The Effects of Physical Activity Level, Sex, and Different Exercise Protocols on Monocyte TLR Expression

by

Marta Oliveira

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

Certificate of Originality

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own except as specified in acknowledgments, and that neither the thesis nor the original work contained therein has been submitted to this or any other institution for any award.

Signed …….

Date: ……

General Abstract

It has been suggested that moderate exercise contributes to protection against the development of chronic diseases by anti-inflammatory mechanisms that include elevations of anti-inflammatory cytokines and also reduction of the expression of Toll-Like Receptors (TLRs). However, prolonged strenuous exercise has been shown to reduce the function of some immune cells, decrease virus protection and consequently may account for the reason athletes appear more vulnerable to catching Upper Respiratory Tract Infections (URTI). Although it has been proven that some exercise is better than no exercise, it is not clear yet what is the right amount of exercise to elicit beneficial immune responses and to help prevent the development of diseases. Therefore, the general aim of the studies in this thesis was to evaluate the impact of different types of exercise on monocyte TLR expression in participants with different fitness levels. It was found that different acute exercise protocols elicit different changes in TLR2 and TLR4 expression, where an acute bout of strenuous exercise reduced TLR4 expression for a few hours after the completion of the exercise (Chapter 5); however, short two bouts of exhaustive exercise separated by 2 hours did not change TLR4 expression (Chapter 6). In addition, changes in TLR4 expression were related to sex and the physical activity level of the participants (Chapter 4), and should therefore be considered separately when analysing TLR4 expression. Furthermore, high-intensity intermittent training improves participants' aerobic capacity and modifies the monocyte subpopulation concentration in the blood, with no changes in TLR4 expression. Further research needs to be done in this area to achieve a conclusive finding about changes in TLR4 expression and monocyte subsets after different training protocols, and possible relationships to cytokine production.

Key words: TLR4, exercise, URTI, sex, monocyte, immune system.

Acknowledgments

I would like to thank Professor Mike Gleeson for his supervision, support and encouragement though these years. Also, I would like to thank He and Dr. Lettie Bishop for giving me the opportunity to be involved in different projects in the Exercise Immunology Laboratory, where I could acquire numerous skills that will certainly be very useful in my future.

I would also like to thank the participants and several people that were involved in broader projects which provided part of the data used for this thesis: Dr. Tracey McCauley and Dr Pedro Tauler for their help in the data collection for Chapter 4; Dr. Deborah Fletcher for allowing me to use the blood samples for the resting study in Chapter 5; Mr. John Hough and Miss Melanie Leggate for giving me the opportunity to join them and share some data for Chapters 6 and 7; and the MSc students: Chris Toms (Chapter 5) and Kurt Rumley (Chapter 8) for their help in data collection and looking after the participants, enabling me to collect more data and improve the quality of my work.

Finally, I would like to thank some of my friends: John Hough for the friendship and constant encouragement; helping me to manage a full-time job, a part-time PhD and a sub-wardenship!; my brother Luis Carlos for his presence and support; my fiancé Dan Child for relaxing times together, for teaching me how to format the thesis and, together with Alison Burrows, for proof reading the whole thesis; and my good friend Sean Mitchell, who spent many hours helping me, whilst also showing me the value of life and work, and how to succeed in both – I would not have done it without you! Last but not least, thanks be to God for His generous love and grace in my life.

Publications

Oliveira, M. and Gleeson, M., 2010. The influence of prolonged cycling on monocyte Toll-like receptor 2 and 4 expression in healthy men. *European journal of applied physiology,* 109(2), 251-257

Conference contributions

Oliveira, M., Toms, C., Barrett, S. and Gleeson, M., 2009. The Influence Of Prolonged Cycling On Monocyte Toll-like Receptor 2 And 4 Expression In Healthy Men". *Proceeding of the American College of Sports Medicine Conference* (ACSM) Abstract Number: 2273.

Oliveira, M. and Gleeson, M., 2009. No effect of age, gender and physical activity level on human monocyte Toll-like receptor 4 expression in an active population. *Proceedings of the 14th annual Congress of the European College of Sport Science*, P115.

List of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
APCs	Antigen presenting cells
BAS	Basophil
BD	Becton–Dickinson
BMI	Body mass index
BSA	Bovine serum albumin
CD	Cluster of differentiation
CHD	Coronary heart disease
CO_2	Carbon dioxide
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
Cy5	Cyanine dye
ºC	Degrees Celsius
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EOS	Eosinophil
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GMFI	Geometric mean fluorescence intensity
h	Hour
HB	Haemoglobin
HBSS	Hank's buffered salt solution
HCT	Haematocrit
HIIT	High-intensity interval training
HLA.DR	Human Leukocyte Antigen DR-1
HSP	Heat shock protein
IFN-γ	Interferon gamma
IFN-β	Interferon beta
IgA	Immunoglobulin A
IKK	Nuclear factor-κB kinase

IKKi	TRIF–TBK1 combination
IL	Interleukin
IPAQ	International physical activity questionnaire
IRAK	IL-1r-associated kinase
IRF3	Interferon-regulatory factor 3
ISO	Isotype control
K_3EDTA	Potassium Ethylene Diamine Tetra-acetic Acid
km	Kilometre
LPS	lipopolysaccharide
LRR	Leucine-rich repeat
LYM	Lymphocyte
M	Molar
MAPK	Mitogen-activated protein kinase
MD-2	Lymphocyte antigen 96
MET	Metabolic equivalent
MHC	Major histocompatibility complex
min	Minutes
ml	millilitres
mmHg	Millimetre of mercury
MON	Monocyte
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
NEU	Neutrophil
NF-κB	Nuclear factor kappa B
NOD	Nnucleotide-binding oligomerization domain
O_2	Oxygen
PAMPs	pathogen-associated molecular patterns
PASW	Predictive Analytics SoftWare
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
pg	picograms
pH	negative logarithm of the hydrogen ion concentration
RER	Respiratory exchange ratio

RPE	Rating of perceived exertion
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences
SSC	Side scatter
TAB	TAK-binding protein
TAK1	Transforming-growth-factor-β-activated kinase 1
TBK1	TANK-binding kinase 1
TBS	Trisbuffered saline
TBS-T	Trisbuffered saline and Tween 20 mixture
Th1	T helper cells type 1
Th2	T helper cells type 2
TIR	Toll/IL-1r domain
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRAF6	Tumour-necrosis-factor-receptor-associated-factor-6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing IFNs
URTI	Upper respiratory tract infection
VO_{2peak}	Peak maximum oxygen uptake
W	Watts
WBC	White blood cell
WHO	World Health Organization
µl	Microlitre

1. Introduction

It is well established that exercise contributes to the protection against the development of chronic diseases such as cardiovascular disease (CVD) and diabetes (Hardman and Stensel 2009, Gleeson et al. 2011c). Explanations for this phenomena include the fact that exercise helps to reduce visceral fat mass, increases anti-inflammatory cytokine and myokine production (cytokines produced by contractile muscle), and reduces the expression of Toll-Like Receptors (TLRs on monocytes) (Flynn and McFarlin 2006, Gleeson et al. 2011c). Moreover, increased fitness results in an augmented basic functional capacity, which consequently results in a longer and better quality of life. The increased interest in physical exercise regimens as a means of preventing the development of chronic disease can be something beneficial not only for the individual but also for the economy. In the UK, around £30.7 billion per year are spent on the treatment and care of people with CVD (British Heart Foundation 2008). However, although it might be suggested that a cardiac rehabilitation programme through physical exercise would cost less money annually than treating the disease with medication or surgery, no cost estimate for such an exercise regimen has yet been reported. Besides, prevention is better than cure.

Exercise has been shown to induce elevations of anti-inflammatory cytokines such as Interleukin (IL)-10 (Gleeson et al. 2011c), and also to reduce the expression of TLRs (Flynn et al. 2003). It has therefore been suggested that reduction in the TLR expression and consequent reduction in cytokine release should be the next step forward in research for the decrease of CVDs (McFarlin et al. 2006). To date, few studies have been done in this area. Flynn et al. (2003) observed that 10 weeks of resistance training reduced $CD14^+$ monocyte TLR4 expression in elderly women and, from this group, the

low TLR4 “expressers” had lower cytokine production compared with the high TLR4 “expressers”. In addition, McFarlin et al. (2004) showed that monocyte TLR4 expression was lower in resistance-trained women and was associated with lower pro-inflammatory cytokines, and that physical activity status (but not age) influenced both TLR4 expression and IL-6 production (McFarlin et al. 2006).

However, several studies have also shown that prolonged strenuous exercise reduces the function and expression of some immune cells such as monocytes, neutrophils, T lymphocytes and natural killer cells, suggesting that strenuous exercise may reduce the hosts capacity to act against invading pathogens (Bermon 2007, Gleeson and Bishop 2005, Nieman 1995). This could represent an ‘open window’ for pathogens, allowing upper respiratory tract infection (URTI) and other common infections to gain a foot-hold (Nieman 1994). The changes of several inflammatory markers – including TLR expression – in this context can decrease protection against virus and may consequently account for why athletes appear to be more vulnerable to catching URTI. In response to the needs of athletes and coaches, the prevention of illnesses during training and competition is highly coveted by sport scientists, since infection almost inevitably negatively affects performance (West et al. 2009). Although factors such as previous URTI episodes and environmental aspects may have influenced the prevalence of infections, more research is required to determine the causes of immune system function diminution and its positive and negative impacts on human health.

In 1994, a “J”-shaped curve was proposed by Nieman, which described the relationship between exercise load and susceptibility to infections, where, on one hand, regular moderate physical activity reduces the risk of URTI below the sedentary individual’s risk. On the other hand, performing a high exercise training load is associated with an

above average risk of URTI. But although it is seems that there is a common knowledge that a moderate amount of exercise is better than no exercise, and that too much exercise with little rest can be detrimental to health, there is not a definite consensus in the literature on the right amount of exercise that would elicit increased well-being response, whilst helping to prevent the development of disease without compromising the immune system. It is important also for the athletic population to know how much daily exercise can be done in order to improve performance, whilst also avoiding URTI.

Therefore, the general aim of the studies in this thesis was to evaluate the impact of different types of exercise on monocyte TLR expression on participants with different levels of fitness. Since TLR is a relatively new immunological 'marker' used in the exercise field, it was hoped that this studies would provide some insights on how exercise elicits a positive or negative response on monocyte TLR and some other immunological markers. The main purposes of the present research studies were to:

1) Investigate the reproducibility of blood measurements, the validity of the methods and coefficient of variation of the flow cytometry analysis.
2) Determine the resting values of monocyte TLR4 expression in sedentary and active people. In addition, analyse the effect of sex and physical activity level on monocyte TLR4 expression.
3) Investigate the influence of prolonged cycling exercise at 75% VO_{2peak} on monocyte TLR2 and TLR4 expression in healthy men.
4) Investigate the influence of repeated high-intensity exercise (two VO_{2peak} tests) separated by 2 hours on monocyte TLR4 expression in healthy men.

5) Examine the effects of 2 and 4-weeks of high-intensity interval training on the monocyte subpopulations, TLR4 expression and plasma IL-6 in sedentary and in obese individuals.

It was hypothesised that:

- TLR4 expressions would be different according to training status.
- A single bout of high-intensity exercise would reduce TLR2 and TLR4 expression for several hours after exercise.
- An exercise training regimen would reduce TLR4 expression in a sedentary population of both normal and high body mass index (BMI) participants.

An overview of the nine Chapters included in this thesis is given as follows:

Chapter 1: Provides a general introduction to the research developed.

Chapter 2: Contains the main literature review relevant for the studies described in the thesis.

Chapter 3: Details the general materials and methods used in the experimental part of the studies. Any specific methodology is described in the subsequent relevant Chapters. This Chapter also presents the methods and results of reproducibility tests of blood measurements and the coefficient of variation of the flow cytometry and cell count analysis.

Chapter 4: Presents results from the first study. This Chapter focuses mainly on training and sex differences in TLR4 expression and its possible relationship with respiratory infection incidence.

Chapter 5: Presents results from the second study. It describes the influence of prolonged cycling exercise at 75% VO_{2peak} on monocyte TLR2 and TLR4 expression in healthy men.

Chapter 6: Presents results of the third study, developed due to results obtained in the previous Chapters. This study investigates the monocyte $CD14^{+}$ TLR4 responses after two VO_{2peak} tests separated by 2 hours.

Chapter 7: Presents results from the fourth study. This Chapter analyses the effects of two weeks of high-intensity training on monocyte TLR2 and TLR4 expression in high BMI sedentary men.

Chapter 8: Provides results of the fifth study, developed due to results obtained in the previous Chapter. This study investigates the effects of a 4-week period of high-intensity intermittent training (HIIT) on monocyte TLR4 expression in low activity level participants.

Chapter 9: Contains a general discussion, conclusions and the limitations of the research conducted. It also suggests further research questions and potential areas for future study.

2. Literature Review

2.1. Role of the Immune System

The immune system recognises, controls, regulates and generates memory against infection. When a foreign body reaches the human body, an immediate response is generated by the innate immune system, which is the organism's first line of defence. Innate immunity is instantly available to battle a wide range of pathogens but it is not specific and does not lead to long-lasting immunity. Innate immunity can be separated into three main mechanisms that prevent the entry of foreign agents into the body: (1) the physical barriers such as skin and mucosa; (2) chemical barriers such as enzymes and low pH in the gut and (3) phagocytic white blood cells (Gleeson 2006, Murphy et al. 2008).

Monocytes, macrophages, neutrophils and dendritic cells are the major phagocytic cells of the immune system and have as their main function the capacity to engulf and ingest foreign material. In addition, neutrophils and monocytes can migrate from the circulation into tissues if the tissue is damaged or infected.

When an infection is recurrent or the innate immune system fails to resist the micro-organism, the adaptive immune system is activated to act against the pathogen. The adaptive immune system consists of T- and B-lymphocytes and it is developed during the lifetime of an individual as an adaptation to infection with specific pathogens, creating an immunological memory (Murphy et al. 2008).

Although the immune system is comprised of two different divisions, they act cooperatively to combat pathogens. Some phagocytic cells from the innate immune

system form a link to the adaptive immune system by acting as antigen presenting cells (APCs). In short, after the phagocytic cell ingests and processes the foreign body, they release cytokines and also present parts of the pathogen's protein molecules to different cells of the adaptive immune system. This process leads to the initiation of specific responses. When the connection is made with B-cells, these lymphocytes will multiply and differentiate into plasma cells: a cell type that produces antibodies and combats the extracellular pathogens. When the link is with T-cells, three different connections can be made: (1) connections with T-cytotoxic cells, which are capable of killing infected cells; (2) connections with T-helper cells, which send signals to produce and release more cytokines, activate B-cells and macrophages and (3) connections with T-regulatory cells, which control the activity of other immune cells and help to orchestrate the immune response. (Gleeson 2006, Murphy et al. 2008). This harmonic cross-communication and co-ordination between the two branches of the immune system is a critical factor in the body's response to cell damage and infection.

2.2. Monocyte Subsets

Monocytes are one of the main APCs found in the circulatory system and the activation of these cells leads to inflammatory responses. Diversity in the expression of proteins on the human monocyte cell surface defines different monocyte subsets. According to the amount of cluster of differentiation (CD)14 and CD16 receptors on its surface, three subpopulations of monocyte can be defined: $CD14^{++}CD16^{-}$ cells (also known as classical monocytes); and more mature subpopulations consisted of $CD14^{++}CD16^{+}$ cells and $CD14^{+}CD16^{+}$ (known as pro-inflammatory monocytes). The classical monocytes consist of around 80-90% of the total circulatory monocyte population, while together

both pro-inflammatory populations consist of only 10-20% (Rogacev et al. 2010, Ziegler-Heitbrock 2007).

It is known that monocyte maturation results in the formation of macrophages and dendritic cells. Randolph et al. (2002) suggested that pro-inflammatory monocytes are precursors of dendritic cells while only a small percentage of classical monocytes can develop into dendritic cells. In addition, pro-inflammatory monocytes have been shown to be prodigious producers of pro-inflammatory cytokines (e.g. IL-1 and tumour necrosis factor (TNF)-α) and low producers of IL-10 compared with classical monocytes (Ziegler-Heitbrock 2007). Augmented numbers of pro-inflammatory monocytes have been observed in several inflammatory conditions such as sepsis and some diseases such as atherosclerosis and coronary heart disease (CHD, Rogacev et al. 2010). By contrast, other studies have revealed no relationship between pro-inflammatory monocytes and diseases such as diabetes and rheumatoid arthritis (Ziegler-Heitbrock 2007).

