2: Lymphocytes and their benign disorders. In *Essential Haematology* pp. 161-185. Blackwell Scientific Publications, Cambridge, MA.

120. Hoffman-Goetz, L., Keir, R., Thorne, R., Houston, M. E., & Young, C. (1986). Chronic exercise stress in mice depresses splenic T lymphocyte mitogenesis in vitro. *Clin Exp.Immunol.* **66**, 551-557.

121. Hoffman-Goetz, L., Simpson, J. R., Cipp, N., Arumugam, Y., & Houston, M. E. (1990). Lymphocyte subset responses to repeated submaximal exercise in men. *J.Appl.Physiol* **68**, 1069-1074.

122. Hogan, D., Morrow, J. D., Smith, E. M., & Opp, M. R. (2003). Interleukin-6 alters sleep of rats. *J.Neuroimmunol.* **137**, 59-66.

123. Hoogeveen, A. R. & Zonderland, M. L. (1996). Relationships between testosterone, cortisol and performance in professional cyclists. *Int J Sports Med* **17**, 423-428.

124. Hooper, S. L., Mackinnon, L. T., Gordon, R. D., & Bachmann, A. W. (1993). Hormonal responses of elite swimmers to overtraining. *Med.Sci.Sports Exerc.* **25**, 741-747.

125. Hu, Y., Cardounel, A., Gursoy, E., Anderson, P., & Kalimi, M. (2000). Anti-stress effects of dehydroepiandrosterone: protection of rats against repeated immobilization stress-induced weight loss, glucocorticoid receptor production, and lipid peroxidation. *Biochem Pharmacol.* **59**, 753-762.

126. Hurwitz, E. L. & Morgenstern, H. (2001). Immediate and longterm effects of immune stimulation: hypothesis linking the immune response to subsequent physical and psychological wellbeing. *Med.Hypotheses* **56**, 620-624.

127. Ibfelt, T., Petersen, E. W., Bruunsgaard, H., Sandmand, M., & Pedersen, B. K. (2002). Exercise-induced change in type 1 cytokine-producing CD8(+) T cells is related to a decrease in memory T cells. *J Appl.Physiol* **93**, 645-648.

128. Ishikawa, I., Kitamura, H., Kimura, K., & Saito, M. (2001). Brain interleukin-1 is involved in blood interleukin-6 response to immobilization stress in rats. *Jpn.J.Vet.Res.* **49**, 19-25.

129. Jeukendrup, A. E., Vet-Joop, K., Sturk, A., Stegen, J. H. J. C., Senden, J., Saris, W. H. M., & Wagenmakers, J. M. (2000). Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase response reaction during and after a long-distance triathlon in highly trained men. *Clinical Science* **98**, 47-55.

130. Jodar, E., Valdepenas, M. P., Martinez, G., Jara, A., & Hawkins, F. (2003). Long-term follow-up of bone mineral density in Addison's disease. *Clin.Endocrinol.(Oxf)* **58**, 617-620.

131. Jonsdottir, I. H., Schjerling, P., Ostrowski, K., Asp, S., Richter, E. A., & Pedersen, B. K. (2000). Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles. *J.Physiol* **528 Pt 1**, 157-163.

132. Jordan, M., Otterness, I. G., Ng, R., Gessner, A., Rollinghoff, M., & Beuscher, H. U. (1995). Neutralization of endogenous IL-6 suppresses induction of IL-1 receptor antagonist. *J.Immunol.* **154**, 4081-4090.

133. Kahn, C. R., Goldfine, I. D., Neville, D. M., Jr., & De Meyts, P. (1978). Alterations in insulin binding induced by changes in vivo in the levels of glucocorticoids and growth hormone. *Endocrinology* **103**, 1054-1066.

134. Kajiura, J. S., MacDougall, J. D., Ernst, P. B., & Younglai, E. V. (1995). Immune response to changes in training intensity and volume in runners. *Med Sci Sports Exerc* **27**, 1111-1117.

135. Keizer, H. A., Kuipers, H., de Haan, J., Janssen, G. M., Beckers, E., Habets, L., van Kranenburg, G., & Geurten, P. (1987). Effect of a 3-month endurance training program on metabolic and multiple hormonal responses to exercise. *Int.J.Sports Med.* **8 Suppl 3**, 154-160.

136. Kellendonk, C., Gass, P., Kretz, O., Schutz, G., & Tronche, F. (2002). Corticosteroid receptors in the brain: gene targeting studies. *Brain Res.Bull.* **57**, 73-83.

137. Ketner, J. & Mellion, M. (1995). The overtraining syndrome: a review of presentation, pathophysiology, and treatment. *Med Exerc Nutr Health* **4**, 136-145.

138. Kiecolt-Glaser, J. K., Preacher, K. J., MacCallum, R. C., Atkinson, C., Malarkey, W. B., & Glaser, R. (2003). Chronic stress and age-related increases in the proinflammatory cytokine IL-6. *Proc.Natl.Acad.Sci.U.S.A* **100**, 9090-9095.

139. Kohut, M. L., Boehm, G. W., & Moynihan, J. A. (2001). Prolonged exercise suppresses antigen-specific cytokine response to upper respiratory infection. *J.Appl.Physiol* **90**, 678-684.

140. Kostic, T. S., Andric, S. A., Maric, D., & Kovacevic, R. Z. (2000). Inhibitory effects of stress-activated nitric oxide on antioxidant enzymes and testicular steroidogenesis. *J.Steroid Biochem.Mol.Biol.* **75**, 299-306.

141. Kotler, D. P. (1994). Wasting syndrome: nutritional support in HIV infection. *AIDS Res.Hum.Retroviruses* **10**, 931-934.

142. Kotler, D. P., Tierney, A. R., Wang, J., & Pierson, R. N., Jr. (1989). Magnitude of body-cell-mass depletion and the timing of death from wasting in AIDS. *Am J Clin Nutr* **50**, 444-447.

143. Kraemer, W. J., Fleck, S. J., Callister, R., Shealy, M., Dudley, G. A., Maresh, C. M., Marchitelli, L., Cruthirds, C., Murray, T., & Falkel, J. E. (1989). Training responses of plasma beta-endorphin, adrenocorticotropin, and cortisol. *Med Sci Sports Exerc* **21**, 146-153.

144. Krebs, C. J. (1999). Repeatability. In *Ecological Methodology* pp. 554-558. Benjamin-Cummings, Menlo Park.

145. Kreutz, M., Ackermann, U., Hauschildt, S., Krause, S. W., Riedel, D., Bessler, W., & Andreesen, R. (1997). A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and Staphyloccous aureus in human monocytes. *Immunology* **92**, 396-401.

146. Krowka, J. F., Cuevas, B., Maron, D. C., Steimer, K. S., Ascher, M. S., & Sheppard, H. W. (1996). Expression of CD69 after in vitro stimulation: a rapid method for quantitating impaired lymphocyte responses in HIV-infected individuals. *J Acquir.Immune.Defic.Syndr.Hum.Retrovirol.* **11**, 95-104.