Monocyte capability of producing cytokines is highly related to TLR ligation (Ziegler-Heitbrock 2007). This topic will be discussed in depth in the further sections as it has been suggested that pro-inflammatory monocytes have a greater expression of TLR, especially TLR2 and TLR4 compared with classical monocytes (Ziegler-Heitbrock 2007, Timmerman et al. 2008). Therefore, it is sensible to suggest that reduction of monocyte TLR expression may reduce the incidence of some diseases that are associated with chronic low-grade inflammation.

2.3. Toll-Like Receptors

TLRs are trans-membrane proteins located in APCs such as monocytes, macrophages and dendritic cells. They are available to recognise an array of pathogen-associated molecular patterns (PAMPs) and activate immune responses via intracellular signalling (Akira 2003a, Akira and Hemmi 2003, Parker et al. 2007). Each human TLR, from a family of at least 10, has specific ligands and functions, as shown in Table 2.1. In addition to the recognition role, TLRs are associated with the increased release of cytokines and the stimulation of antimicrobial activity by both the innate and acquired immune system.

The discovery of TLRs changed the scientific community's views on the human body's discrimination between self and non-self. However, it is worth mentioning that the innate immune system also has some other means to recognise and interact with microbial pathogens, such as the use of complement proteins, NOD proteins and cell surface receptors on natural killer cells (Hoebe et al. 2004). Nevertheless, the activation of TLRs results in the effective production of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α and also results in the activation of the adaptive immune system by the presentation of pathogen particles to T cells (Gleeson et al. 2006, Medzhitov 1997), which will be discussed later. These multiple immune responses are proven to be a rapid and efficient way for the human body to deal with infection. However, augmented production of inflammatory cytokines is related to the incidence of diseases; but little is known of the reasons for this. Explanations have included TLR gene mutation due to ageing or simply TLR failure to regulate cytokine production (Gleeson et al. 2006, Hoebe et al. 2004).

Table 2.1 - TLR location and ligands

	Location	Examples of ligands	Reference
TLR1/TLR2 TLR2/TLR6	Cell surface	Peptidoglycan; lipoproteins, Zymozan	Akira 2003a, Murphy et al. 2008
TLR3	Intracellular	Double-stranded RNA	Brown et al. 2010
TLR4	Cell surface	LPS, RSV	Akira 2003, Monick et al. 2003
TLR5	Cell surface	Flagelin	Brown et al. 2010, Testro and Visvanathan 2009
TLR7 & TLR8	Intracellular	Single-stranded RNA	Murphy et al. 2008, Brown et al. 2010
TLR9	Intracellular	CpG DNA, hemozoin	Brown et al. 2010, Murphy et al. 2008, Testro and Visvanathan 2009
TLR10/TLR2	Cell surface	Microbes and fungi	Guan et al. 2010, Parker et al. 2007

2.3.1. TLR2 and TLR4 Pathways

TLR2 and TLR4 are located on the cell surface of APCs and their activation stimulates a range of intracellular signalling pathways that coordinate the extent, form and duration of the inflammatory response. They are trans-membrane receptors that possess an external ligand-binding domain consisting of leucine-rich repeats (LRR) and a cytoplasmic region containing a toll/IL-1r domain (TIR), which is essential to activate the downstream response in the TLR pathway. TLR2, when connected with its heterodimers TLR1, TLR6 and TLR10[1], recognises lipoproteins directly via the LRR region: a capability that TLR4 lacks.

The gram-negative bacteria that have lipopolysaccharide (LPS) on their surface are firstly recognised in the body by the LPS-binding protein, a specific lipid transfer protein that delivers LPS to the CD14 receptor present on the surface of phagocytic cells. This interaction is followed by the presentation of the LPS by CD14 to the TLR4 and the link between TLR4 and the protein MD-2 (Akira 2003a, Figure 2.1a). After

[1] TLR10 has been recently discovered and has been associated to TLR1 and TLR2, recognising microbes and fungi; however more research in the area is required to confirm these findings (Brown et al. 2010).

that, the interaction of the intercellular TIR domain with either TIRAP (TIR-domain-containing adaptor protein) and myeloid differentiation factor 88 (MyD88) or TRAM (TRIF-related adaptor molecule) and TRIF (TIR-domain-containing adaptor inducing IFNs) is the next step in this downstream response (Akira 2003a, Akira et al. 2003, Akira and Takeda 2004).

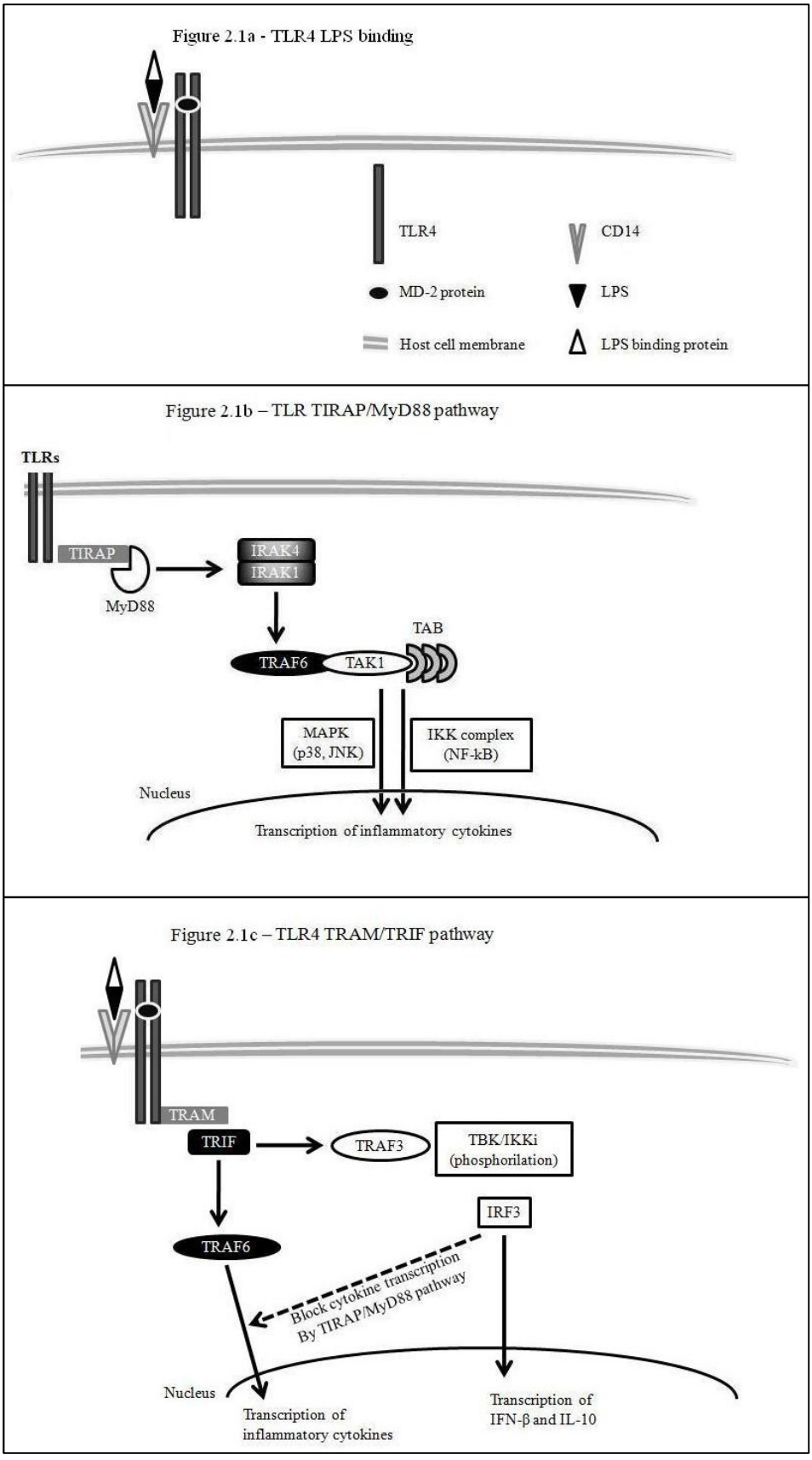

Figure 2.1- TLR pathways

MyD88 interacts with IL-1r-associated kinase (IRAK)-4 which activates other members of the IRAK family. This process results in the activation of TRAF6 (tumour-necrosis-factor-receptor-associated-factor-6) and the activation of transforming-growth-factor-β-activated kinase 1 (TAK1) and the TAK1-binding protein (TAB1), followed by TAB2 and 3. The activation of this signalling triggers both MAPK (mitogen-activated protein kinases) and nuclear factor-κB (NF-κB) signalling pathways. The activation of the inhibitor of nuclear factor-κB kinase (IKK) complex mediates the phosphorylation of IκB-α, which is degraded and stimulates the NF-κB to move to the nucleus and initiate the transcription of inflammatory cytokines and interferon gamma (IFN-γ) (Akira and Hemmi 2003, Brown et al. 2010). Similarly, the MAPK pathway activates kinases that stimulate cytokine gene expression inside the cell nucleus and subsequent cytokine synthesis (Figure 2.1b).

Most of TLRs, including TLR2, use the TIRAP + MyD88 route to activate the production of IFN-γ, a cytokine responsible for controlling bacterial and viral infection. TLR4, however, can also start a pathway through the link with TRAM, following the activation with TRIF, which can either associate with TRAF6 or TRAF3. Next, the TRAF3 pathway is used and a link is established with TBK1 (TANK-binding kinase 1) and IKKi (a TRIF–TBK1 combination) to phosphorylate interferon-regulatory factor 3 (IRF3), resulting in the production of IFN-β (which is involved in the innate immune response activation) and the anti-inflammatory cytokine IL-10 (Brown et al. 2010) (Figure 2.1c). It is important to note that the specificity of each TLR with the different adaptor will influence the type, magnitude and duration of the inflammatory response. For example, the TLR4/MyD88 signalling pathway is used to induce the expression of pro-inflammatory cytokines, while TLR2/MyD88 stimulates the production of Th2 cells (Brown et al. 2010).

Bacteria that are phagocytosed by APCs are degraded to peptide fragments and presented to T cells (T helper cells – Th) through the major histocompatibility complex (MHC) class II. When Th cells are activated, they differentiate into Th1 or Th2, and stimulate the production of cytokines (Gleeson et al. 2006). The Th cells help to activate an immune response that includes inflammation and leukocyte infiltration due to the recruitment of phagocytes to the affected area (Murphy et al. 2008).

The final product of TLR activation on APCs such as monocytes is therefore the secretion of cytokines (e.g. IL-1, IL-6, IL-12, TNF-α) which leads to subsequent immune responses. A similar pathway is followed by other TLRs against bacterial and viral infections, fungal infections, and infections with parasites. Thus, TLRs have been shown to have an important role not only in the innate immune system process but also in the enrolment of adaptive immunity.

2.3.2. Cytokines

Cytokines are protein molecules synthesised under different stimuli by some immune cells such as monocytes, macrophages, dendritic cells and lymphocytes. They regulate cellular function through cell survival, apoptosis, proliferation and differentiation (Fulop et al. 2006). They can be classified as pro-inflammatory (e.g. IFN-γ, IL-1, IL-6) or anti-inflammatory cytokines (e.g. IL-2, IL-4, IL-5, IL-10). With factors such as ageing or obesity, a reduction in the anti-inflammatory cytokine (e.g. IL-2) production can occur. Moreover, an increase in the pro-inflammatory cytokine production (e.g. TNF-α, IFN-γ, IL-6) can also occur, which can create an unbalanced immune system, and may therefore contribute to the occurrence of autoimmune disorders and of chronic inflammation processes, leading to the development of metabolic and cardiovascular

diseases, including Type II diabetes and atherosclerosis (Fischer et al. 2007, Fulop et al. 2006).

IL-6 is a cytokine that can be considered to be both pro- and anti-inflammatory. It is produced by monocytes, macrophages and by contracting skeletal muscle (Petersen and Pedersen 2006, Pedersen 2009). IL-6 production can negatively affect TNF-α production, while activating IL-10 synthesis. Conversely, high circulating IL-6 levels are associated with (and are a predictor of risk for) low-grade chronic inflammation and the prevalence of numerous diseases. TNF-α is a pro-inflammatory cytokine as its function is to induce inflammation. Together with IL-6, TNF-α has been linked to several human diseases caused by low-grade chronic inflammation. IL-10 is an anti-inflammatory cytokine produced by monocytes and Th2 cells that inhibits pro-inflammatory cytokine synthesis and stimulates antibody production.

2.3.3. Methods of TLR Analysis and Variability in TLR

Since TLRs were discovered an increasing number of studies have been conducted in the area utilising different methods for analysing TLR expression. Reverse transcription polymerase chain reaction (RT-PCR) (Zanchi et al. 2010), western blot (Frisard et al. 2010) and flow cytometry (Booth et al. 2010, Coen et al. 2010, Simpson et al. 2009, Timmerman et al. 2008) are currently the most common methods in current use. In addition, both human subjects and mice have been used in exercise-related studies. The use of mice has been very common in the analysis of TLR responses in different organs (such as lungs) and different APCs (such as dendritic cells), as they can be use for *in vivo* analysis and can also be sacrificed for a thorough analysis of the various immune system components. However, due to its specificity and applicability it is more reliable to use human participants for sport-related research concerned with human responses,

although in humans, only monocyte APCs can be analysed through blood sampling. Other important APCs such as macrophages can only be analysed from muscle or adipose tissue biopsies (Francaux 2009, Frisard et al. 2010).

The flow cytometer is a user-friendly instrument capable of quickly distinguishing the size and complexity of cells, as well as differentiating cells according to their phenotype. Cells can be easily staining with specific antibodies and a selective analysis can be done using an user friendly computer program. In addition, before acquiring the data, the electronics of the instrument can be optimised for the particular samples studied, increasing the reliability of the data.

2.4. Exercise and the Immune System

2.4.1. Exercise and Infection Risk

Several studies have shown that prolonged exercise reduces the function and expression of some immune cells such as lymphocytes and natural killer cells, supporting the suggestion that strenuous exercise may reduce the host capacity to act against invading pathogens (Bermon 2007, Gleeson and Bishop 2005, Brines et al. 1996, Lancaster et al. 2005, Moreira et al. 2009, Nieman 1995). This could represent an 'open window' for pathogens, allowing URTI and other common infections to gain a foot-hold (Nieman 1994). The changes of several inflammatory markers in this context can decrease virus protection and consequently may possibly account for why athletes appear to be more vulnerable to catching URTI. Moreira et al. (2009) showed in a systematic review that in the majority of the studies analysed athletes demonstrated a greater rate of URTI when compared with non athletes, and that the risk of infection increases as the training load increases.

A “J”-shaped curve has been proposed by Nieman in 1994, which describes the relationship between exercise load and susceptibility to infections, where, on one hand, regular moderate physical activity reduces the risk of URTI below the sedentary individual’s risk. On the other hand, performing a high exercise training load is associated with an above average risk of URTI (Figure 2.2).