147. Kuipers, H. & Keizer, H. A. (1988). Overtraining in elite athletes. Review and directions for the future. *Sports Med* **6**, 79-92.

148. Kunz-Ebrecht, S. R., Mohamed-Ali, V., Feldman, P. J., Kirschbaum, C., & Steptoe, A. (2003). Cortisol responses to mild psychological stress are inversely associated with proinflammatory cytokines. *Brain Behav.Immun.* **17**, 373-383.

149. Kuoppasalmi, K. (1980). Plasma testosterone and sex-hormone-binding globulin capacity in physical exercise. *Scand J Clin Lab Invest* **40**, 411-418.

150. Kuoppasalmi, K., Naveri, H., Harkonen, M., & Adlercreutz, H. (1980). Plasma cortisol, androstenedione, testosterone and luteinizing hormone in running exercise of different intensities. *Scand J Clin Lab Invest* **40**, 403-409.

151. Lacey, K., Zaharia, M. D., Griffiths, J., Ravindran, A. V., Merali, Z., & Anisman, H. (2000). A prospective study of neuroendocrine and immune alterations associated with the stress of an oral academic examination among graduate students. *Psychoneuroendocrinology* **25**, 339-356.

152. Lehmann, M., Foster, C., Dickhuth, H. H., & Gastmann, U. (1998). Autonomic imbalance hypothesis and overtraining syndrome. *Med Sci.Sports Exerc* **30**, 1140-1145.

153. Lenczowski, M. J., Van Dam, A. M., Poole, S., Larrick, J. W., & Tilders, F. J. (1997). Role of circulating endotoxin and interleukin-6 in the ACTH and corticosterone response to intraperitoneal LPS. *Am.J.Physiol* **273**, R1870-R1877.

154. Lessells, C. M. & Boag, P. T. (1987). Unrepeatable repeatabilities: a common mistake. *The Auk* **104**, 116-121.

155. Levy, B., Valtier, M., de Chillou, C., Bollaert, P. E., Cane, D., & Mallie, J. P. (1999). Beneficial effects of L-canavanine, a selective inhibitor of inducible nitric oxide synthase, on lactate metabolism and muscle high energy phosphates during endotoxic shock in rats. *Shock* **11**, 98-103.

156. Lewis, D. E. & Rickman, W. J. (1992). Methodology and quality control for flow cytometry: cell phenotyping by flow cytometry. In *Manual of Clinical Laboratory Immunology* pp. 164-173. American Society for Microbiology, Washington, DC.

157. Li, K. S., Liege, S., Moze, E., & Neveu, P. J. (2000). Plasma corticosterone and immune reactivity in restrained female C3H mice. *Stress.* **3**, 285-298.

158. Li, S., Cong, B., Yan, Y., Yao, Y., Ma, C., & Ling, Y. (2002). Cholecystokinin octapeptide inhibits the in vitro expression of CD14 in rat pulmonary interstitial macrophages induced by lipopolysaccharide. *Chin Med.J.(Engl.)* **115**, 276-279.

159. Lindsay, F. H., Hawley, J. A., Myburgh, K. H., Schomer, H. H., Noakes, T. D., & Dennis, S. C. (1996). Improved athletic performance in highly trained cyclists after interval training. *Med.Sci.Sports Exerc.* **28**, 1427-1434.

160. Llera, A. S., Viedma, F., Sanchez-Madrid, F., & Tormo, J. (2001). Crystal structure of the C-type lectin-like domain from the human hematopoietic cell receptor CD69. *J.Biol.Chem.* **276**, 7312-7319.

161. Llovera, M., Lopez-Soriano, F. J., & Argiles, J. M. (1993). Effects of tumor necrosis factor-alpha on muscle-protein turnover in female Wistar rats. *J Natl Cancer Inst.* **85**, 1334-1339.

162. Lund, T. D., Munson, D. J., Haldy, M. E., & Handa, R. J. (2004). Dihydrotestosterone may inhibit hypothalamo-pituitary-adrenal activity by acting through estrogen receptor in the male mouse. *Neurosci.Lett.* **365**, 43-47.

163. Mackinnon, L. T. (1992). *Exercise and Immunology*, 2 ed. Human Kinetics, Champaign.

164. Mackinnon, L. T. (1999). *Advances in exercise immunology* Human Kinetics, Champaign.

165. Madrigal, J. L., Hurtado, O., Moro, M. A., Lizasoain, I., Lorenzo, P., Castrillo, A., Bosca, L., & Leza, J. C. (2002). The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. *Neuropsychopharmacology* **26**, 155-163.

166. Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., Bosmans, E., De, M., I, Benoy, I., Neels, H., Demedts, P., Janca, A., Scharpe, S., & Smith, R. S. (1998). The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. *Cytokine* **10**, 313-318.

167. Maino, V. C., Suni, M. A., & Ruitenberg, J. J. (1995). Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry* **20**, 127-133.

168. Malkin, C. J., Pugh, P. J., Jones, R. D., Jones, T. H., & Channer, K. S. (2003). Testosterone as a protective factor against atherosclerosis--immunomodulation and influence upon plaque development and stability. *J.Endocrinol.* **178**, 373-380.

169. Malm, C. (2002). Exercise Immunology: a skeletal muscle perspective. *Exercise Immunology Review* **8**, 116-167.

170. 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* **529 Pt 1**, 243-262.

171. Marby, D., Lockhart, G. R., Raymond, R., & Linakis, J. G. (2001). Anti-interleukin-6 antibodies attenuate inflammation in a rat meningitis model. *Acad.Emerg.Med.* **8**, 946-949.

172. Mardiney, M., III, Brown, M. R., & Fleisher, T. A. (1996). Measurement of T-cell CD69 expression: a rapid and efficient means to assess mitogen- or antigen-induced proliferative capacity in normals. *Cytometry* **26**, 305-310.

173. Marti, O., Garcia, A., Velles, A., Harbuz, M. S., & Armario, A. (2001). Evidence that a single exposure to aversive stimuli triggers long- lasting effects in the hypothalamus-pituitary-adrenal axis that consolidate with time. *Eur.J Neurosci.* **13**, 129-136.

174. Mastronardi, C. A., Yu, W. H., & McCann, S. M. (2001). Comparisons of the effects of anesthesia and stress on release of tumor necrosis factor-alpha, leptin, and nitric oxide in adult male rats. *Exp.Biol Med (Maywood.)* **226**, 296-300.

175. Matalka, K. (2003). Neuroendocrine and cytokine-induced responses to minutes, hours, and days of mental stress. *Neuroendocrinology Letters* **24**, 285-294.

176. Matthys, P., Mitera, T., Heremans, H., Van Damme, J., & Billiau, A. (1995). Anti-gamma interferon and anti-interleukin-6 antibodies affect staphylococcal enterotoxin B-induced weight loss, hypoglycemia, and cytokine release in D-galactosamine-sensitized and unsensitized mice. *Infect.Immun.* **63**, 1158-1164.