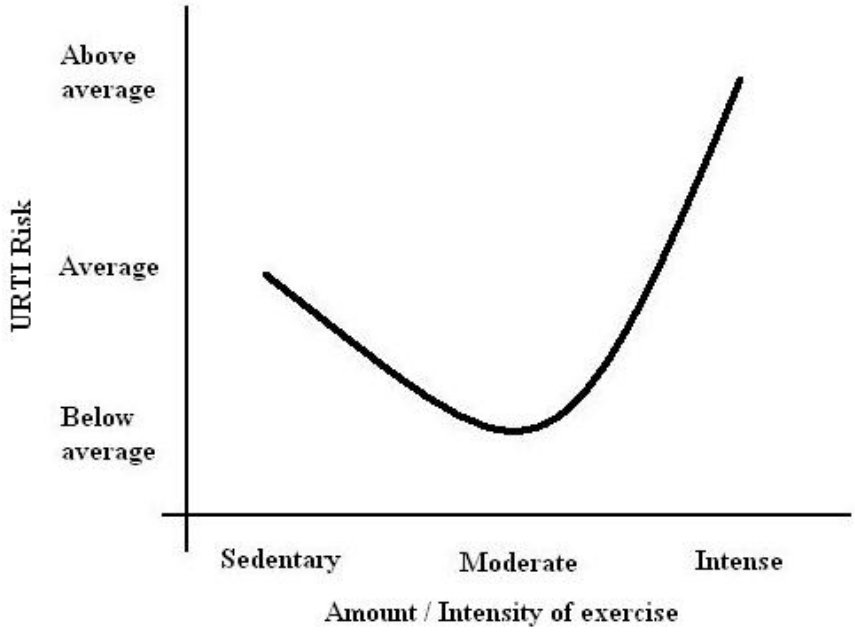

Figure 2.2- J-shaped curve (Adapted from Nieman 1994)

In addition, it has been seen that intense exercise decreases the expression of TLR (Lancaster et al. 2005), which may lead to a less powerful cell-mediated immune response and inflammation, due to a diminished monocyte/macrophage and Th-1 cell cytokine production. The prevention of illnesses during training and competition is highly sought after by sport scientists in response to the urging of athletes and their coaches, since infection almost inevitably negatively affects performance (West et al. 2009). Although factors such as previous URTI episodes and environmental aspects (such as pollutants in the road and chlorine in the water) can influence the results found so far, more research is required to determine what causes the diminution of the

immune system function, and to what degree this reduction has positive or negative effects on human health.

2.4.2. Exercise and the Prevention of Diseases

Factors such as poor diet, obesity (especially visceral fat accumulation), age and low levels of physical activity or fitness have been suggested to be linked to the increased incidence of CVDs such as atherosclerosis, coronary heart disease (narrowing of the coronary arteries), and stroke, as well as diabetes and cancer (Hardman and Stensel 2009). In addition, poor diet (i.e. ingestion of too much energy, fat and sugary foods) has been linked to the increased prevalence of obese people especially in Western society, which can subsequently lead to the development of hypertension, diabetes and hypercholesterolaemia (Hardman and Stensel 2009). In 2002, the World Health Organization (WHO) produced a report showing that more than 60% of the world's population is sedentary, and that if they do exercise, people are not often sufficiently active to gain any health benefits from it. In the UK physical activity levels are below the European average, although the numbers of adults meeting the recommended levels of physical activity increased between 1997 and 2006 in England. Inactivity has been fairly recently identified as a major risk factor for CHD, where 20% of CHD in developed countries is due to low (often zero) levels of physical activity. This statement is also true in relation to stroke, where 10% of stroke incidents in developed countries were due to sedentarism (World Health Organization 2002).

One of the possible explanations regarding the relationship between risk factors and the development of CHD and other forms of CVD include chronically high levels of inflammatory markers in the body. Nicklas et al. (2005) suggested that risk factors are linked with low-grade chronic inflammation, which has been shown to be related to

complications such as atherosclerosis, oxidative stress and endothelial dysfunction – precursors of adverse health conditions. In addition, some studies have shown that persistent high levels of circulatory inflammation markers such as C-reactive protein (CRP), TNF-α and IL-6 are risk factors for CVD not only in the elderly but also in the middle-aged population (Rost et al. 2001, Cesari et al. 2003).

Myers et al. (2004) showed that low levels of physical fitness and all-cause mortality risk are strongly related. According to this study, sedentary people have a greater risk of dying prematurely compared with physically active people. The mechanisms by which exercise reduces the incidence of CVD are not yet fully understood; however, suggestions are being made that exercise may influence lipids and lipoprotein metabolism, reduce high blood pressure, improve myocardial perfusion and oxygen delivery to the heart and reduce inflammation (Nicklas et al. 2005). Therefore, an increasing number of studies are now suggesting a strong association between CVD, CHD and inflammation.

In support of this idea, evidence from observational studies suggests that both decreasing body weight and increasing physical activity levels are effective as treatment for reducing overall inflammation. For example, Colbert et al. (2004) examined 3,075 well-functioning, elderly men and women of mixed ethnicity in relation to exercise level and the association with circulating levels of CRP, IL-6 and TNF-α. As a result, it was found that high levels of physical exercise are related to low levels of all variables analysed when compared with non-physically active people. In relation to body fat mass, fat location and markers of chronic inflammation, Festa et al. (2001) analysed a large multi-ethnic population in relation to body fat mass and fat distribution with circulating CRP levels. A strong correlation was found between plasma CRP concentration and BMI, waist circumference and adipose body mass, suggesting that

inflammatory markers are elevated in populations with a higher adipose mass. It is known that adipose tissue releases cytokines due to the reversible infiltration of inflammatory cells into the adipose tissue; therefore, weight loss either through diet or exercise may reduce inflammation to some extent (Nicklas et al. 2005).

Moreover, increased fitness results in an augmented basic functional capacity, which consequently results in a better and longer quality of life. Therefore, the increased interest in physical exercise regimens as a means of preventing the development of chronic disease can be something beneficial not only for the individual but also for the economy. In the UK, around £30.7 billion per year are spent on the treatment and care of people with CVD. However, although it might be suggested that a cardiac rehabilitation programme through physical exercise would cost less money annually than treating the disease with medication or surgery, no cost estimate for such an exercise regimen has been reported as yet (British Heart Foundation 2008).

In addition, a dysfunctional immune system is also found in the elderly population which may be linked with memory loss and degeneration of neurones in older people (Fulop et al. 2006). Several studies suggest that the continuous exposure to pro-inflammatory cytokines could be responsible for the amplified pro-inflammatory status related to the malfunctioning and unbalancing of the immune system in both elderly and obese populations (Petersen and Pedersen 2005). Nevertheless, exercise has been shown to reduce this chronic low-grade inflammation. For example, Stewart et al. (2005) showed a reduction in inflammation markers in young and old participants after 12 weeks of resistance training by assessing circulating levels of IL-1β, IL-6 and TNF- α. In support of this research, a cross sectional study by McFarlin et al. (2006) showed that a physically active lifestyle may possess anti-inflammatory properties regardless of sex or age. Therefore, exercise-induced elevations of anti-inflammatory cytokines such as

IL-10, combined with training-induced lowering the expression of APC receptors such as toll-like receptor (TLRs) could play an important role in the link between exercise and reduced inflammation (Flynn et al. 2003; Gleeson 2007, Gleeson et al. 2011c).

Reduction in the TLR expression and consequent reduction in cytokine release has therefore, been suggested to be the next step forward in research for the decrease of CVDs. Few studies in this area have been done so far. Flynn et al. (2003) observed that 10 weeks of resistance training reduced $CD14^+$ monocyte TLR4 expression in elderly women and, from this group, the low TLR4 "expressers" had lower cytokine production compared with the high TLR4 "expressers". In addition, McFarlin et al. (2004) showed that monocyte TLR4 expression was lower in resistance trained women and was associated with lower inflammatory cytokines. Subsequent research showed that physical activity status (but not age) influenced both TLR4 expression and IL-6 production (McFarlin et al. 2006).

2.5. Exercise and TLR Response

2.5.1. Acute Strenuous Exercise and TLR2 and TLR4 Responses

Several studies have indicated that different types of physical activity may play a role in modifying TLR expression and downstream responses (Flynn et al. 2003, McFarlin et al. 2004, McFarlin et al. 2006, Stewart et al. 2005). Furthermore, it has been suggested that a large number of immune system aspects, including TLR expression and function can be influenced by strenuous exercise (Lancaster et al. 2005), but results have not always been consistent. TLR2 and TLR4 expression have been shown to be reduced after an acute bout of strenuous cycling exercise (Lancaster et al. 2005) but other studies reported no change in TLR2 after 45 minutes of running (Simpson et al. 2009)

and even an increase in both TLR2 and TLR4 expression after cycling a 60-km time trial (Booth et al. 2010). From the decline in TLR function reported by Lancaster et al. (2005) and Simpson et al. (2009), it was suggested that reductions in accessory signal molecule expression (e.g. Human Leukocyte Antigen DR-1, HLA.DR), costimulatory molecule expression (e.g. CD80 and CD86) and APC activation after acute exercise may occur, which may impair immune function and increase susceptibility to infection such as URTI (Gleeson 2006). Other factors that also may be related to the reduction in cell surface expression of TLR2 and TLR4 after an acute bout of strenuous exercise are the heat shock proteins (HSPs), the changes in HLA.DR expression and the circulating concentration of cytokines such as IL-6 and IL-10 (Gleeson et al. 2006, Simpson et al. 2009). The inconsistent results may be due to the difference in participant fitness levels, exercise type, duration and intensity and methods used to analyse the variables; therefore, care must be taken to interpret these results.

2.5.2. Chronic Exercise and TLR2 and TLR4 Responses

There have been several studies (both cross-sectional and longitudinal) showing that people who engage in regular moderate-intensity physical activity maintain a lower risk of self reported respiratory symptoms than both the sedentary and athletic population (Martin et al. 2009, Matthews et al. 2002, Nieman 1997, Nieman et al. 2010). In addition, it has been suggested that TLR2 and TLR4 responses can be modified with physical training such as endurance and resistance exercises (Stewart et al. 2005). Two studies suggested that exercise-induced changes in monocyte TLR2 and TLR4 may be cumulative over time since 12 weeks of resistance and aerobic exercise training has been shown to reduce messenger RNA (mRNA) for TLR4 and cell surface TLR4

expression (Flynn et al. 2003, Stewart et al. 2005), although research to confirm these findings is limited to date.

Increased levels of monocyte TLR expression have shown to be linked with higher cytokine production possibly leading to chronic inflammation (Stewart et al. 2005, Timmerman et al. 2008, Vitseva et al. 2008). As mentioned previously, chronic inflammation is related to the development and progress of several diseases (Parker et al. 2007); therefore the reduction in TLR2 and TLR4 expression in sedentary populations through exercise may be a crucial key in reducing the severity, or even preventing, the occurrence of chronic diseases.

2.5.3. The Influence of Sex and Training levels on TLR2 and TLR4 Expression

To date, only two studies have compared the TLR4 expression between a young and old population. According to Stewart et al. (2005), TLR4 expression was reduced equally after 12 weeks of exercise in both young (18-35 year olds) and old (65-85 year olds) subject groups. Similarly, McFarlin et al. (2006) showed that TLR4 expression is related to physical activity status but not age. Although the authors used both male and female participants, there are no studies as yet comparing the TLR2 and TLR4 expression between sexes.

A study conducted by Gleeson et al. (2011a) examined sex differences in several immunological markers and URTI incidence. Higher salivary flow rates, and higher immunoglobulin A (IgA, an antibody related to mucosal immunity) concentration and secretion rates were observed in males compared with females who engaged in a similar volume (average 10h/week) of endurance-based exercise training. Although this study showed no sex difference in the number URTI episodes, leukocyte counts, and antigen-

stimulated cytokine production by whole blood culture, monocyte TLR expression was not analysed, suggesting the need for research in this area.

2.6. Overview

This Chapter presents a review of the role of the immune system, with a focus on monocyte subsets, TLR and cytokines. It has been suggested that monocyte capability in producing cytokines is highly related to TLR stimulation and that pro-inflammatory monocytes have a greater expression of TLR2 and TLR4 compared with classical monocytes. The activation of TLRs results in rapid multiple immune responses such as cytokine production, proven to be a competent way for the human body to deal with infection. On the contrary, augmented production of inflammatory cytokines is related to the incidence of chronic diseases that may have been caused by TLR gene mutation or failure to regulate cytokine production.

This Chapter also describes how physical exercise has been related to positive (prevention of chronic diseases) and negative (increased susceptibility to URTI) effects on the immune system, and how this may be related to the influence of exercise on TLR2 and TLR4 expression, resulting in subsequent changes in cytokine production. Moreover, it draws attention to the available research that has investigated factors such as the amount, intensity, duration and type of exercise, and their influence on TLR responses, leading either to a positive or a negative outcome. Little research has been done to analyse the influence of sex on TLR2 and TLR4 expression for an active population and its influence on the immune system. This indicates that the TLR response to exercise requires more attention and, in conjunction with that, the responses

of other immunological markers (such as IL-6 and IL-10) and their association with TLR expression must also be investigated.

3. General Methods

3.1. Introduction

This Chapter is separated into five main sections, describing the common material and methodology used in the studies. Any other specific methods and participants' characterization is discussed in more detail in the appropriate Chapter. Section 3.2 describes the recruitment criteria for the selection of participants. The general exercise protocols are described in section 3.3. The biochemical assays and any other method used for analyses of the participants' whole blood and plasma are described in section 3.4. Section 3.5 outlines the statistical analysis and section 3.6 presents the methods and results of reproducibility tests of blood measurements and the coefficient of variation of the flow cytometry and cell count analyses.

3.2. Recruitment Criteria for the Selection of Participants

The recruitment criteria varied according to each study and it will be described in detail in the respective Chapters. As a common criterion, before voluntarily giving their informed consent, participants were fully informed (verbally and in writing) of the nature of the experimental procedures and possible risks of the experiments. All participants were asked to complete a general health screening and a physical activity questionnaire in which they described their weekly training levels (see Appendices). The health screen questionnaire was checked to ensure that they were suitable to participate. In addition, they were made aware of their right to withdraw from the study at any time without justification. Participants then signed an informed consent form. Ethical approval for all studies was obtained from Loughborough University ethics committee and studies were conducted in accordance with the Declaration of Helsinki.

The main criteria for the direct exclusion of participants from the studies were:

- Having any of their family members, otherwise healthy, under the age of 35 that died suddenly during or soon after exercise;
- Smoking;
- Problems with joints, muscles or tendons that would compromise their performance in exercise studies;
- Any illness or diseases that could influence the immunological results (including cold or flu in the previous 4 weeks prior to the study);
- Those having donated more than 500 ml of blood in the three months preceding the study;
- Taking medications known to influence (directly or indirectly) leukocyte function or inflammation,
- Having a VO_{2peak} result above or below the range specified for the study.

3.3. Exercise Protocol

No exercise was conducted in one study described in this thesis (Chapter 4). In this study, participants were asked to maintain their regular daily activity and physical activity routines, but to refrain from physical activity the day before attending the laboratory. The monitoring and recording of their physical activity levels was undertaken using an international physical activity questionnaire (IPAQ, http://www.ipaq.ki.se/downloads.htm) providing quantitative information on training loads in metabolic equivalent (MET)-h/week (Craig et al. 2003). In Chapter 5, all participants completed three exercise bouts: two preliminary trials (VO_{2peak} determination and Familiarisation) and one main trial. In Chapter 6 participants performed one VO_{2peak} test, then two VO_{2peak} tests separate by two hours in the

following week. Chapter 7 describes a 2-week training study which required two preliminary exercise bouts (VO_{2peak} determination and Familiarisation) and one main trial, followed then by 2 weeks of exercise training and then the repetition of the main trial and VO_{2peak} determination. Chapter 8 had the same preliminary exercises as Chapter 7, followed then by 4 weeks of exercise training and then the repetition of the VO_{2peak} determination and main trial. All main trials were only performed if participants had avoided strenuous physical activity the day before coming to the laboratory.

3.3.1. VO_{2peak} Determination

Participants completed a continuous incremental exercise test to volitional exhaustion on a cycle ergometer. Different protocols were used in different studies.

- For Chapters 5 and 6 participants cycled on a Monark 874E ergometer. After a short warm-up (no more than 5 min with a load of no more than 60 W), participants began cycling at 95 W. The work rate was increased by 35 W every 3 min, until volitional exhaustion. A Douglas bag was used to collect the expired gas during the final minute of each stage. Also at this time, rating of perceived exertion (RPE, using the 6 – 20 Borg Scale) and heart rate (using a Polar A1 telemetric device, Polar Electro, Kempele, Finland) were recorded. When the participant signalled that they could continue for only 1 more minute at the required intensity, a new expired gas collection was commenced and verbal encouragement was given, and the test was ended after the minute was complete. Expired gas was analysed by a Servomex O_2 and CO_2 analyser (series 1400, Crowborough, UK), and the gas volume was measured using a dry gas

meter (Harvard Instruments, Edenbridge, UK), allowing determination of the VO_2 at each work rate.