177. May, L. T., Ghrayeb, J., Santhanam, U., Tatter, S. B., Sthoeger, Z., Helfgott, D. C., Chiorazzi, N., Grieninger, G., & Sehgal, P. B. (1988). Synthesis and secretion of multiple forms of beta 2-interferon/B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes. *J Biol Chem.* **263**, 7760-7766.

178. May, L. T., Torcia, G., Cozzolino, F., Ray, A., Tatter, S. B., Santhanam, U., Sehgal, P. B., & Stern, D. (1989). Interleukin-6 gene expression in human endothelial cells: RNA start sites, multiple IL-6 proteins and inhibition of proliferation. *Biochem Biophys.Res.Commun.* **159**, 991-998.

179. Mazzeo, R. S., Rajkumar, C., Rolland, J., Blaher, B., Jennings, G., & Esler, M. (1998). Immune response to a single bout of exercise in young and elderly subjects. *Mech.Ageing Dev.* **100**, 121-132.

180. McCarthy, D. A. & Dale, M. M. (1988). The leucocytosis of exercise. A review and model. *Sports Med* **6**, 333-363.

181. McEwen, B. S. (2002). Sex, stress and the hippocampus: allostasis, allostatic load and the aging process. *Neurobiol.Aging* **23**, 921-939.

182. McEwen, B. S. & Seeman, T. (1999). Protective and damaging effects of mediators of stress. Elaborating and testing the concepts of allostasis and allostatic load. *Ann.N.Y.Acad.Sci.* **896**, 30-47.

183. Meyer, T., Gabriel, H. H., Ratz, M., Muller, H. J., & Kindermann, W. (2001). Anaerobic exercise induces moderate acute phase response. *Med.Sci.Sports Exerc.* **33**, 549-555.

184. Miller, A. H., Spencer, R. L., Pearce, B. D., Pisell, T. L., Azrieli, Y., Tanapat, P., Moday, H., Rhee, R., & McEwen, B. S. (1998). Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. *Cell Immunol.* **186**, 45-54.

185. Miller, G. E., Cohen, S., & Ritchey, A. K. (2002). Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. *Health Psychol.* **21**, 531-541.

186. Misao, R., Hori, M., Ichigo, S., Fujimoto, J., & Tamaya, T. (1994). Corticosteroid-binding globulin mRNA levels in human uterine endometrium. *Steroids* **59**, 603-607.

187. Misra, M. & Klibanski, A. (2002). Evaluation and treatment of low bone density in anorexia nervosa. *Nutr.Clin.Care* **5**, 298-308.

188. Mohan, P. F. & Cleary, M. P. (1988). Effect of short-term DHEA administration on liver metabolism of lean and obese rats. *Am J Physiol* **255**, E1-E8.

189. Moldoveanu, A. I., Shephard, R. J., & Shek, P. N. (2000). Exercise elevates plasma levels but not gene expression of IL-1beta, IL- 6, and TNF-alpha in blood mononuclear cells. *J.Appl.Physiol* **89**, 1499-1504.

190. Morgan, W. P., O'Connor, P. J., Sparling, P. B., & Pate, R. R. (1987). Psychological characterization of the elite female distance runner. *Int.J.Sports Med.* **8 Suppl 2**, 124-131.

191. Moshe, D. A biosystematic study of the genus Sutherlandia Br. R. (Fabaceae:Galegeae). 1999. Rand Afrikaans University, Auckland Park, South Africa.
Ref Type: Thesis/Dissertation

192. Mosmann, T. R. & Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu.Rev.Immunol.* **7**, 145-173.

193. Mujika, I., Goya, A., Ruiz, E., Grijalba, A., Santisteban, J., & Padilla, S. (2002). Physiological and performance responses to a 6-day taper in middle-distance runners: influence of training frequency. *Int.J.Sports Med.* **23**, 367-373.

194. Myburgh, K. H., Viljoen, A., & Tereblanche, S. (2001). Plasma lactate concentrations for self-selected maximal effort lasting 1 h. *Med.Sci.Sports Exerc.* **33**, 152-156.

195. Natale, V. M., Brenner, I. K., Moldoveanu, A. I., Vasiliou, P., Shek, P., & Shephard, R. J. (2003). Effects of three different types of exercise on blood leukocyte count during and following exercise. *Sao Paulo Med.J.* **121**, 9-14.

196. Nehlsen-Cannarella, S. L., Nieman, D. C., Balk-Lamberton, A. J., Markoff, P. A., Chritton, D. B., Gusewitch, G., & Lee, J. W. (1991a). The effects of moderate exercise training on immune response. *Med.Sci.Sports Exerc.* **23**, 64-70.

197. Nehlsen-Cannarella, S. L., Nieman, D. C., Fagoaga, O. R., Kelln, W. J., Henson, D. A., Shannon, M., & Davis, J. M. (2000). Saliva immunoglobulins in elite women rowers. *Eur.J.Appl.Physiol* **81**, 222-228.

198. Nehlsen-Cannarella, S. L., Nieman, D. C., Jessen, J., Chang, L., Gusewitch, G., Blix, G. G., & Ashley, E. (1991b). The effects of acute moderate exercise on lymphocyte function and serum immunoglobulin levels. *Int.J.Sports Med.* **12**, 391-398.

199. Newsholme, E. A. (1994). Biochemical mechanisms to explain immunosuppression in well-trained and overtrained athletes. *Int.J.Sports Med.* **15 Suppl 3**, S142-S147.

200. Newsholme, E. A., Calder, P., & Yaqoob, P. (1993). The regulatory, informational, and immunomodulatory roles of fat fuels. *Am.J.Clin.Nutr.* **57**, 738S-750S.

201. Nieman, D. C. (1994). Exercise, upper respiratory tract infection, and the immune system. *Med.Sci.Sports Exerc.* **26**, 128-139.

202. Nieman, D. C. (1995). Upper respiratory tract infections and exercise. *Thorax* **50**, 1229-1231.

203. Nieman, D. C. (2000). Is infection risk linked to exercise workload? *Med.Sci.Sports Exerc.* **32**, S406-S411.

204. Nieman, D. C., Berk, L. S., Simpson-Westerberg, M., Arabatzis, K., Youngberg, S., Tan, S. A., Lee, J. W., & Eby, W. C. (1989a). Effects of long-endurance running on immune system parameters and lymphocyte function in experienced marathoners. *Int.J.Sports Med.* **10**, 317-323.

205. Nieman, D. C., Johanssen, L. M., & Lee, J. W. (1989b). Infectious episodes in runners before and after a roadrace. *J.Sports Med.Phys.Fitness* **29**, 289-296.