- For Chapter 7 an Excalibur electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) was used. After a short warm-up (no more than 5 min with the load no more than 60 W), participants began cycling at 100 W and had the work rate increased by 30 W every 3 min with participants cycling at 70 rpm. The work rate was increased by 35 W every 3 min, until volitional exhaustion. An online breath by breath analyser (Ultima CPX, MedGraphics, MN, USA) was used to measure expired gas. Heart rate and RPE (Polar RS200, Polar Electro, Kempele, Finland) were recorded every 3 minutes.
- For Chapter 8, an Excalibur electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) was used following the same protocol described above. When female participants were recruited, the work load started at 75 W and was increased by 25 W every 3 min.

In order to confirm that subjects performed at their maximum effort, all test results were checked to see if at least two out of the three following criteria were met: subjects achieved within 10 bpm of their age predicted maximum heart rate (where maximum heart rate = 220 beats.min^{-1}- participant's age); participants' respiratory exchange ratio (RER) was >1.15 or they indicated a score of ≥ 19 on the RPE scale (Whaley et al. 2005).

3.3.2. High-Intensity Intermittent Training – HIIT

The high-intensity intermittent training protocol was used in Chapters 7 and 8. In this, participants performed ten 4-min cycling intervals each separated by a 2-min rest period

at a workload corresponding to that which elicited 90% of their VO_{2peak}. Heart rate and RPE measurements were made during the last 30 s of each 4-min stage.

3.4. Blood Sample Collection, Plasma Separation and Biochemical Assays

3.4.1. Blood Sample Collection and Plasma Separation

Samples of whole blood were collected in 4 ml vacuum tubes containing EDTA and in 6 ml vacuum tubes containing sodium heparin as anticoagulant (Becton-Dickinson, Oxford, UK). Whole blood was collected from the antecubital vein by venepuncture. Blood EDTA samples were used to measure the total blood count using an automated haematology analyser (AC·T 5diff, Beckman Coulter, Buckinghamshire, UK) and then centrifuged for 10 min at 1500 *g* at 4ºC (Allegra 21R, Beckman Coulter, Buckinghamshire, UK). Aliquots of plasma were immediately stored within a -80 ºC freezer for future analysis. Any changes in plasma volume before and after exercise trials were calculated from measurements of haemoglobin and haematocrit according to the method described by Dill and Costill (1974). Heparinised blood samples were used to determine the cell surface expression of TLR on circulating monocytes (described in section 3.4.2).

3.4.2. $CD14^+$ Monocyte and TLR Expression

In order to determine the cell surface expression of TLR on circulating $CD14^+$ monocytes 80 µl of heparinized blood was surface stained with monoclonal antibodies containing different fluorochromes: 20 µl CD14-Fluorescein isothiocyanate (FITC) (Becton– Dickinson Bioscience, Oxford, UK) and antihuman phycoerythrin (PE)-conjugated TLR2 (clone TL2.1, 20 µl), PE-TLR4 (clone HTA125, 20 µl) or PE-Isotype

control (Mouse IgG2k, 20 µl) (e-Bioscience, San Diego, CA). All the tubes were incubated at room temperature for 20 min in darkness. To lyse the red blood cells, tubes were then filled with a lysis buffer solution (10x dilution, FACS lyse, BD Biosciences, Oxford, UK) and re-incubated for 10 min under the same conditions. All samples were then centrifuged at 1000 *g* for 6 min (Allegra 21R centrifuge, Beckman Coulter, Buckinghamshire, UK). Supernatants were aspirated and cell pellets were re-suspended in phosphate buffered saline (PBS) containing 0.1% of bovine serum albumin (BSA) and 2 mM EDTA and centrifuged as previously described. Finally, supernatants were aspirated again, and cell pellets were re-suspended in 500 µl of PBS/BSA/EDTA buffer and transferred to FACS tubes for analysis.

Samples were analysed on a flow cytometer (BD FACSCalibur) equipped with the CellQuest software package (BD Biosciences, Oxford, UK). The FACSCalibur flow cytometer is equipped with a blue laser that emits light at a wavelength of 488 nm. The light emitted by this laser is directed at cells as they pass through in single file flow. The diffracted, refracted and reflected light emitted from the cells is then passed through various filters and sensed by optical filters. The detected light signals are then transformed into comparative electronic signals and digitised (as dots) on the computer screen.

Before acquiring the data, the electronics of the instrument were optimised for the particular samples studied. Instrument compensation adjustments for overlap in the emission spectra of the fluorochromes was performed to make sure that each detector was only identifying one fluorochrome and that only the cell populations of interest came into view on the data plots. To ensure that the instrument settings were stable and

to allow consistent conditions over the course of the studies, FACS comp (the in-built quality control software) was performed before optimising any instrument settings.

Cells were gated according to side scatter and CD14-FITC expression (which characterises monocytes, Figure 3.1a), and the geometric mean fluorescence intensity (GMFI) of the Isotype control (ISO), and of TLR2 and TLR4 antibodies in the $CD14^+$ cell gated population was obtained to quantify TLR expression (Figure Figure 3.1b, total of 3,000 cells acquired for each sample). Isotype control GMFI was used to correct values for non-specific binding. Chapters 7 and 8 present slight differences in this protocol (such as the analysis of the monocyte subsets), which will be described in the appropriated Chapters.

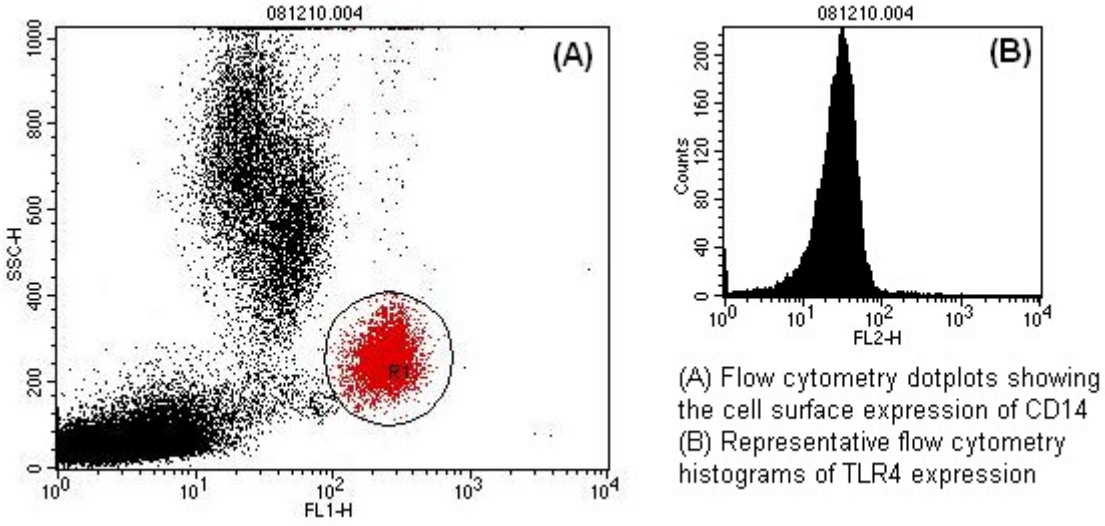

Figure 3.1- Flow cytometer results exemplified

One of the main problems in immunology research is the considerable variability that exists within and between people. Therefore, a reproducibility study was carried out to ensure that the methods applied using the flow cytometer were valid and reliable for each individual and for the group, as no study covering that has been shown yet. Moreover, as some of the studies mentioned in this thesis differ from the thesis'

methodology for TLR analysis, it is suggested that the interpretation of the results may be taken with caution since direct comparisons between studies may be difficult.

3.5. Statistical Analysis

Normality tests were carried out on all data. The following tests were used according to normality results and the number of variables:

- Independent or paired sample t-test for normally distributed data;
- Mann-Whitney U-test for not normally distributed data;
- For the repeated not normally distributed sample, Friedman's ANOVA was used; if a statistically significant difference was found, a Mann-Whitney U-test (corrected for the time points) was carried out to locate the difference;
- One-way ANOVA with repeated measures for normally distributed data;
- Pearson's Correlation, where 0.0 to 0.3 is a weak correlation, 0.4 to 0.6 is medium and 0.7 to 1.0 is high correlation (in both ± direction).
- The program G*Power 3.1.3 (Faul et al. 2007) was used to determine the number of participants needed to achieve a power of between 0.6 and 0.8. All studies aimed for an effect size of at least 0.5.

In order to calculate these statistics, PASW version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used. Statistical significance was accepted at $P < 0.05$. All results are presented as mean ± standard deviation (SD).

3.6. The Reproducibility of the Flow Cytometry and Cell Count Analysis

3.6.1. Participants

For involvement in this small study participants had to be between 18 and 55 years old and non-smokers. Ten males and five females were recruited (35 ± 9 years; 71.3 ± 9.3 kg; 174 ± 10 cm; BMI 23.7 ± 3.0) when comparing the automated blood count machine with the manual white blood cell count and for the TLR4 expression reproducibility analysis. For the reproducibility of the results produced by the automated blood analyser, blood from 49 subjects were used. All participants were healthy and were not taking any medication.

3.6.2. Experimental Procedures

Participants were asked to come to the Exercise Immunology Laboratory for one visit only. In an overnight fasted state they had their body mass and height measured, and gave a venous blood sample. Blood samples were collected as described above in Section 3.4.

Cell Count Analysis

EDTA blood samples (N=49) were analysed in duplicate for assessment of the reproducibility and coefficient of variation (CV) of the total cell count, using an automated haematology analyser (AC·T 5diff, Beckman Coulter, Buckinghamshire, UK). In addition, smears were created by placing 10 µl of blood onto a slide and then staining using the *Wright's staining* protocol (N=20). Firstly, the blood smear was dipped into 100% methanol and left to dry. Once dried, slides were then dipped into Wright's stain (Sigma-Aldrich, Dorset, UK) for 2 min. Subsequently the slides were

washed with deionised water and left to dry. Slides were analysed for total white blood cell count using a microscope with a 100x lens. A total of 100 white blood cells were counted and differentiated by staining characteristics and morphology and results were presented as a percentage.

$CD14^+$ Monocyte and TLR Expression

Heparinised blood samples were used to determine the reproducibility and the CV of the cell surface expression of TLR on circulating $CD14^+$ monocytes. All samples were analysed in duplicate using the staining method explained in Section 3.4.

3.6.3. The Reproducibility of the Cell Count Analysis – Results

All variables were normally distributed. A paired sample t-test ($P < 0.05$) was used to compare the results of the white blood cell counts from the automated analyser with the manual counting of the stained slides. The duplicate analysis of the blood samples using an automated machine is shown in Table 3.1.

Table 3.1 – Blood haematological analysis

	Analysis	**CV**
RBC	4.7 ± 0.04	1%
HCT (%)	40.4 ± 0.3	1%
HB (g/dL)	13.3 ± 0.08	1%
WBC ($x10^9$/L)	5.9 ± 0.06	1%
NEU ($x10^9$/L)	3.0 ± 0.04	1%
LYM ($x10^9$/L)	2.2 ± 0.5	2%
MON ($x10^9$/L)	0.5 ± 0.03	6%
EOS ($x10^9$/L)	0.2 ± 0.02	12%

(RBC = Red blood cells; HCT = Haematocrit; HB = Haemoglobin; WBC = white blood cell; NEU = neutrophil; LYM = Lymphocyte; MON = Monocyte; EUS = Eosinophil); all values $x10^9$/L of blood

There was no difference in the white blood cell count when comparing the automated and the manual cell count, as shown in Table 3.2.

Table 3.2 –White blood cell count method comparison of leukocyte subsets (% of total)

%	**Automated analyser**	**Slide analysis**	***P*value**
WBC	100	100	
NEU	48 ± 9	52 ± 4	0.050
LYM	38 ± 8	37 ± 7	0.684
MON	9 ± 2	7 ± 4	0.073
EOS	3 ± 2	3 ± 2	0.511
BAS	0 ± 1	1 ± 1	0.334

(WBC = white blood cell; NEU = neutrophil; LYM = Lymphocyte; MON = Monocyte; EUS = Eosinophil; BAS = Basophil); all values $x10^9$/L of blood; N= 20.

3.6.4. The Reproducibility of the Flow Cytometry Analysis – Results

Geometric mean fluorescence intensity (GMFI) was 29.1 ± 3.7 for TLR2 and 19.1 ± 2.0 for TLR4, with a coefficient of variation of the analysis was 10% and 11% respectively.

4. Chapter 4 - Training and Sex Differences in TLR4 Expression: Relationship with Respiratory Infection Incidence?

Abstract: The purpose of this study was to determine whether URTI incidence, $CD14^+$ monocyte TLR4 expression and cytokine production differed between subjects who practiced regular moderate amounts of exercise (MED, N=39) compared with those who engaged in very little exercise (LOW, N=13) or more than 9 hours of endurance-based training per week (HIGH, N=40). In addition, a second aim was to determine if sex differences exist in resting $CD14^+$ monocyte TLR4 expression and cytokine production by antigen-stimulated whole blood culture in these populations (N=52 males and N=40 females). TLR4 expression was shown to be higher in females compared with males (40 ± 23 and 28 ± 11 GMFI respectively) and in the population with a low compared with a high level of physical activity (39 ± 26 and 32 ± 17 GMFI respectively). No differences were found between sexes for stimulated cytokine production and URTI symptoms. Different levels of physical activity did not result in difference in pro-inflammatory cytokine production, but a difference in the IL-10 anti-inflammatory cytokine production was found between the physically active groups, despite no difference in URTI incidence and no correlation with TLR4 expression. Therefore, sex differences and physical activity levels in immune function need to be considered in future mixed-gender studies on exercise when analysing TLR4 expression.

4.1. Introduction

Changes in the immune system have been shown to be modulated by exercise and to be dependent on intensity and duration (Gleeson 2007). As suggested by Nieman's "J-

shape" relationship theory (Nieman 1994), engaging in chronic moderate activity may improve immune function above sedentary levels, while high amounts of high-intensity prolonged exercise may weaken immune function and increase susceptibility to URTI. Little clinical evidence supports the idea of a real difference in immune function between the sedentary and physically active population. However, the fact that athletes engaged in high amounts of training present higher upper respiratory tract infection symptoms than athletes that exercise less hours is confirmed by several studies (Neiman et al. 2010, Gleeson et al. 2011b). However, it has been suggested that, in the true resting state, immune function in the sedentary individuals and athletes is generally similar (Gleeson, 2007). According to Gleeson (2007), a possible reason why prolonged exercise increases susceptibility to infection in athletes is due to the decrease of TLR expression, with the consequent reduction of cytokine production by macrophages and Th1 cells. This may weaken cell-mediated immunity and increase the chances of infection. So far, no studies have examined the TLR expression and its relationship with URTI episodes in an athletic population; however, it can be suggested that this impairment in the immune system may be concerning for athletes, as it can lead to impaired exercise performance and persistent fatigue (Gleeson 2007).

In addition, other variables such as age, sex and lifestyle habits may affect the capacity of the immune system to defend against common infections such as URTI (Gleeson et al. 2011a, Gleeson et al. 2011b, Gillum et al. 2011). Although few studies found a sex difference in some immunological markers such as IL-1, IL-4 and IL-10, others suggest that males and females are equally susceptible to infection at rest, and produce similar concentrations of pro- and anti-inflammatory cytokines when challenged (Gillum et al. 2011). For example, Gleeson et al. (2011a) examined sex differences in several immunological markers and URTI incidence in a physically active population. Higher

salivary flow rates, and higher saliva immunoglobulin A (IgA, an antibody related to mucosal immunity) concentration and secretion rates were observed in males compared with females that engaged in a similar volume (average 10h/week) of endurance-based exercise training. Although this study showed no sex difference in the number of URTI episodes, blood leukocyte counts, and antigen-stimulated cytokine production by whole blood culture, monocyte TLR expression were not analysed, suggesting the need for research in this area.