206. Nieman, D. C., Miller, A. R., Henson, D. A., Warren, B. J., Gusewitch, G., Johnson, R. L., Davis, J. M., Butterworth, D. E., Herring, J. L., & Nehlsen-Cannarella, S. L. (1994). Effect of high- versus moderate-intensity exercise on lymphocyte subpopulations and proliferative response. *Int.J.Sports Med* **15**, 199-206.

207. Nieman, D. C., Miller, A. R., Henson, D. A., Warren, B. J., Gusewitch, G., Johnson, R. L., Davis, J. M., Butterworth, D. E., & Nehlsen-Cannarella, S. L. (1993). Effects of high- vs moderate-intensity exercise on natural killer cell activity. *Med.Sci.Sports Exerc.* **25**, 1126-1134.

208. Nieman, D. C., Nehlsen-Cannarella, S. L., Markoff, P. A., Balk-Lamberton, A. J., Yang, H., Chritton, D. B., Lee, J. W., & Arabatzis, K. (1990). The effects of moderate exercise training on natural killer cells and acute upper respiratory tract infections. *Int.J.Sports Med.* **11**, 467-473.

209. Nieman, D. C. & Pedersen, B. K. (1999). Exercise and immune function. Recent developments. *Sports Med.* **27**, 73-80.

210. Nieman, D. C., Simandle, S., Henson, D. A., Warren, B. J., Suttles, J., Davis, J. M., Buckley, K. S., Ahle, J. C., Butterworth, D. E., Fagoaga, O. R., & . (1995). Lymphocyte proliferative response to 2.5 hours of running. *Int.J.Sports Med* **16**, 404-409.

211. Nieman, D. C., Tan, S. A., Lee, J. W., & Berk, L. S. (1989c). Complement and immunoglobulin levels in athletes and sedentary controls. *Int.J.Sports Med.* **10**, 124-128.

212. Northoff, H., Weinstock, C., & Berg, A. (1994). The cytokine response to strenuous exercise. *Int.J Sports Med* **15 Suppl 3**, S167-S171.

213. Nukina, H., Sudo, N., Komaki, G., Yu, X., Mine, K., & Kubo, C. (1998). The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-alpha level. *Neuroimmunomodulation.* **5**, 323-327.

214. Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., & Pedersen, B. K. (1999). Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J.Physiol* **515 (Pt 1)**, 287-291.

215. Ostrowski, K., Rohde, T., Zacho, M., Asp, S., & Pedersen, B. K. (1998). Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J.Physiol* **508 (Pt 3)**, 949-953.

216. Owen, N. & Steptoe, A. (2003). Natural killer cell and proinflammatory cytokine responses to mental stress: associations with heart rate and heart rate variability. *Biol.Psychol.* **63**, 101-115.

217. Palacios, A., Campfield, L. A., McClure, R. D., Steiner, B., & Swerdloff, R. S. (1983). Effect of testosterone enanthate on hematopoiesis in normal men. *Fertil.Steril.* **40**, 100-104.

218. Parker, L. N., Levin, E. R., & Lifrak, E. T. (1985). Evidence for adrenocortical adaptation to severe illness. *J Clin Endocrinol Metab* **60**, 947-952.

219. Parry-Billings, M., Budgett, R., Koutedakis, Y., Blomstrand, E., Brooks, S., Williams, C., Calder, P. C., Pilling, S., Baigrie, R., & Newsholme, E. A. (1992). Plasma amino acid concentrations in the overtraining syndrome: possible effects on the immune system. *Med.Sci.Sports Exerc.* **24**, 1353-1358.

220. Pattison, J. S., Folk, L. C., Madsen, R. W., & Booth, F. W. (2003). Selected Contribution: Identification of differentially expressed genes between young and old rat soleus muscle during recovery from immobilization-induced atrophy. *J.Appl.Physiol* **95**, 2171-2179.

221. Pedersen, B. K. & Hoffman-Goetz, L. (2000). Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev.* **80**, 1055-1081.

222. Pedersen, B. K., Steensberg, A., & Schjerling, P. (2001). Exercise and interleukin-6. *Curr.Opin.Hematol.* **8**, 137-141.

223. Pellegrini, A., Grieco, M., Materazzi, G., Gesi, M., & Ricciardi, M. P. (1998). Stress-induced morphohistochemical and functional changes in rat adrenal cortex, testis and major salivary glands. *Histochem.J.* **30**, 695-701.

224. Pemberton, P. A., Stein, P. E., Pepys, M. B., Potter, J. M., & Carrell, R. W. (1988). Hormone binding globulins undergo serpin conformational change in inflammation. *Nature* **336**, 257-258.

225. Pestell, R. G., Hurley, D. M., & Vandongen, R. (1989). Biochemical and hormonal changes during a 1000 km ultramarathon. *Clin Exp.Pharmacol.Physiol* **16**, 353-361.

226. Peters, A., Schweiger, U., Pellerin, L., Hubold, C., Oltmanns, K. M., Conrad, M., Schultes, B., Born, J., & Fehm, H. L. (2004). The selfish brain: competition for energy resources. *Neurosci.Biobehav.Rev.* **28**, 143-180.

227. Peters, E. M. & Bateman, E. D. (1983). Ultramarathon running and upper respiratory tract infections. An epidemiological survey. *S.Afr.Med.J.* **64**, 582-584.

228. Petersen, E. W., Carey, A. L., Sacchetti, M., Steinberg, G. R., Macaulay, S. L., Febbraio, M. A., & Pedersen, B. K. (2004). Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vitro. *Am.J.Physiol Endocrinol.Metab*.

229. Petrides, J. S., Gold, P. W., Mueller, G. P., Singh, A., Stratakis, C., Chrousos, G. P., & Deuster, P. A. (1997). Marked differences in functioning of the hypothalamic-pituitary-adrenal axis between groups of men. *J.Appl.Physiol* **82**, 1979-1988.

230. Pickup, J. C., Mattock, M. B., Chusney, G. D., & Burt, D. (1997). NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* **40**, 1286-1292.

231. Pitman, D. L., Ottenweller, J. E., & Natelson, B. H. (1988). Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: chronic stress and habituation. *Physiol Behav.* **43**, 47-55.

232. Pizza, F. X., Flynn, M. G., Sawyer, T., Brolinson, P. G., Starling, R. D., & Andres, F. F. (1995a). Run training versus cross-training: effect of increased training on circulating leukocyte subsets. *Med.Sci.Sports Exerc.* **27**, 355-362.

233. Pizza, F. X., Mitchell, J. B., Davis, B. H., Starling, R. D., Holtz, R. W., & Bigelow, N. (1995b). Exercise-induced muscle damage: effect on circulating leukocyte and lymphocyte subsets. *Med Sci.Sports Exerc* **27**, 363-370.

234. Pool, E. J. The effect of pyrogens on interleukin-6 (IL-6) secretion by whole blood cultures. 1999. University of Stellenbosch.
Ref Type: Thesis/Dissertation

235. Pool, E. J., Johaar, G., James, S., Petersen, I., & Bouic, P. (1998). The detection of pyrogens in blood products using an ex vivo whole blood culture assay. *J Immunoassay* **19**, 95-111.