The aim of this study was therefore to examine URTI incidence and its possible association with resting values of $CD14^+$ monocyte TLR4 expression and cytokine production by antigen-stimulated whole blood culture in an athletic population. In particular, it was to be determined whether URTI incidence and any immune variables differed between subjects who practiced regular moderate amounts of exercise (defined as 3-8 hours of exercise per week) compared with those who engaged in very little exercise (0-2 h/week, i.e., essentially sedentary people) or more than 9 hours of endurance-based training per week. It was hypothesised that high volume training would be associated with a higher incidence of URTI and an impaired pro-inflammatory cytokine response and/or an elevated anti-inflammatory cytokine response to the multi-antigen challenge compared with subjects engaged in lower levels of physical activity.

In addition, a second aim was to determine if sex differences exist in resting $CD14^+$ monocyte TLR4 expression and cytokine production by antigen-stimulated whole blood culture in these populations. It was hypothesised that the incidence of URTI and the production of pro- and anti-inflammatory cytokine response to the multi-antigen challenge would be different between sexes.

4.2. Methods

Some of the data for this Chapter were collected as part of a project that led to publications such as Gleeson et al. 2011a, Gleeson et al. 2011b. Therefore, most aspects of the methodology, such as the participants of the study and the protocol are similar to the described in these papers. Tests performed specifically in this study and that were not described in Chapter 3 are fully explained.

4.2.1. Participants

The physically active population were composed of 46 males and 33 females (18 to 35 year olds) who practiced at least 3 h/week of endurance based activities. Additionally, 6 males and 7 females (18 to 45 year olds) were recruited as part of the sedentary group (Table 4.1). For data analysis, subjects were allocated to one of three groups according to their self-reported hours of weekly training: 0-2 h/week, 3-8 h/week and 9 or more h/week, designated as low (LOW), medium (MED) and high (HIGH) volume training groups, respectively. All participants were asked to complete a general health screen questionnaire and a short version of the IPAQ (see Appendices) where they described their weekly training levels.

Table 4.1 – Participants general characteristics

	Male (N=52)	**Female (N=40)**
IPAQ score ((MET)-h/week)	64.3 ± 39.2	54.5 ± 27.6
Training (h/wk)	8.7 ± 5.2	7.6 ± 4.5
Age (years)	23 ± 5	22 ± 4
Height (m)	1.8 ± 0.1	1.7 ± 0.1
Body mass (kg)	79.5 ± 11.7	62.6 ± 5.8
BMI (kg/(m)^2)	24.2 ± 2.8	22.4 ± 2.5

4.2.2. Experimental Procedures

Participants were asked to come to the Exercise Immunology Laboratory in an overnight fasted state. Body mass and height were measured, and a resting venous blood sample was taken.

Blood Sample Collection

Samples of whole blood were collected from an antecubital vein into tubes containing EDTA and sodium heparin as anticoagulant (Becton-Dickinson, Oxford, UK).

Cell Count Analysis

EDTA samples were analysed for assessment of the total and differential leukocyte count, using an automated haematology analyser (AC·T 5diff, Beckman Coulter, Buckinghamshire, UK).

$CD14^+$ Monocyte and TLR Expression

Heparinised samples were used to determine the cell surface expression of TLR on circulating $CD14^+$ monocytes. All samples were analysed for TLR4 expression using the staining method explained in Chapter 3.

Antigen-Stimulated Cytokine Production

Stimulated whole blood culture production of cytokines (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6, IL-8 and IL-10) was determined as described in Gleeson et al. (2011a) only for the physically active groups. The stimulant was a commercially available multi-antigen vaccine (Pediacel Vaccine, Sanofi Pasteur, UK) containing diphtheria, tetanus, acellular pertussis, poliomyelitis and haemophilus influenzae type b antigens. Briefly,

heparinised whole blood was cultured with vaccine (1:4000 dilution) at 37 °C and 5% CO_2 for 24 h. After centrifugation at 1500 *g* for 10 min at 4ºC, supernatants were collected and stored frozen at -80°C prior to the analysis of cytokine concentrations using an Evidence Investigator System using the high sensitivity cytokine biochip array EV3513 (Randox, County Antrim, UK). The intra-assay coefficient of variation for all measured cytokines was less than 5.0%.

Questionnaires

During the 4-month subsequent study period subjects on the physically active groups were requested to continue with their normal training programmes whilst completing a health (URTI symptoms) questionnaire on a weekly basis. Supplements (vitamins and minerals, etc.) were not permitted during this period. Subjects were not required to abstain from medication when they were suffering from illness symptoms but they were required to report any unprescribed medications taken and visits to the doctor.
The illness symptoms listed on the questionnaire were: sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains, weakness, headache and loss of sleep. The non-numerical ratings of light, moderate or severe (L, M or S, respectively) of severity of symptoms were scored as 1, 2 or 3, respectively to provide a quantitative means of data analysis (Fricker et al., 2005) and the total symptom score for every subject each week was calculated by multiplying the total number of days each symptom was experienced by the numerical symptom severity rating. A single URTI episode was defined as a period during which the weekly total symptom score was ≥12 and separated by at least one week from another week with a total symptom score ≥12. Subjects were also asked to rate the impact of illness symptoms on their ability to train (normal training maintained, training

reduced or training discontinued; L, M or S, respectively). This score was chosen as to achieve it, a subject would have to record at least 3 moderate symptoms lasting for 2 days or 2 moderate symptoms lasting for at least 3 days in a given week. Subjects were also asked to fill in a standard short form International Physical Activity Questionnaire (IPAQ) at weekly intervals, thus providing quantitative information on training loads in metabolic equivalent (MET)-h/week (Craig et al. 2003).

Statistical Analysis

Normality tests were carried out on the data. Independent samples t-test and Mann-Whitney U test were used to compare any sex difference on TLR4 expression and all the other variables. The results of the non-parametric test showed similar results to t-tests, and so only t-test values ($P \leq 0.05$) will be presented in the Results section. One way ANOVA ($P \leq 0.05$) was used to calculate any difference in the TLR4 expression according to the physical activity level. Relationships between variables were examined using Pearson's correlation.

4.3. Results

4.3.1. Training Load and URTI Incidence

Participants were divided into three different groups according to their level of physical activity (0 to 2h·week^{-1}[LOW, N=13], 3 to 8 h·week^{-1} [MED, N=39] or ≥ 9 h·week^{-1}[HIGH, N=40]). The LOW, MED and HIGH groups participated in moderate-vigorous exercise for 1.2 ± 0.9, 6.1 ± 1.3 and 12.6 ± 3.7 h·week^{-1}, respectively ($P < 0.001$). Analysis of the IPAQ questionnaires showed that the weekly training loads were substantially higher in the HIGH group compared with the MED and LOW groups, and also higher on the MED compared with LOW (Table 4.2). The IPAQ scores in MET-

h/week correlated significantly with subjects' self-reported training loads at the start of the study (r = 0.6, $P < 0.001$, n=87).

Analysis of the URTI symptom questionnaires could only be performed in the MED and HIGH groups. Results indicated that the HIGH and MED groups experienced 2.5 ± 2.7 and 1.5 ± 2.5 episodes respectively during the 4-month period but they were not significantly different ($P = 0.142$). However, the proportion of subjects who suffered one or more episodes of URTI was higher in the HIGH (68%) compared with the MED (59%) group.

In relation to the haematological values, the LOW group presented lower levels of haematocrit and high numbers of lymphocytes compared with MED and HIGH groups and higher white blood cell and monocyte counts compared with the HIGH group (Table 4.2).

Table 4.2 - Anthropometric and haematological characteristics of the low (LOW), medium (MED) and high (HIGH) volume training groups

	LOW (N=13)	MED (N=39)	HIGH (N=40)	*P* value
IPAQ (MET-h/week)	20.1 ± 8.9	51.9 ± 26.5*	78.3 ± 34.5*$^{\$}$	0.001
Training (h/week)	1.2 ± 1.0	6.1 ± 1.3	12.6 ± 3.7*$^{\$}$	0.001
Age (years)	25 ± 5	23 ± 4	22 ± 8	0.086
Height	1.7 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	0.741
Body Mass	75.5 ± 18.3	70.8 ± 10.7	72.6 ± 12.7	0.505
BMI	24.8 ± 3.9	23.1 ± 2.6	23.4 ± 2.5	0.162
RBC	4.4 ± 0.8	4.7 ± 0.5	4.7 ± 0.5	0.072
HCT	37.6 ± 6.6	41.2 ± 3.5*	40.9 ± 3.1*	0.013
HB	14.5 ± 1.7	13.9 ± 1.3	13.8 ± 1.2	0.239
WBC	7.0 ± 1.2	6.0 ± 1.5	5.6 ± 1.3*	0.008
NEU	3.4 ± 0.8	3.1 ± 1.2	2.8 ± 1.0	0.185
LYM	2.7 ± 0.9	2.1 ± 0.5*	2.0 ± 0.7*	0.006
MON	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.1*	0.046
EUS	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.804

* Significant difference from LOW, $^{\$}$ significant difference from MED; Mean ± SD; (RBC = Red blood cells; HCT = Haematocrit; HB = Haemoglobin; WBC = white blood cell; NEU = neutrophil; LYM = Lymphocyte; MON = Monocyte; EUS = Eosinophil);

4.3.2. The Resting Values of the Monocyte TRL4 Expression in Different Levels of Physical Activity.

TLR4 expression at rest was higher in the LOW compared with the HIGH group (P = 0.03), and a trend of a higher TLR4 expression during rest was found for the LOW group when compared with the MED group (P = 0.08). Results can be seen in Figure 4.1. There was no TLR expression difference between the MED and HIGH groups (P = 1.00) and no difference of TLR4 expression between the sexes within groups (P > 0.32).

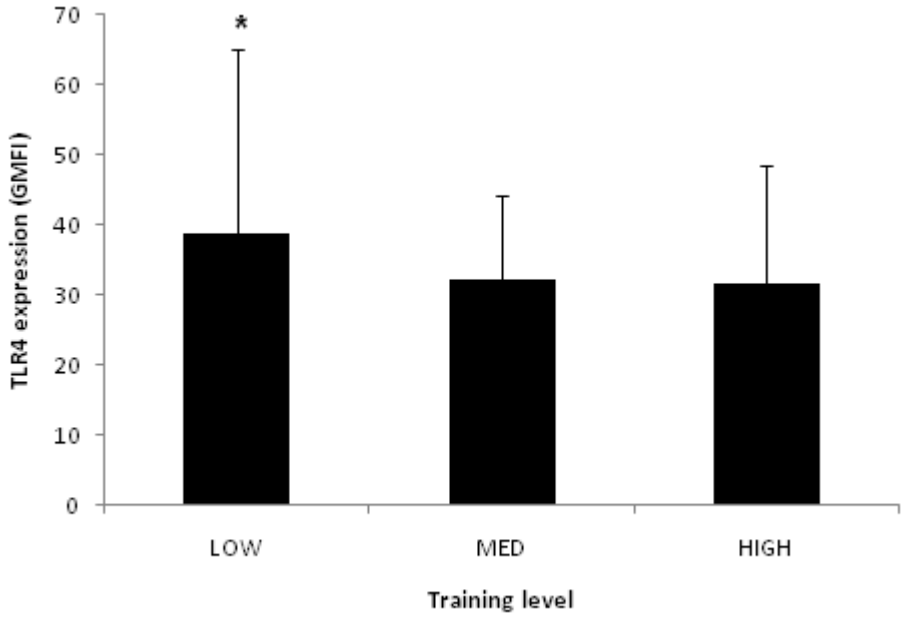

Figure 4.1 – Resting TLR4 expression according to physical activity level.
* Significantly different from the HIGH group

4.3.3. Influence of Training Load on Blood Immune Variables

Analysis of the stimulated whole blood culture production of cytokines could only be performed in the MED and HIGH groups. The HIGH group showed higher IL-10 production after incubation when compared with the MED group. A trend for a significant difference was found between MED and HIGH IFN-γ production (P =

0.066), where the HIGH group presented a higher cytokine production after incubation compared with the MED. No significant differences were found in any of the other cytokine analysed (TNF-α, IL-1β, IL-1α, IL-2, IL-4, IL-6 and IL-8, Table 4.3). In addition, none of the cytokines were significantly correlated with TLR4 expression (P > 0.05).

Table 4.3 – Stimulated whole blood cytokine production in medium (MED) and high (HIGH) physically active population

(pg/ml)	MED (n=39)	HIGH (N=38)	*P* values
IL-2 production	93.3 ± 177.6	156.55 ± 181	0.127
IL-4 production	3.7 ± 3.8	4.0 ± 4.6	0.811
IL-6 production	147.5 ± 127.9	157.3 ± 134.4	0.743
IL-8 production	1034.8 ± 718.1	1050.1 ± 718.0	0.926
IL-10 production	2.4 ± 2.1*	5.4 ± 6.6	0.008
IFN-γ production	15.8 ± 19.0	26.1 ± 28.0	0.066
TNF-α production	17.3 ± 27.2	28.8 ± 49.0	0.207
IL-1α production	1.2 ± 1.6	1.4 ± 2.4	0.629
IL-1β production	8.0 ± 9.7	7.6 ± 6.9	0.850

* Statistically different from HIGH; IL= interleukin; IFN-γ = interferon gamma; TNF-α = Tumour necrosis factor

4.3.4. Sex and URTI Incidence

Analysis of the URTI symptom questionnaires was performed according to sex. Results indicated that the males and females experienced 1.7 ± 2.2 and 2.3 ± 2.5 episodes respectively during the 4-month period but this was not significantly different (*P* = 0.284). However, the proportion of subjects who suffered one or more episodes of URTI was higher in the female (70%) compared with the male (57%) group.

4.3.5. Sex Differences in Resting Values of the Monocyte TRL4 Expression

Participants' $CD14^+$ monocyte TLR4 expression was analyzed according to sex (Figure 4.2). Males presented lower TLR4 expression (28 ± 11 GMFI) when compared with females (40 ± 23 GMFI) at $P = 0.01$.

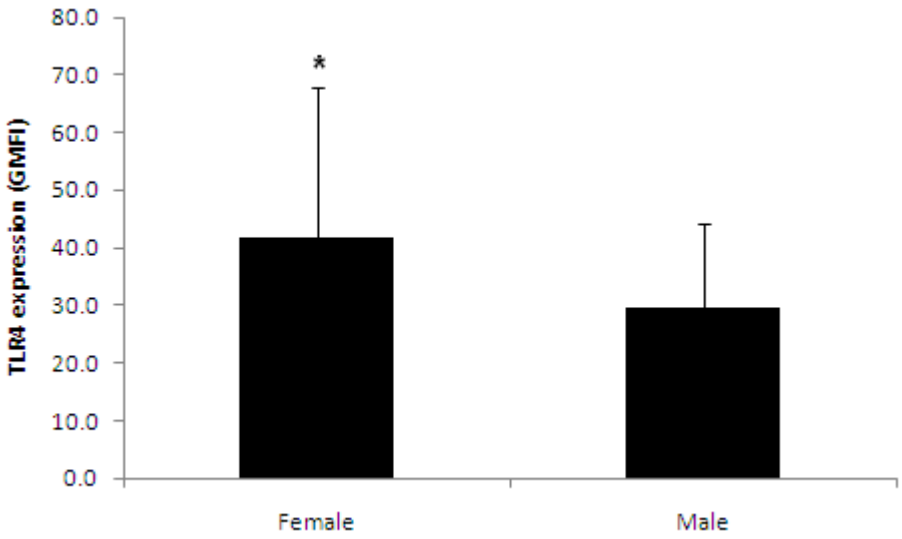

Figure 4.2 – Resting TLR4 expression according to sex.
* Significant difference compared with males (Female N=33, Male N=46; P < 0.05)

4.3.6. Sex Differences on Anthropometric and Haematological characteristics

Males were taller ($P = 0.001$), heavier ($P = 0.001$) and, therefore, had a slightly higher BMI (24 ± 3 and 23 ± 2 $kg.m^{-2}$ respectively, $P = 0.003$) compared with females. There was no significant difference in the hours exercised per week between the sexes, where males exercised 8.7 ± 5.2 h per week and females exercised 7.4 ± 4.5 h per week ($P = 0.213$). There was also no difference in age between sexes ($P = 0.425$).

In relation to the haematological values, males presented higher numbers of red blood cells, haematocrit and haemoglobin concentration compared with females, as shown in Figure 4.3.