236. Pool, E. J., Robson, P. J., Smith, C., Van Wyk, J. H., & Myburgh, K. H. (2002). In vitro interleukin-6 release in whole blood cultures in samples taken at rest from triathletes and professional rugby players. *Eur.J Appl.Physiol* **87**, 233-237.

237. Pritchard, J., Despres, J. P., Gagnon, J., Tchernof, A., Nadeau, A., Tremblay, A., & Bouchard, C. (1999). Plasma adrenal, gonadal, and conjugated steroids following long-term exercise-induced negative energy balance in identical twins. *Metabolism* **48**, 1120-1127.

238. Regelson, W., Loria, R., & Kalimi, M. (1994). Dehydroepiandrosterone (DHEA)--the "mother steroid". I. Immunologic action. *Ann.N.Y.Acad Sci* **719**, 553-563.

239. Rhind, S. G., Castellani, J. W., Brenner, I. K., Shephard, R. J., Zamecnik, J., Montain, S. J., Young, A. J., & Shek, P. N. (2001). Intracellular monocyte and serum cytokine expression is modulated by exhausting exercise and cold exposure. *Am.J.Physiol Regul.Integr.Comp Physiol* **281**, R66-R75.

240. Rhind, S. G., Gannon, G. A., Shek, P. N., Brenner, I. K., Severs, Y., Zamecnik, J., Buguet, A., Natale, V. M., Shephard, R. J., & Radomski, M. W. (1999). Contribution of exertional hyperthermia to sympathoadrenal-mediated lymphocyte subset redistribution. *J.Appl.Physiol* **87**, 1178-1185.

241. Rinaldi, S., Geay, A., Dechaud, H., Biessy, C., Zeleniuch-Jacquotte, A., Akhmedkhanov, A., Shore, R. E., Riboli, E., Toniolo, P., & Kaaks, R. (2002). Validity of free testosterone and free estradiol determinations in serum samples from postmenopausal women by theoretical calculations. *Cancer Epidemiol.Biomarkers Prev.* **11**, 1065-1071.

242. Rivier, A., Pene, J., Chanez, P., Anselme, F., Caillaud, C., Prefaut, C., Godard, P., & Bousquet, J. (1994). Release of cytokines by blood monocytes during strenuous exercise. *Int J Sports Med* **15**, 192-198.

243. Robson, P. (2003). Elucidating the unexplained underperformance syndrome in endurance athletes : the interleukin-6 hypothesis. *Sports Med.* **33**, 771-781.

244. Robson, P. J., Blannin, A. K., Walsh, N. P., Castell, L. M., & Gleeson, M. (1999). Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes. *Int.J.Sports Med.* **20**, 128-135.

245. Rohde, T., MacLean, D. A., Hartkopp, A., & Pedersen, B. K. (1996). The immune system and serum glutamine during a triathlon. *Eur.J.Appl.Physiol Occup.Physiol* **74**, 428-434.

246. Roitt, I. Essential Immunology. Eight edition. 1994. Oxford, Blackwell Scientific Publications.
Ref Type: Catalog

247. Ronsen, O., Haug, E., Pedersen, B. K., & Bahr, R. (2001a). Increased neuroendocrine response to a repeated bout of endurance exercise. *Med Sci Sports Exerc* **33**, 568-575.

248. Ronsen, O., Holm, K., Staff, H., Opstad, P. K., Pedersen, B. K., & Bahr, R. (2001b). No effect of seasonal variation in training load on immuno-endocrine responses to acute exhaustive exercise. *Scand J Med Sci Sports* **11**, 141-148.

249. Ronsen, O., Kjeldsen-Kragh, J., Haug, E., Bahr, R., & Pedersen, B. K. (2002). Recovery time affects immunoendocrine responses to a second bout of endurance exercise. *Am J Physiol Cell Physiol* **283**, C1612-C1620.

250. Ronsen, O., Pedersen, B. K., Oritsland, T. R., Bahr, R., & Kjeldsen-Kragh, J. (2001c). Leukocyte counts and lymphocyte responsiveness associated with repeated bouts of strenuous endurance exercise. *J.Appl.Physiol* **91**, 425-434.

251. Rood, B. (1994). *Uit die veldapteek (Out of the field-pharmacy)* Tafelberg, Cape Town.

252. Rosmond, R., Dallman, M. F., & Bjorntorp, P. (1998). Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J.Clin.Endocrinol.Metab* **83**, 1853-1859.

253. Rowbottom, D. G., Keast, D., Goodman, C., & Morton, A. R. (1995). The haematological, biochemical and immunological profile of athletes suffering from the overtraining syndrome. *Eur.J.Appl.Physiol* **70**, 502-509.

254. Saag, K. G. (2004). Prevention of glucocorticoid-induced osteoporosis. *South.Med.J.* **97**, 555-558.

255. Sakakima, H., Yoshida, Y., Sakae, K., & Morimoto, N. (2004). Different frequency treadmill running in immobilization-induced muscle atrophy and ankle joint contracture of rats. *Scand.J.Med.Sci.Sports* **14**, 186-192.

256. Sancho, D., Santis, A. G., Alonso-Lebrero, J. L., Viedma, F., Tejedor, R., & Sanchez-Madrid, F. (2000). Functional analysis of ligand-binding and signal transduction domains of CD69 and CD23 C-type lectin leukocyte receptors. *J.Immunol.* **165**, 3868-3875.

257. Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr.Rev.* **21**, 55-89.

258. Saunders, L. C. Psychological and endocrine indicators of stress: health and management implications. 2002. University of Stellenbosch.
Ref Type: Thesis/Dissertation

259. Sautebin, L., Ianaro, A., Rombola, L., Ialenti, A., Sala, A., & Di Rosa, M. (1999). Cyclooxygenase-2-dependent generation of 8-epiprostaglandin F2alpha by lipopolysaccharide-activated J774 macrophages. *Inflamm.Res.* **48**, 503-508.

260. Schmid-Ott, G., Jaeger, B., Meyer, S., Stephan, E., Kapp, A., & Werfel, T. (2001). Different expression of cytokine and membrane molecules by circulating lymphocytes on acute mental stress in patients with atopic dermatitis in comparison with healthy controls. *J Allergy Clin Immunol.* **108**, 455-462.

261. Scott, L. V., Svec, F., & Dinan, T. (2000). A preliminary study of dehydroepiandrosterone response to low-dose ACTH in chronic fatigue syndrome and in healthy subjects. *Psychiatry Res.* **97**, 21-28.

262. Seier, J. V. http://www.sahealthinfo.org/traditionalmeds/firststudy.htm. 2002.
Ref Type: Generic

263. Seier, J. V., Mdhluli, M., Dhansay, M., Loza, J., & Laubscher, R. A toxicity study of Sutherlandia leaf powder (Sutherlandia microphylla) consumption. 2002. 2002.
Ref Type: Report

264. Selye, H. (1978). *The stress of life* McGraw-Hill, New York.

265. Shapiro, H. M. (1995). Parameters and probes: Extrinsic parameters. In *Practical Flow Cytometry* pp. 229-238. Wiley-Liss, New York.

266. Shephard, R. J. (2002). Cytokine responses to physical activity, with particular reference to IL-6: sources, actions, and clinical implications. *Crit Rev.Immunol.* **22**, 165-182.

267. Shephard, R. J. & Shek, P. N. (1993). Athletic Competition and Susceptibility to Infection. *Clinical Journal of Sport Medicine* **3**, 75-77.

268. Shephard, R. & Shek, P. (1996). Exercise and CD4+/CD8+ cell counts: influence of various contributing factors in health and HIV infection. *Exercise Immunology Review* **2**, 65-83.

269. Shore, S., Shinkai, S., Rhind, S., & Shephard, R. J. (1999). Immune responses to training: how critical is training volume? *J.Sports Med Phys.Fitness* **39**, 1-11.

270. Silberman, D. M., Ayelli-Edgar, V., Zorrilla-Zubilete, M., Zieher, L. M., & Genaro, A. M. (2004). Impaired T-cell dependent humoral response and its relationship with T lymphocyte sensitivity to stress hormones in a chronic mild stress model of depression. *Brain Behav.Immun.* **18**, 81-90.

271. Singh, R. K., Pandey, B. L., Tripathi, M., & Pandey, V. B. (2001). Anti-inflammatory effect of (+)-pinitol. *Fitoterapia* **72**, 168-170.

272. Sinha-Hikim, I., Roth, S. M., Lee, M. I., & Bhasin, S. (2003). Testosterone-induced muscle hypertrophy is associated with an increase in satellite cell number in healthy, young men. *Am.J.Physiol Endocrinol.Metab* **285**, E197-E205.

273. Smith, L. L. (2000). Cytokine hypothesis of overtraining: a physiological adaptation to excessive stress? *Med Sci Sports Exerc* **32**, 317-331.

274. Smith, L. L. (2003). Overtraining, excessive exercise, and altered immunity: is this a T helper-1 versus T helper-2 lymphocyte response? *Sports Med.* **33**, 347-364.

275. Solomon, E. A., Schmidt, R. R., & Adragna, P. J. (1990). The body;'s defense mechanisms: immunity. In *Human Anatomy & Physiology*, ed. Field, C., pp. 790-819. Saunders College Publishing, Division of Rinehart and Winston, Orlando, Florida.

276. Spencer, R. L., Miller, A. H., Moday, H., McEwen, B. S., Blanchard, R. J., Blanchard, D. C., & Sakai, R. R. (1996). Chronic social stress produces reductions in available splenic type II corticosteroid receptor binding and plasma corticosteroid binding globulin levels. *Psychoneuroendocrinology* **21**, 95-109.

277. Sprenger, H., Jacobs, C., Nain, M., Gressner, A. M., Prinz, H., Wesemann, W., & Gemsa, D. (1992). Enhanced release of cytokines, interleukin-2 receptors, and neopterin after long-distance running. *Clin.Immunol.Immunopathol.* **63**, 188-195.

278. Spurgeon, A., Harrington, J. M., & Cooper, C. L. (1997). Health and safety problems associated with long working hours: a review of the current position. *Occup.Environ.Med.* **54**, 367-375.

279. Starkie, R. L., Rolland, J., Angus, D. J., Anderson, M. J., & Febbraio, M. A. (2001a). Circulating monocytes are not the source of elevations in plasma IL-6 and TNF-alpha levels after prolonged running. *Am J Physiol Cell Physiol* **280**, C769-C774.

280. Starkie, R. L., Rolland, J., & Febbraio, M. A. (2001b). Effect of adrenergic blockade on lymphocyte cytokine production at rest and during exercise. *Am J Physiol Cell Physiol* **281**, C1233-C1240.

281. Steen, H. B. (1990). Characteristics of flow cytometers. In *Flow cytometry and sorting*, eds. Melamed.M.R, Lindmo, T., & Mendelsohn, M. L., pp. 12. Wiley-Liss, New York.

282. Steensberg, A., Toft, A. D., Bruunsgaard, H., Sandmand, M., Halkjaer-Kristensen, J., & Pedersen, B. K. (2001a). Strenuous exercise decreases the percentage of type 1 T cells in the circulation. *J.Appl.Physiol* **91**, 1708-1712.

283. Steensberg, A., Toft, A. D., Schjerling, P., Halkjaer, K., & Pedersen, B. K. (2001b). Plasma interleukin-6 during strenuous exercise: role of epinephrine. *Am J Physiol Cell Physiol* **281**, C1001-C1004.

284. Steensberg, A., van Hall, G., Osada, T., Sacchetti, M., Saltin, B., & Klarlund, P. B. (2000). Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J.Physiol* **529 Pt 1**, 237-242.

285. Stefanski, V. (1998). Social stress in loser rats: opposite immunological effects in submissive and subdominant males. *Physiol Behav.* **63**, 605-613.

286. Stefanski, V. (2000). Social stress in laboratory rats: hormonal responses and immune cell distribution. *Psychoneuroendocrinology* **25**, 389-406.

287. Stefanski, V. & Engler, H. (1998). Effects of acute and chronic social stress on blood cellular immunity in rats. *Physiol Behav.* **64**, 733-741.

288. Stefanski, V. & Engler, H. (1999). Social stress, dominance and blood cellular immunity. *J.Neuroimmunol.* **94**, 144-152.

289. Stepto, N. K., Hawley, J. A., Dennis, S. C., & Hopkins, W. G. (1999). Effects of different interval-training programs on cycling time-trial performance. *Med.Sci.Sports Exerc.* **31**, 736-741.

290. Straub, R. H., Schuld, A., Mullington, J., Haack, M., SchOLmerich, J., & PollmACher, T. (2002). The endotoxin-induced increase of cytokines is followed by an increase of cortisol relative to dehydroepiandrosterone (dhea) in healthy male subjects. *J Endocrinol* **175**, 467-474.

291. Strausbaugh, H. J., Dallman, M. F., & Levine, J. D. (1999). Repeated, but not acute, stress suppresses inflammatory plasma extravasation. *Proc Natl Acad Sci U S A* **96**, 14629-14634.

292. Strickland, I., Kisich, K., Hauk, P. J., Vottero, A., Chrousos, G. P., Klemm, D. J., & Leung, D. Y. (2001). High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *J.Exp.Med.* **193**, 585-593.

293. Swaffar, D. S., Ang, C. Y., Desai, P. B., Rosenthal, G. A., Thomas, D. A., Crooks, P. A., & John, W. J. (1995). Combination therapy with 5-fluorouracil and L-canavanine: in vitro and in vivo studies. *Anticancer Drugs* **6**, 586-593.

294. Takaki, A., Huang, Q. H., Somogyvari-Vigh, A., & Arimura, A. (1994). Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines. *Neuroimmunomodulation.* **1**, 335-342.