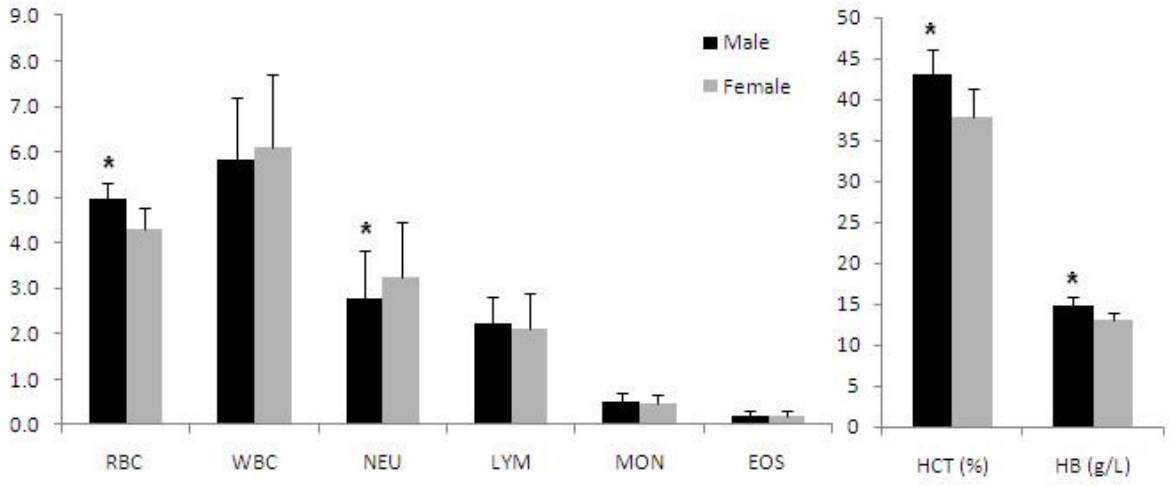

Figure 4.3 – Haematological values
* Significant difference from female group; Mean ± SD; RBC = Red blood cells ($x10^{12}$/L); WBC = white blood cell ($x10^{9}$/L); NEU = neutrophil ($x10^{9}$/L); LYM = Lymphocyte ($x10^{9}$/L); MON = Monocyte ($x10^{9}$/L); EOS = Eosinophil ($x10^{9}$/L); HCT = Haematocrit (%); HB = Haemoglobin (g/L)

4.3.7. Influence of Sex on Blood Immune Variables

Analysis of the stimulated whole blood culture production of cytokines was performed in the male and female groups. No significant differences were found in any of the cytokines analysed (IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8 and IL-10, Table 4.4), although there was a trend for higher IL-1β and IL-8 cytokine production in males. None of the variables were correlated to TLR4 expression within the sexes (all $P > 0.05$).

Table 4.4 - Stimulated whole blood cytokine production in males and females

(pg/ml)	Males (n=45)	Females (N=33)	*P* values
IL-2 production	113.8 ± 136.8	135.8 ± 229	0.625
IL-4 production	3.4 ± 4.1	4.3 ± 7.5	0.514
IL-6 production	163 ± 132.4	135.9 ± 126.2	0.355
IL-8 production	1162 ± 739.0	867.5 ± 639.9	0.063
IL-10 production	3.9 ± 5.4	3.7 ± 4.7	0.840
IFN-γ production	28.9 ± 58.8	26.1 ± 54.3	0.833
TNF-α production	27.6 ± 46.8	16.1 ± 25.5	0.169
IL-1α production	1.5 ± 2.4	1.0 ± 1.2	0.177
IL-1β production	9.1 ± 10.1	5.9 ± 4.9	0.073

* Statistically different from HIGH; IL= interleukin; IFN-γ = interferon gamma; TNF-α = Tumour necrosis factor

4.4. Discussion

The main finding of the present study was that males and females present similar aspects of immunity but females have a higher $CD14^+$ monocyte TLR4 expression compared with males. However, levels of TLR4 expression were not related to URTI incidence, and therefore this difference seems to not be significant enough to considerably affect susceptibility to URTI. There were no significant differences in the cytokine production by antigen-stimulated whole blood culture in these populations. The hypothesis that the incidence of URTI and the production of pro- and anti-inflammatory cytokine response to the multi-antigen challenge were different between sexes was therefore not verified. Males presented higher values for red blood cells, haematocrit and haemoglobin compared with females; however all the values were within the normal healthy range. Blood leukocyte, neutrophil, monocyte and lymphocyte counts were similar in men and women so it is important to emphasise that most aspects of immunity measured in this study were not different between the sexes.

A limitation of the present study is that the phase of the menstrual cycle was not determined when the blood sample was taken and it was not established whether or not the females were taking oral contraceptives. Immune functions in females are subject to

endogenous oestrogenic effects (Gillum et al. 2011), so it is possible that some of the female endurance athletes in our study could be amenorrhoic (have lower oestrogen levels) due to the high training loads, making their immune variables more similar to that of men. Some studies found however that the menstrual cycle did not affect some immunological parameters in trained athletes (Burrows et al. 2002, Gillum et al. 2011). Sex differences in immune function among athletes probably do not therefore need to be considered in future mixed gender studies on exercise, infection and immune function unless TLR4 expression has been analysed.

In addition, this study examined the URTI incidence and its possible association with resting values of $CD14^+$ monocyte TLR4 expression and cytokine production by antigen-stimulated whole blood culture in an athletic population. Results showed that URTI incidence and most of the immune variables analysed were not different between the MED and HIGH activity groups, and did not correlate with TLR4 expression. However, the anti-inflammatory cytokine IL-10 production was higher after stimulation in the HIGH group compared with the MED. Cytokine production was not analysed in the LOW group, which impaired the comparison with the MED and HIGH groups. Therefore, only the hypothesis that high volume training would be associated with an elevated anti-inflammatory cytokine response compared with subjects engaged in lower levels of physical activity was verified. When comparing the three groups according to their TLR4 expression the low physical activity group had higher $CD14^+$ monocyte TLR4 expression when compared with the HIGH group. Even though there was a difference in the number of males and females in each group, it is worth noting that there was no difference in TLR4 expression between the sexes within groups.

It has been suggested that IL-10 is able to impair the ability of APCs to promote the differentiation and proliferation of $CD4^+$ T cells, thereby regulating both initiation and perpetuation of adaptive T-cell responses (Murphy et al. 2008). This happens through a downregulation of MHC antigens, the intercellular adhesion molecule-1, as well as the costimulatory molecules CD80 and CD86 on antigen presenting cells. In addition, IL-10 has been shown to promote differentiation of dendritic cells expressing low levels of MHC class II, CD80, and CD86 (Maynard & Weaver, 2008). That could potentially increase the risk of URTI; however, no difference was found between the MED and HIGH group, although the HIGH group had 9% more participants reporting URTI symptoms compared with the MED.

As suggested by Lancaster et al. (2005), higher TLR expression on monocytes was considered one possible reason for an elevated cytokine production in response to an antigen challenge. In addition, it has been seen that lower TLR4 expression can be found in trained compared with sedentary subjects in both cross sectional (McFarlin et al. 2006) and longitudinal training studies (Stewart et al. 2005). Similar findings were obtained in the present study, where low levels of training resulted in higher monocyte TLR4 expression. A limitation of the present study is that vaccine stimulated cytokine production was not analysed for the LOW group, making the interpretation of the data for the LOW group limited. Nevertheless, the production of none of the measured cytokines correlated with monocyte TLR4 expression in the MED or HIGH groups. A few small differences were found between the groups in relation to the haematological values and white blood cell counts. However all the values are within the normal healthy range.

Thus, TLR4 expression has been shown to be higher in females compared with males and in the population with a low compared with a high level of physical activity. No differences were found between sexes for stimulated cytokine production and URTI symptoms. Different levels of physical activity do not result in difference in pro-inflammatory cytokine production, but a difference in the IL-10 anti-inflammatory cytokine production was found between the physically active groups, despite no difference in URTI incidence and no correlation with TLR4 expression. Therefore, sex differences and physical activity levels in immune function need to be considered in future mixed-gender studies on exercise when analysing TLR4 expression.

5. The Influence of Prolonged Cycling Exercise at 75% Vo_{2peak} on Monocyte TLR2 and TLR4 Expression in Healthy Men

Abstract: Several studies have reported that some immune cell functions including monocyte TLR expression and antigen presentation are temporarily impaired following acute bouts of strenuous exercise, which could represent an 'open window' to URTI. However, the time course of effects of acute exercise on human monocyte TLR expression was not yet known. The purpose of the present study was to examine the effects of 1.5 h cycling at 75% VO_{2peak} on human monocyte TLR2 and TLR4 expression and how long it takes for TLR expression to return to pre-exercise values. Nine healthy endurance trained males (age 25 ± 5 years) had blood samples taken before and for up to 24 h after exercise and analysed using flow cytometry. Although there was an increase in the total monocyte cell count at 0, 1 and 4 h post-exercise ($P < 0.01$) compared to pre-exercise values. Monocyte TLR4 expression was reduced (geometric mean fluorescence intensity, corrected for non-specific binding; $P < 0.05$) by 32% and 45% at 0 and 1 h post-exercise, respectively, returning to baseline values by 4 h post-exercise. No statistically significant changes were found in TLR2 expression after exercise. In addition, a control resting study was conducted on six healthy endurance trained men (age 25 ± 2 years) to analyse any diurnal changes on monocyte TLR2 and TLR4 expression but no changes were found across time ($P > 0.05$). This study showed that prolonged cycling at 75% VO_{2peak} temporarily reduces TLR4 expression, which may, in part, be responsible for post-exercise immunodepression.

5. .Introduction

Reduced immune cell functions after prolonged exercise could represent an 'open window' for pathogens, allowing URTI and other common infections to gain a foot-hold (Nieman 1994). Several studies have indicated that both chronic exercise training (combined resistance and aerobic regimens) and acute bouts of prolonged exercise may play a role in modifying TLR4 expression and downstream responses (Flynn et al. 2003; McFarlin et al. 2004, 2006; Stewart et al. 2005). A study by Lancaster et al. (2005) reported that TLR2 and TLR4 expression on $CD14^+$ blood monocytes was reduced following 1.5 h of cycling at 65% VO_2max in the heat. Simpson et al. (2009) showed that TLR4 (but not TLR2) expression on $CD14^+$ blood monocytes was reduced after running for 45 min at 75% of VO_{2max}. The reasons why there are changes in TLR expression after a single bout of exercise is not yet known. Nevertheless, suggestions have been made regarding how the augmented level of heat shock proteins (HSPs) and cytokines in the blood might affect the TLR responses, but more research in this area is needed (Gleeson et al. 2006). The acute effects of exercise on TLR expression in the study by Lancaster et al. (2005) may have been influenced by increases in core temperature, yet it is not clear if acute post-exercise depression of TLR expression can be mediated by cycling exercise in thermoneutral conditions. Additionally, it is presently unknown how long monocyte TLR expression takes to recover after an acute bout of prolonged exercise.

Therefore, the aim of the present study was to determine the influence of 1.5 h of cycling exercise at 75% VO_{2peak} in thermoneutral conditions on monocyte $CD14^+$ TLR2 and TLR4 expression in healthy men and the time course of any changes over the following 24 h. It was hypothesised that acute strenuous cycling exercise would influence the expression of TLR2 and TLR4 for several hours following exercise.

Although numerous studies have reported changes in TLR2 and TLR4 after exercise, no study to date has analysed the time course of TLR2 and TLR4 responses beyond 2 h post-exercise after a single bout of cycling exercise.

5.2. Methods

This Chapter describes the influence of cycling exercise on monocyte TLR2 and TLR4 expressions in healthy men. A control study was conducted in parallel with the exercise study in order to check for any changes in the TLR2 and TLR4 expressions over time with subjects in the resting state, with these results also presented in this Chapter. Detailed descriptions of the methods are given in Chapter 3. Any methodology that was used specially in this study is fully explained in this section, with methods previously described are briefly summarised.

5.2.1. Participants

Nine healthy endurance trained men (age 25 ± 5 years; body mass 76.8 ± 8.1 kg; VO_{2peak} 58.5 ± 5.6 $ml \cdot kg^{-1} \cdot min^{-1}$, BMI 23.8 ± 1.4 $kg \cdot m^{-2}$) participated in the exercise study. The control resting study was completed by six healthy endurance trained men (age 25 ± 2 years; body mass 75.5 ± 5.8 kg; BMI 23.4 ± 0.8 $kg \cdot m^{-2}$).

The participants were all non-smokers, were not taking any medication and had remained free of symptoms of respiratory infection for at least 4 weeks prior to participation in the study. They were asked to abstain from alcohol, caffeine and heavy exercise for at least 1 day prior to their visit and the 24 h after the end of the exercise trial. The same recommendations and exclusion criteria applied to the resting control study. For the exercise study, participants had to achieve a criterion VO_{2peak} minimum value of 50 $ml \cdot kg^{-1} \cdot min^{-1}$.

Participants completed a food record diary for the 24-h period before a Familiarisation trial and were required to follow the same diet during the 24 h prior to the main trial. They also recorded their food intake in the 24 h after the main trial. The food diary was used to ensure that subjects avoided intake of caffeine (or caffeine containing foods such as chocolate) prior to the exercise trial, since caffeine has been shown to suppress some immune responses (Horrigan et al. 2006).

5.2.2. Experimental Procedures

All participants completed three exercise bouts: two preliminary trials (VO_{2peak} determination and Familiarisation) and one main trial. All trials were separated by at least 1 week.

VO_{2peak} Determination

Participants were tested as described in Chapter 3. In brief, they completed a continuous incremental exercise test to volitional exhaustion on a cycle ergometer (Monark 874E, Sweden). When the participant signalled that they could continue for only one more minute at the required intensity, a new expired gas collection was commenced and verbal encouragement was given, and the test was ended. In order to confirm that subjects performed at their maximum effort, all test results were checked to see if at least two out of the three criteria were met: subjects achieved within 10 bpm of their age predicted maximum heart rate; participants' RER was >1.15 or they indicated a score of >19 on the RPE scale.

Familiarisation

Participants returned to the lab approximately 1 week after the VO_{2peak} test. Two hours before arriving in the lab, they consumed their normal breakfast (e.g. either porridge, muesli, or toasted bread and orange juice), which averaged (±SD) 1.8 ± 0.3 MJ, 14.8 ± 0.4 g of protein, 12.2 ± 3.8 g of fat and 69.5 ± 22.6 g of carbohydrate. After eating, participants cycled for 1.5 h at the work rate equivalent to 75% of their VO_{2peak}. 200 ml of water was consumed every 20 min during exercise. A 60-s sample of expired gas was collected using Douglas bags every 10 min during the exercise and measured as described previously. Heart rate and RPE measurements were taken every 20 min of exercise. The purpose of this Familiarisation trial was threefold: First, to habituate the participants to the physical stress of the exercise and to the methods involved in the trial; second, to ensure that the participants were able to maintain the selected intensity for 1.5 h; and third, to ensure that the selected work rate actually did elicit a relative intensity of 75% VO_{2peak}.

Main Trial

Participants exercised under similar conditions to those described for the Familiarisation trial. They arrived at the laboratory at 09:30, 2 h after having eaten the same breakfast as for the Familiarisation ride, for measurement of body mass. After 5 min seated rest and immediately before beginning to cycle, a resting (PRE) blood sample was taken (as described in section 3.4).

During exercise, expired gas was collected into Douglas bags (60-s sample), after 30, 60 and 90 min of exercise, for analysis of VO_2 and RER. Heart rate and RPE were recorded every 20 min during exercise and subjects consumed 200 ml of water every 20 min during exercise. Body mass was measured again after exercise. Further venous

blood samples were taken immediately post-exercise and at 1, 4 and 24 h after exercise. All participants remained seated after exercise until after the 1 h post-exercise blood sample had been taken. Subsequently, participants were given a standardized lunch [consisting of two ham and cheese sandwiches, a sports drink, a piece of fruit (apple or banana) and a cereal bar, with a total energy of 2.0 MJ] to eat and were asked to return to the laboratory at 4 and 24 h after exercise. Laboratory ambient conditions during the trials were 20.7 ± 2.0 °C whit a relative humidity of 48 ± 7%.

Resting Control Protocol

All participants in the control study visited the lab after an overnight fast. They arrived at the laboratory at 09:20. After a 10 min seated rest, a resting blood sample was taken as described in Chapter 3. Participants remained seated for 2.5 h, and further blood samples were taken after 1.5 h (equivalent to the post-exercise sample in the exercise protocol) and 2.5 h (equivalent to the 1 h post-exercise sample in the exercise protocol).

5.2.3. Biochemical Analysis

Blood Analysis

Haematocrit, haemoglobin, total white blood cell (leukocyte) and monocyte counts were determined from the K_3EDTA blood at each time point during the main trial (PRE, 0, 1, 4 and 24 h post-exercise and the equivalent for PRE, 0 and 1 h post at the resting study) using an automated haematology analyser (AC.T 5diV, Beckman Coulter, Buckinghamshire, UK). Changes in plasma volume were calculated from measurements of haemoglobin and haematocrit according to the method described by Dill and Costill (1974).

$CD14^+$ Monocyte and TLR Expression

In order to determine the cell surface expression of TLR on circulating monocytes, heparinized blood was surface stained, using the methods described in Chapter 3. In short, blood aliquots were stained with CD14- FITC and PE-conjugated TLR2, TLR4 or Isotype control. After the treatment previously described, cell pellets were re-suspended in 500 µl of PBS/BSA/EDTA buffer and transferred to FACS tubes for analysis. Samples were analysed on a flow cytometer (BD FACSCalibur) equipped with the CellQuest software package (BD Biosciences, Oxford, UK). Cells were gated according to side scatter and CD14-FITC expression (which characterises monocytes; Figure 3.1), and the geometric mean fluorescence intensity (GMFI) of the Isotype control (ISO), and of TLR2 and TLR4 antibodies in the $CD14^+$ cell gated population was obtained to quantify TLR expression (a total of 3,000 cells acquired for each sample).

5.2.4. Statistical Analysis

Normality tests were carried out on all data. Total leukocyte count, monocyte count and monocyte TLR2 and TLR4 expression results were not normally distributed and therefore were analysed using Friedman's ANOVA ($P < 0.05$). If a statistically significant difference was found, a Wilcoxon signed-rank test (corrected for the time points) was carried out to locate the difference ($P < 0.01$). A paired *t* test ($P < 0.05$) was used for before and after body mass measurements, as these values were normally distributed. One-way ANOVA with repeated measures was used to compare plasma volume results with Bonferroni correction if *P* values were ≤ 0.05. In order to calculate these statistics, SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used. All results are presented as mean ± SD.

5.3. Results

Mean heart rate, exercise intensity and RPE during exercise are detailed in Table 5.1.

Table 5.1 - Heart rate, $\%VO_{2peak}$ and RPE at 30, 60 and 90 min of exercise.

	30 min	60 min	90 min
Heart Rate (bpm)	155 ± 5	158 ± 6	161 ± 9
$\%VO_{2peak}$	73 ± 5	75 ± 3	76 ± 4
RPE	14 ± 1	15 ± 2	16 ± 2

Participants lost approximately 0.9 kg of body mass during exercise and plasma volume was unchanged immediately post-exercise compared with PRE ($P > 0.05$). Plasma volume was also unchanged at the other timepoints compared with PRE.

Blood monocyte count was increased immediately after exercise, as well as at 1 h and 4 h post-exercise (all $P < 0.01$). Values returned close to resting values after 24 h ($P = 0.375$ vs PRE). Similarly, there was an increase in the total leukocyte count at all time points up to 4 h post exercise (all $P < 0.01$), with values returning to normal after 24 h ($P = 0.367$ vs PRE) (Figure 5.1).

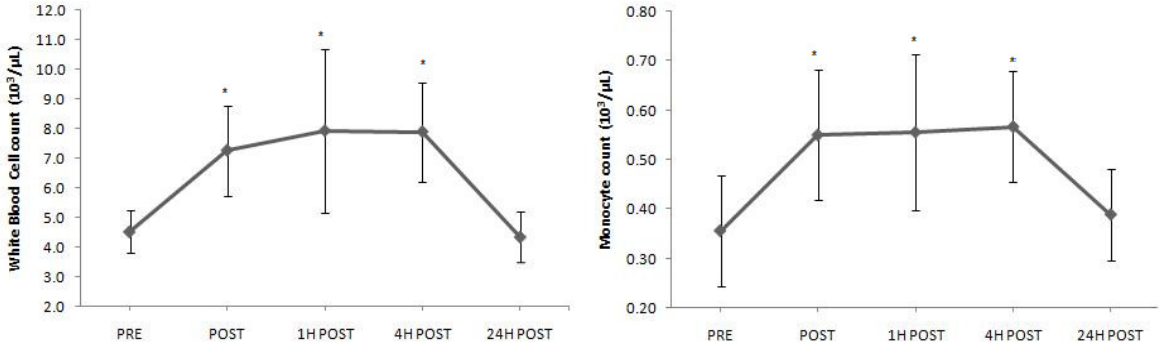

Figure 5.1 - Total white blood cell (WBC) and monocyte counts before (PRE) and up to 24 h after exercise.
* Significantly different from PRE ($P < 0.01$).

5.3.1. TLR Expression

Monocyte cell surface TLR2 expression showed no change across time ($X^2(4) = 4.178$, $P = 0.402$; Figure 5.2a). However, monocyte TLR4 expression was significantly lower than PRE at 0 and 1 h post-exercise ($X^2(4) = 9.511$, $P < 0.05$), which corresponded to a 32% reduction at 0 h post-exercise and a 45% reduction at 1 h post-exercise compared with PRE values. Values had returned to normal at 4 h post-exercise ($P = 0.074$), as can be seen in Figure 5.2b.

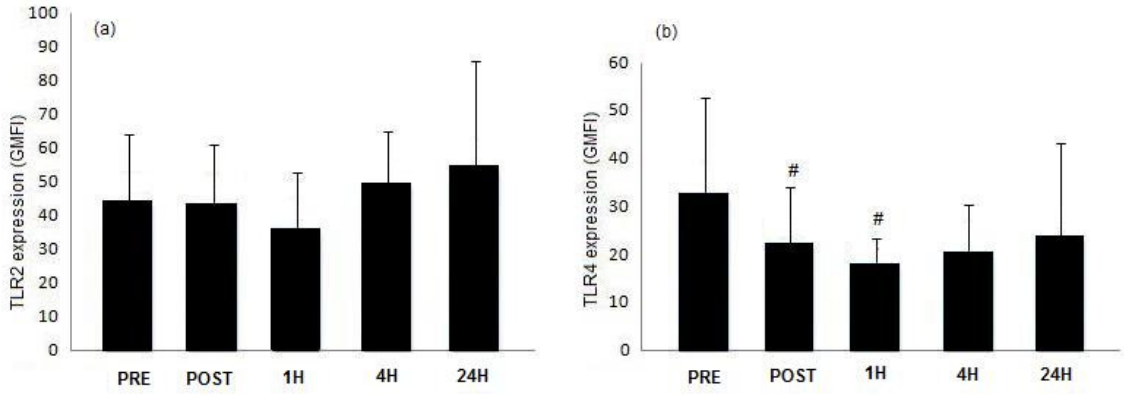

Figure 5.2 - (a) $CD14^+$ Monocyte TLR2 and (b) TLR4 expression (GMFI) after exercise.
Significantly different from PRE ($P < 0.05$)

In the resting control study, monocyte cell surface TLR2 and TLR4 expression showed no change across time. The GMFI for TLR4 was 26.3 ± 12.5, 22.4 ± 14.1 and 22.4 ± 16.6 at the 0, 1.5 h and 2.5 h time points, respectively; ($X2(2) = 2.333$, $P = 0.429$). The GMFI for TLR2 was 32.1 ± 19.6, 29.6 ± 18.5 and 29.8 ± 15.1 at the 0, 1.5 h and 2.5 h time points, respectively ($X2(2) = 1.334$, $P = 0.570$).

5.4. Discussion

The present study provides information about changes in the $CD14^+$ monocyte TLR2 and TLR4 expression after a single bout of 1.5 h of cycling exercise at 75% of VO_{2peak} and the time course of recovery. Although there was an increase in the blood monocyte count after exercise, TLR4 expression fell by 32% and 45% at 0 and 1 h post-exercise but returned to pre-exercise levels within 4 h post-exercise. In contrast, TLR2 expression showed no statistically significant changes after exercise. Neither TLR2 nor TLR4 expression changed over time in the resting control study.

The findings of the present study corroborate a recent study (Simpson et al. 2009), where a reduction in TLR4 expression at 0 and 1.5 h after exercise was found. Simpson et al. (2009) analysed the TLR expression on monocyte subpopulations (e.g. $CD14^+/CD16^-$ and $CD14^+/CD16^+$) after 15 volunteers ran for 1.5 h at 75% VO_2max. Interestingly, as in this study, no difference was found in $CD14^+$ monocyte TLR2 expression after exercise. In addition, the changes in TLR4 expression after exercise found in the present study are in accordance with the findings of Lancaster et al. (2005) who reported a reduction in TLR4 expression immediately post-exercise and a further reduction at 2 h after exercise in a hot environment, independent of changes in immunomodulatory hormones such as cortisol.

In contrast to the present study, Lancaster et al. (2005) observed a reduction in TLR2 expression after 1.5 h of cycling in the heat. Exercise in the heat is known to be more stressful than in thermoneutral conditions (Galloway and Maughan 1997; Walsh and Whitham 2006) and it is possible that monocyte TLR2 expression may be affected only by a more severe exercise stress stimulus compared with TLR4.

The changes that occurred in the TLR expression in this study are likely to have been due to the effects of exercise, as the resting control study on six healthy subjects showed that both TLR2 and TLR4 expression did not change when analysed at the same time points (equivalent to pre-, post- and1 h post-exercise) as those in the exercise study. Furthermore, Lancaster et al. (2005) have previously shown no influence of circadian variation in monocyte TLR expression in healthy humans. However, the reason why TLR4 expression is reduced after exercise is not fully understood. Researchers have suggested a relationship between endogenous ligands (e.g. cytokines, necrotic cell products and HSPs) and TLR expression, indicating that both microbial and endogenous agents might use the same pathway to activate the adaptive immune system (Beg 2002). The changes that occur in TLR expression after exercise may also be related to the production and secretion of IL, such as IL-6 and IL-10 (Gleeson et al. 2006). It is well established that circulating IL-6 levels are elevated after a single bout of exercise, and it has been suggested that IL-6 plays an important role in down regulating TLR expression in different conditions (Lancaster et al. 2005). Plasma IL-6 concentration increases after exercise, with little increase in other cytokines such as IL-1β and TNF-α (Ostrowski et al. 1999; Pedersen and Febbraio 2008). Pedersen and Febbraio (2008) have established that intramuscular IL-6 production and IL-6 release into the circulation are augmented during prolonged exercise. As TLR activation also results in the production of IL-6 through the NF-kB signalling pathway, it is hypothesised that high levels of IL-6 in the blood after exercise down regulate the expression of TLRs. The decline in TLR expression is associated with reductions in accessory signal molecule expression (e.g. CD80 and CD86) and antigen presenting cell activation after acute exercise. This may impair immune function and increase susceptibility to URTI (Lancaster et al. 2005; Gleeson et al. 2006).

Another important endogenous ligand that may influence TLR expression is the HSPs. These are proteins presented in all the cells and are up-regulated during physiological stress. The HSPs act as chaperones to guide the synthesis, transportation and degradation of some proteins (Kilmartin and Reen 2004). In addition, it has been suggested that HSPs (e.g. HSP60 and HSP72), in a similar manner to LPS, act as activators of TLR4. Kilmartin and Reen (2004) showed that repeated monocyte exposure to HSP60 induces a tolerance to HSP and a 'cross-tolerance' to LPS stimulation, subsequently reducing TLR4 activation. Strenuous exercise results in physiological stress due to increased body temperature and muscle damage, so it is hypothesised that HSPs in the extracellular environment are increased after exercise and could therefore reduce TLR expression. Repeated exposure to HSPs could play a critical role in monocyte deactivation and contribute to the increased susceptibility to URTI in endurance athletes.

Nevertheless, the results of the present study showed that monocyte TLR4 expression had essentially returned to normal within 4 h following exercise, suggesting that the effects of a single bout of exercise in healthy participants is present only for a short time. In conclusion, 1.5 h of cycling exercise at 75% VO_{2peak} reduced $CD14^{+}$ monocyte TLR4 expression in healthy men for a few hours, but no changes in TLR2 expression at any time point after exercise were found.

6. The Monocyte $CD14^+$ TLR4 Responses After Two VO_{2peak} Tests Separated by 2 Hours.

Abstract: Although it has been shown that strenuous bouts of prolonged exercise (45 min to 1.5 hours) reduce TLR4 expression, it has not yet been found if short duration, incremental exercise would elicit similar reduction in TLR4 expression. The aim of this study was to analyse the influence of two consecutive VO_{2peak} tests on monocyte TLR4 expression in healthy men. Nine healthy endurance trained men (age 25 ± 5 years; body mass 75.3 ± 8.2 kg; VO_{2peak} 56 ± 3.6 ml kg^{-1} min^{-1}, mean ± SD) performed two incremental VO_{2peak} test until volitional exhaustion separated by 2-h. Venous blood samples were taken at rest (PRE) and immediately post-exercise (POST) both tests for analysis of haematocrit, haemoglobin, total white blood cell (leukocyte), monocyte counts and monocyte $CD14^+$ TLR4 expression. No difference was found for any variable analysed at any time point. Thus, two subsequent incremental tests to exhaustion did not reduce $CD14^+$ monocyte TLR4 expression, which may suggest that the duration or overall load (where overall load = intensity x duration) of the exercise is not high enough to induce a depressive immunological response in an active population.

6.1. Introduction

The reduction in immune cell function after prolonged strenuous exercise may be associated with URTI occurrence (Nieman 1994, Gleeson et al. 2011b). However, the mechanism of how exercise affects the immune cell response is not fully understood. Suggestions have been made that exercise is linked to the lowering of TLR expression and consequently to the lower capacity of the immune system to act against infection. A study by Lancaster et al. (2005) reported that TLR2 and TLR4 expression on $CD14^+$

blood monocytes was reduced following a single bout of 1.5 h of cycling at 65% VO_{2max} in the heat. In addition Simpson et al. (2009) showed that TLR4 expression on $CD14^+$ blood monocytes was reduced after running for 45 min at 75% of VO_{2max}. But although it has been shown that strenuous bouts of prolonged exercise (45 min to 1.5 hours) reduce TLR4 expression, it has not yet been found if short duration, incremental exercise would elicit similar reduction in TLR4 expression. Therefore the aim of this study was to analyse the influence of two consecutive VO_{2peak} tests on monocyte TLR4 expression in healthy men. It was hypothesised that a single bout of incremental exercise to exhaustion would reduce TLR4 expression, and a further reduction would be seen after the second bout of exercise.

6.2. Methods

This was a short study which is part of a main study published elsewhere. This Chapter describes the influence of two consecutive VO_{2peak} tests on monocyte TLR4 expression in healthy men. Detailed descriptions of the methods are given in Chapter 3. Any methodology that was used specifically in this study is fully explained in this section, whilst the methods previously described are summarised.

6.2.1. Participants

Nine healthy endurance trained men (age 25 ± 5 years; body mass 75.3 ± 8.2 kg; VO_{2peak} 56 ± 3.6 ml kg^{-1} min^{-1}) participated in the study. Participants were all non-smokers, were not taking any medication and had remained free of symptoms of respiratory infection for at least 4 weeks prior to participation in the study. They were asked to abstain from alcohol, caffeine and heavy exercise for at least 1 day prior to their visit.

6.2.2. Experimental Procedures

All participants completed two exercise bouts: one preliminary trial (VO_{2peak} determination) and one main trial. Trials were separated by at least 1 week.

VO_{2peak} Determination

Participants were tested as described in Chapter 3. In brief, they completed a continuous incremental exercise test to volitional exhaustion on a cycle ergometer (Monark 874E, Sweden). The workload started at 95 W and 35 W was added at each 3-min stage. When the participant signalled that they could continue for only 1 more minute at the required intensity, a new expired gas collection was commenced, verbal encouragement was given, and the test was ended. In order to confirm that subjects performed at their maximum effort, all test results were checked to see if at least two out of the three criteria were met: subjects achieved within 10 bpm of their age predicted maximum heart rate; participants' RER was >1.15 or they indicated a score of >19 on the RPE scale.