295. Tanriverdi, F., Silveira, L. F., MacColl, G. S., & Bouloux, P. M. (2003). The hypothalamic-pituitary-gonadal axis: immune function and autoimmunity. *J.Endocrinol.* **176**, 293-304.

296. Tantak, Y. S., Selkirk, S., Bristow, A. F., Carpenter, A., Ball, C., Rafferty, B., & Poole, S. (1991). Assay of pyrogens by interleukin-6 release from monocytic cell lines. *J Pharm.Pharmacol.* **43**, 578-582.

297. Terblanche, J., Fahlman, A., Myburgh, K. H., & Jackson, S. J. (2004). Measurement reliability of highly variable physiological responses to experimentally-manipulated gas fractions. *Physiological Measurement* **25**, 1189-1197.

298. Tinnikov, A. A. (1999). Responses of serum corticosterone and corticosteroid-binding globulin to acute and prolonged stress in the rat. *Endocrine.* **11**, 145-150.

299. Tisdale, M. J. (2001). Cancer anorexia and cachexia. *Nutrition* **17**, 438-442.

300. Tomasi, T. B., Trudeau, F. B., Czerwinski, D., & Erredge, S. (1982). Immune parameters in athletes before and after strenuous exercise. *J.Clin.Immunol.* **2**, 173-178.

301. Tsirpanlis, G., Bagos, P., Ioannou, D., Bleta, A., Marinou, I., Lagouranis, A., Chatzipanagiotou, S., & Nicolaou, C. (2004). The variability and accurate assessment of microinflammation in haemodialysis patients. *Nephrol.Dial.Transplant.* **19**, 150-157.

302. Ullum, H., Haahr, P. M., Diamant, M., Palmo, J., Halkjaer-Kristensen, J., & Pedersen, B. K. (1994). Bicycle exercise enhances plasma IL-6 but does not change

IL-1 alpha, IL-1 beta, IL-6, or TNF-alpha pre-mRNA in BMNC. *J Appl.Physiol* **77**, 93-97.

303. Urhausen, A. & Kindermann, W. (1992). Biochemical monitoring of training. *Clinical Journal of Sport Medicine* **2**, 52-61.

304. Urhausen, A., Gabriel, H., & Kindermann, W. (1995). Blood hormones as markers of training stress and overtraining. *Sports Med* **20**, 251-276.

305. Urhausen, A. & Kindermann, W. (2002). Diagnosis of overtraining: what tools do we have? *Sports Med.* **32**, 95-102.

306. Urhausen, A., Kullmer, T., & Kindermann, W. (1987). A 7-week follow-up study of the behaviour of testosterone and cortisol during the competition period in rowers. *Eur.J.Appl.Physiol Occup.Physiol* **56**, 528-533.

307. Vaartjes, W. J., de Haas, C. G., & Houweling, M. (1990). Acute effects of interleukin 1 alpha and 6 on intermediary metabolism in freshly isolated rat hepatocytes. *Biochem.Biophys.Res.Commun.* **169**, 623-628.

308. Valenti, G. (2002). Adrenopause: an imbalance between dehydroepiandrosterone (DHEA) and cortisol secretion. *J.Endocrinol.Invest* **25**, 29-35.

309. Van Wyk, B. E. & Gericke, N. (2000). *People's plants. A guide to the useful plants of Southern Africa* Briza, Pretoria.

310. Van Wyk, B. E., Van Oudtshoorn, B., & Gericke, N. Medicinal plants of South Africa. 1997. Briza Publications.

Ref Type: Serial (Book,Monograph)

311. Vander, A., Sherman, J., & Luciano, D. (1998a). Defence mechanisms of the body. In *Human Physiology: The mechanisms of body function*, ed. Kane, K. T., pp. 688-734. McGraw-Hill, San Francisco, California.

312. Vander, A., Sherman, J., & Luciano, D. (1998b). Principles of Hormonal Control Systems. In *Human Physiology: The mechanisms of body function*, ed. Kane, K. T., pp. 261-286. McGraw-Hill, San Francisco, California.

313. Vassilakopoulos, T., Karatza, M. H., Katsaounou, P., Kollintza, A., Zakynthinos, S., & Roussos, C. (2003). Antioxidants attenuate the plasma cytokine response to exercise in humans. *J.Appl.Physiol* **94**, 1025-1032.

314. Verde, T. J., Thomas, S. G., Moore, R. W., Shek, P., & Shephard, R. J. (1992). Immune responses and increased training of the elite athlete. *J Appl.Physiol* **73**, 1494-1499.

315. Vermeulen, A., Verdonck, L., & Kaufman, J. M. (1999). A critical evaluation of simple methods for the estimation of free testosterone in serum. *J.Clin.Endocrinol.Metab* **84**, 3666-3672.

316. Vider, J., Lehtmaa J, Kullisaar T, Vihalemm T, Zilmer K, Kairane C, Landor A, Karu T, & Zilmer M (2001). Acute immune response in respect to exercise-induced oxidative stress. *Pathophysiology* **7**, 263-270.

317. Viljoen, P. T. The oxidation of pinitol and partial identification of a triterpene glycoside from Sutherlandia microphylla Burch. 1969. University of the Free State, Bloemfontein, South Africa. 1969.
Ref Type: Thesis/Dissertation

318. Villette, J. M., Bourin, P., Doinel, C., Mansour, I., Fiet, J., Boudou, P., Dreux, C., Roue, R., Debord, M., & Levi, F. (1990). Circadian variations in plasma levels of hypophyseal, adrenocortical and testicular hormones in men infected with human immunodeficiency virus. *J.Clin.Endocrinol.Metab* **70**, 572-577.

319. Viru, A. (2002). Early contributions of Russian stress and exercise physiologists. *J Appl.Physiol* **92**, 1378-1382.

320. Viru, A. M., Hackney, A. C., Valja, E., Karelson, K., Janson, T., & Viru, M. (2001). Influence of prolonged continuous exercise on hormone responses to subsequent exercise in humans. *Eur.J Appl.Physiol* **85**, 578-585.

321. Vorobiev, D. V., Vetrova, E. G., Larina, I. M., Popova, I. A., & Grigoriev, A. I. (1996). Energy substrates, hormone responses and glucocorticoid binding in lymphocytes during intense physical exercise in humans following phosphocreatine administration. *Eur.J Appl.Physiol Occup.Physiol* **74**, 534-540.

322. Walsh, N. P., Blannin, A. K., Bishop, N. C., Robson, P. J., & Gleeson, M. (2000). Effect of oral glutamine supplementation on human neutrophil lipopolysaccharide-stimulated degranulation following prolonged exercise. *Int.J.Sport Nutr.Exerc.Metab* **10**, 39-50.

323. Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A. M., Snyder, P. J., Weber, T., Berman, N., Hull, L., & Swerdloff, R. S. (2004). Long-term testosterone gel (AndroGel) treatment maintains beneficial effects on sexual function and mood, lean and fat mass, and bone mineral density in hypogonadal men. *J.Clin.Endocrinol.Metab* **89**, 2085-2098.

324. Warren, M. P. & Goodman, L. R. (2003). Exercise-induced endocrine pathologies. *J.Endocrinol.Invest* **26**, 873-878.

325. Watkins, A. D. (1994). Hierarchical cortical control of neuroimmunomodulatory pathways. *Neuropathol.Appl.Neurobiol.* **20**, 423-431.

326. Weinstock, C., Konig, D., Harnischmacher, R., Keul, J., Berg, A., & Northoff, H. (1997). Effect of exhaustive exercise stress on the cytokine response. *Med Sci.Sports Exerc* **29**, 345-354.

327. Werfel, T., Boeker, M., & Kapp, A. (1997). Rapid expression of the CD69 antigen on T cells and natural killer cells upon antigenic stimulation of peripheral blood mononuclear cell suspensions. *Allergy* **52**, 465-469.

328. Westgarth-Taylor, C., Hawley, J. A., Rickard, S., Myburgh, K. H., Noakes, T. D., & Dennis, S. C. (1997). Metabolic and performance adaptations to interval training in endurance-trained cyclists. *Eur.J.Appl.Physiol Occup.Physiol* **75**, 298-304.

329. Wisniewski, T. L., Hilton, C. W., Morse, E. V., & Svec, F. (1993). The relationship of serum DHEA-S and cortisol levels to measures of immune function in human immunodeficiency virus-related illness. *Am J Med Sci* **305**, 79-83.

330. Woolf, P. D. (1992). Hormonal responses to trauma. *Crit Care Med.* **20**, 216-226.

331. Wright, B. E., Porter, J. R., Browne, E. S., & Svec, F. (1992). Antiglucocorticoid action of dehydroepiandrosterone in young obese Zucker rats. *Int J Obes.Relat Metab Disord.* **16**, 579-583.

332. Wughalter, E. H. & Gondola, J. C. (1991). Mood states of professional female tennis players. *Percept.Mot.Skills* **73**, 187-190.

333. Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., & Achong, M. K. (1998). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin.Invest* **101**, 311-320.

334. Yao, G., Liang, J., Han, X., & Hou, Y. (2003). In vivo modulation of the circulating lymphocyte subsets and monocytes by androgen. *Int.Immunopharmacol.* **3**, 1853-1860.

335. Zhou, D., Kusnecov, A. W., Shurin, M. R., DePaoli, M., & Rabin, B. S. (1993). Exposure to physical and psychological stressors elevates plasma interleukin 6: relationship to the activation of hypothalamic-pituitary-adrenal axis. *Endocrinology* **133**, 2523-2530.

336. Zinna, E. M. & Yarasheski, K. E. (2003). Exercise treatment to counteract protein wasting of chronic diseases. *Curr.Opin.Clin Nutr Metab Care* **6**, 87-93.

Pectora roborant cultus recti

Figure 1.1 Lymphocyte subpopulations

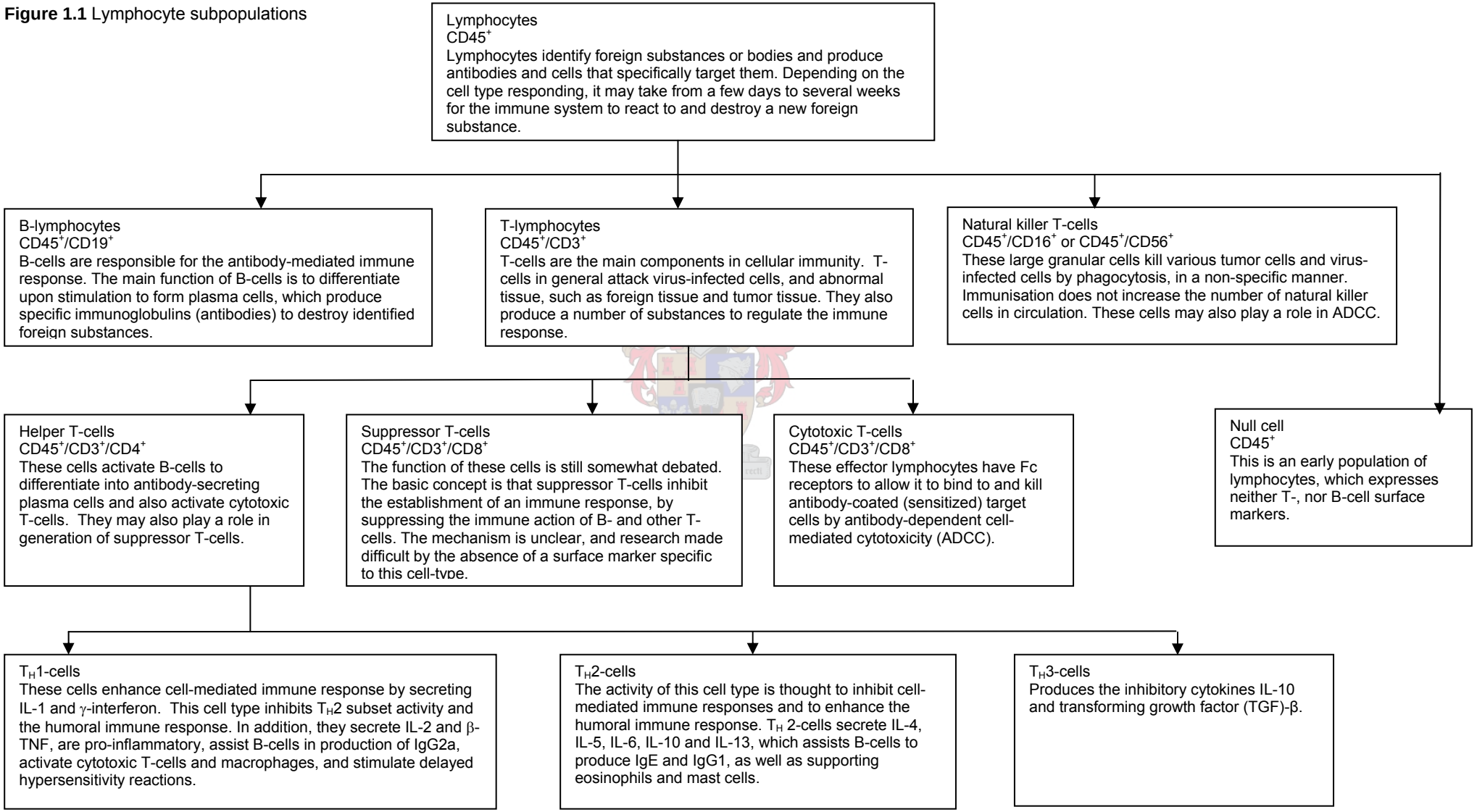


Figure 1.2 Interaction between cytokines and immune cells in the immune response