Main Trial

Participants arrived at the laboratory at 09:30, 2 h after having eating breakfast, for measurement of body mass. After 5 min seated rest and immediately before beginning to cycle, a resting (PRE1) blood sample was taken (as described in section 3.4).

The main trial consisted of an incremental VO_{2peak} test until volitional exhaustion as explained above. No encouragement was given during the test. Venous blood samples were taken immediately post-exercise (POST1). All participants remained seated after

exercise for 2-h, then a second VO_{2peak} test was performed as described, with a pre (PRE2) and a post (POST2) blood taken.

6.2.3. Biochemical Analysis

Blood Analysis

Haematocrit, haemoglobin, total white blood cell (leukocyte) and monocyte counts were determined from the K_3EDTA blood at each time point during the trial (PRE1, POST1, PRE2, POST2) using an automated haematology analyser (AC.T 5diV, Beckman Coulter, Buckinghamshire, UK). Changes in plasma volume were calculated from measurements of haemoglobin and haematocrit according to the method described by Dill and Costill (1974).

CD14+ Monocyte and TLR Expression

In order to determine the cell surface expression of TLR on circulating monocytes, heparinised blood was surface stained as described in Chapter 3. In short, blood aliquots were stained with CD14-FITC and PE-conjugated TLR4 or ISO. After treatment previously described, cell pellets were re-suspended in 500 µl of PBS/BSA/EDTA buffer and transferred to FACS tubes for analysis. Samples were analysed on a flow cytometer (BD FACSCalibur) equipped with the CellQuest software package (BD Biosciences, Oxford, UK). Cells were gated according to side scatter and CD14-FITC expression (which characterises monocytes), and the geometric mean fluorescence intensity (GMFI) of the ISO and TLR4 antibodies in the $CD14^+$ cell gated population was obtained to quantify TLR expression (total of 3,000 cells acquired for each sample).

6.2.4. Statistical Analysis

Normality tests were performed out on all the data. Total leukocyte count, monocyte count and monocyte $CD14^+$ TLR4 expression results were normally distributed and were, therefore analysed using repeated measures ANOVA ($P \leq 0.05$) with a Bonferroni correction. A student t-test was carried out to locate the difference in the case $P < 0.05$. In order to calculate these statistics, PASW version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA) was used. All results are presented as mean ± SD.

6.3. Results

No changes in plasma volume were found after the tests. In addition, no difference was found for $CD14^+$ monocyte TLR4 expression at any time point ($P = 0.57$, Table 6.1). Participants exercised for 23 ± 5 and 22 ± 4 minutes during the first and second tests respectively.

Table 6.1 - TLR4 expression after 2 VO_{2peak} tests separated by 2 hours

	Rest 1	**Post 1**	**Rest 2**	**Post 2**
TLR4 expression (GMFI)	37 ± 14	39 ± 14	43 ± 17	38 ± 14

6.4. Discussion

The main finding of the present study was that short term intense incremental exercise such as VO_{2peak} tests does not influence TLR4 expression in an active male population. Therefore, the hypothesis that a single bout of incremental exercise to exhaustion would reduce TLR4 expression, and a further reduction would be seen after the second exercise bout was not verified.

Although Lancaster et al. (2005) and Simpson et al. (2009) found differences in the TLR4 expression after an acute bout of exercise, differences in time, intensity and duration of the exercise may have contributed to their findings. Although a VO_{2peak} test has been considered a strenuous exhaustive activity, the work load performed in the first half of the activity may not be high enough to stimulate an immunological response in this active population, thus a TLR4 reduction was not seen. Short-period high-intensity exercise has been shown to improve aerobic capacity and fat oxidation even when the total exercise time does not exceed 30 min per session, however no immunological markers were analysed in any of these studies (Cornish et al. 2011, Gibala et al. 2006, Gibala and McGlee 2008, Serpiello et al. 2011, Talanian et al. 2007). Moreover, most of the exercises used in the cited studies consist of high-intensity bouts separated by a small recovery, while the present study used a continuous incremental load activity with approximately 20 min of duration.

To conclude, two subsequent incremental tests to exhaustion did not reduce $CD14^+$ monocyte TLR4 expression, which may suggest that the duration or overall load (where overall load = intensity x duration) of the exercise is not high enough to induce a depressive immunological response in an active population. It is recommended that further research should be conducted to analyse different types and intensity of exercise and the changes in TLR4 responses in active and sedentary populations.

7. Effects of Two Weeks of High-intensity Interval Training (HIIT) on Monocyte TLR2 and TLR4 Expression in High BMI Sedentary Men

Abstract: Monocyte TLR expression has been shown to be reduced after a combination of aerobic and resistance exercise, but more studies considering the influences of different exercise intensities, type and duration on TLR expression are needed. Although there is an agreement about the importance of physical exercise, the minimal optimal amount needed to improve health status is uncertain. Therefore, the aim of this study was to analyse the influence of 2 weeks of high-intensity intermittent exercise training on $CD14^+$ monocyte TLR4 expression in a sedentary, high BMI population. As a secondary purpose, this study covers the influence of exercise on classical and pro-inflammatory monocytes and the TLR4 expression before and after a training period in these monocyte subsets. Six high-intensity interval training (HIIT) sessions over a 2 week period (three sessions per week) were completed by 11 participants (24 ± 5 years old). Blood samples were taken at the beginning and end of the training period for analysis of haematocrit, haemoglobin, total white blood cell (leukocyte), monocyte counts, monocyte $CD14^+$ TLR4 expression and monocyte subsets. Two weeks of high-intensity intermittent exercise training increased VO_{2peak} and total $CD14^+$ monocyte TLR4 expression in a sedentary, high BMI population. There was no influence of training on the proportions of classical and pro-inflammatory monocyte subsets, but TLR4 expression in the majority of these monocyte subsets (apart from $CD14^{++}CD16^+$) was higher after the six training sessions.

7.1. Introduction

It has been suggested that chronic elevation of inflammatory biomarkers is related to low physical function in the elderly population (Brinkley et al. 2009) and to chronic disease conditions such as CVD and diabetes independent of body weight (Fischer et al. 2007, Nicklas and Brinkley 2009). Interleukin-6, together with C-Reactive Protein (CRP), are examples of inflammation biomarkers that have been found to be related to the risk of developing chronic metabolic and CVDs, and exercise training has been shown to reduce these inflammatory markers (Stewart et al. 2005). Several researchers have suggested that exercise-induced reduction of TLR expression and shifts in monocyte phenotype could explain the fall in the inflammatory markers measured (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Stewart et al. 2005, Timmerman et al. 2008).

Stewart et al. (2009) demonstrated that 12 weeks of combined aerobic and resistance exercise (3 times per week) significantly increases aerobic capacity and muscle strength, and reduces TLR4 expression in both the old and young population with no changes in BMI. In addition, stimulated-production of IL-6 was also lower although only in the young group. Similar findings were shown by Timmerman et al. (2008), where 12 weeks of resistance and aerobic training (3 times per week, around 50 min in total) did not change total $CD14^+$ monocyte TLR4 expression but reduced TLR4 expression in pro-inflammatory monocytes ($CD14^+CD16^+$), a monocyte subset reported to highly stimulate the production of pro-inflammatory cytokines (Timmerman et al. 2008).

Aerobic, long duration exercise at moderate intensity (around 60% VO_{2peak}) results in improved aerobic capacity, increased fat oxidation and increased mitochondrial volume, augmenting the capacity to oxidise fat (Chesley et al. 1996, Gibala et al. 2006, Gormley et al. 2008). These positive outcomes can be linked to better insulin sensitivity and general health (Talanian et al. 2007). Nevertheless, studies have shown that HIIT (high power output for a few minutes followed by a resting period) results in similar – if not better – augmentation of aerobic capacity and fat oxidation even when total exercise time does not exceed 30 min per session (Cornish et al. 2011, Gibala et al. 2006, Gibala and McGlee 2008, Serpiello et al. 2011, Talanian et al. 2007). For example, Gibala et al. (2006) demonstrated that high-intensity, short duration repeated exercise (4 to 6 repetitions of 30 s cycling with 4 min recovery) performed 3 times per week for two weeks resulted in a similar improvement in performance for a time trial, and increased muscle oxidative capacity and muscle buffering to endurance training (90 to 120 min at 65%VO_{2peak}) in active men. Likewise, Talanian et al. (2007) demonstrated that 10 repetitions of 4 min at 90% VO_{2peak} followed by 2 min rest increases aerobic capacity, power output, fat oxidation and citrate synthase activity in recreationally active women. These findings suggest that high-intensity intermittent exercise is a powerful training strategy to stimulate adaptations in the skeletal muscle and to improve health in trained and untrained individuals (Gibala et al. 2006, Serpiello et al. 2011, Talanian et al. 2007).

Monocyte TLR expression has shown to be reduced after a combination of aerobic and resistance exercise, but more studies considering the influences of different exercise intensities, type and duration on TLR expression are needed. Although there is an agreement about the importance of physical exercise, the minimal optimal amount needed to improve health status is still uncertain (Gibala and McGlee 2008). In 2002,

the World Health Organisation (WHO) produced a report which showed that more than 60% of the world population is sedentary. In the UK, the physical activity levels are below the European average and it is possible that the majority of people might not exercise due to time constraints. As HIIT lasting up to one hour has been shown to result in greater physiological adaptations than moderate exercise of the same duration, it would be beneficial to know if the high-intensity training protocol can also elicit more positive immunological changes. Therefore, the aim of this study was to analyse the influence of 2 weeks of high-intensity intermittent exercise training on $CD14^+$ monocyte TLR4 expression in a sedentary, high BMI population. As a secondary purpose, this Chapter covers the influence of exercise on classical and pro-inflammatory monocytes and the TLR4 expression before and after a training period in these monocyte subsets.

7.2. Methods

This study was part of a larger study conducted in the School of Sport, Exercise and Health Sciences at Loughborough University. Any methodology that was used specifically in this study is fully described in this section while the methods previously described in Chapter 3 are summarised.

7.2.1. Participants

A total of 11 participants aged 24 ± 5 years old were recruited through word of mouth, emails and advertisements around the Loughborough University area. Participants were all non-smokers, were not taking any medication and had remained free of symptoms of respiratory infection for at least 4 weeks prior to participation in the study. This study used the same selection methods and exclusion criteria used in Chapter 3 and only

sedentary (no more than 2 hours of exercise per week) males were recruited. Participants' anthropometric and fitness characteristics are shown in Table 7.1.

7.2.2. Experimental Procedures

VO_{2peak} Determination

A VO_{2peak} test (as described in Chapter 3) was used to determine participants' maximum aerobic capacity at the beginning and end of the study.

Main Trial

Participants arrived in the laboratory at 08:00 after an overnight fast and rested for 5 min. A blood sample was taken, and this session was repeated 48 h after the end of the two-week training period. After this they performed their first training session as described below.

Training Protocol

Participants completed six training sessions over a 2 week period (three sessions per week). Sessions occurred at any time of the day according to the availability of the participants. The sessions consisted of participants performing ten, 4-min cycling intervals separated by 2-min rest period at a work rate corresponding to that which elicited 90% of a participant's VO_{2peak}. Water was consumed *ad libitum* during exercise. Heart rate and RPE measurements were made at the last 30 s of each 4-min stage. The work rate was adjusted (by about 10 W) during training sessions if participants reported lower RPE and had a lower heart rate value when compared with the values achieved in either the main trial or previous sessions.

7.2.3. Biochemical Analysis

Blood Analysis

Haematocrit, haemoglobin, total white blood cell (leukocyte) and monocyte counts were determined from the K_3EDTA blood using an automated haematology analyser. Heparinised blood was used to analyse monocyte and TLR expression as described below.

Monocyte Subpopulation Determination and TLR Expression

For this study a peripheral blood mononuclear cell (PBMC) method was applied before staining the cells. 4 ml of Histopaque 1077 (Sigma Aldrich, Dorset, UK) was added into a tube and 4 ml of heparinised blood was then carefully layered on top. The tubes were centrifuged at 400 *g* for 30 min at 25ºC. PBMC layer was then aspirated and transferred to another tube containing Hank's buffered salt solution (HBSS, 1:1 dilution) and centrifuged at 300 *g* for 10 minutes at 25ºC. After that the supernatant was aspirated and cell pellets were re-suspended in phosphate buffered saline (PBS) containing 0.1% of bovine serum albumin (BSA) and 2 mM EDTA.

In addition to the TLR2 and TLR4 expression on classical $CD14^+$ monocytes, this study also analysed the $CD16^+$ monocyte subsets (also called pro-inflammatory monocytes) response after exercise. For this, the monoclonal antibody CD16 was included in the staining process. Cell staining procedures were similar to those described for the other studies in Chapter 3. In brief, 0.5 µl of PBMC were surface stained with 20 µl CD14-FITC, 20 µl of CD16-PE-Cy5, and antihuman PE-conjugated TLR2 (20 µl), PE-TLR4 (20 µl) or PE-Isotype control (20 µl). All the tubes were incubated at room temperature for 20 min in the dark. In order to lyse the remaining red blood cells, tubes were then filled with a lysis buffer solution and re-incubated for 10 min in the same conditions.

All samples were then centrifuged at 1000 *g* for 6 min. Supernatants were aspirated and cell pellets were re-suspended in 500 µl of PBS/BSA/EDTA and transferred to FACS tubes for analysis. Samples were analysed on a flow cytometer equipped with the CellQuest software package. Cells were gated according to side scatter and CD14-FITC expression (which characterises monocytes), and the geometric mean fluorescence intensity (GMFI) of the Isotype control (ISO), and of TLR2 and TLR4 antibodies in the $CD14^+$ cell gated population was obtained to quantify TLR expression (total of 20,000 cells acquired for each sample). Isotype control GMFI was used to correct values for non-specific binding. Additional gates were then used to identify the $CD14^{++}CD16^-$, $CD14^+CD16^{++}$ and $CD14^{++}CD16^+$ monocyte populations and their co-expression with TLR2 and TLR4. The expression of TLR2 and TLR4 on all $CD14^+$ monocytes was also analysed.

7.2.4. Statistical Analysis

Student's t-test was used to compare before and after training samples in all data. Variables that were not normally distributed ($CD14^+$ and $CD14^{++}CD16^-$ TLR4 expression after training, $CD14^+$ and $CD14^{++}CD16^-$ TLR2 expression before training and $CD14^+CD16^{++}$ TLR2 expression) were also analysed using the Wilcoxon signed rank test. The results of the non-parametric test showed similar results to t-tests, and so only t-test values will be presented in the results section. Pearson correlation was used to assess relationship between VO_{2peak}, BMI waist-to-hip ratio and TLR expression. Statistical significance was accepted at $P < 0.05$. All results are presented as mean ± SD.

7.3. Results

7.3.1. Physiological Characteristics

Participants performed the main high-intensity intermittent training at ~84% of their VO_{2peak} both before and after the 2-week training programme. VO_{2peak} was increased by 7.6% ($P = 0.05$, Table 7.1). Percentage of heart rate during exercise was 5% lower after the training programme ($P = 0.001$).

Table 7.1 - Participants characteristics before and after the 2-week training programme

	Before training	**After training**
Body Mass (kg)	90.0 ± 7.5	89.9 ± 7.6
Height (m)	1.77 ± 0.05	1.77 ± 0.05
BMI ($kg \cdot m^{-2}$)	28.9 ± 3.2	28.9 ± 3.3
Waist circumference (cm)	96.3 ± 8.4	95.0 ± 8.8
Hip circumference (cm)	109.4 ± 5.3	108.2 ± 5.8
Waist-to-hip ratio	0.88 ± 0.05	0.88 ± 0.05
VO_{2peak} ($l \cdot min^{-1}$)	3.40 ± 0.6	3.66 ± 0.5*

Mean ± SD, BMI: Body Mass Index; * Statistical difference found compared with after ($P < 0.05$)

7.3.2. Biochemical Analysis

Blood Cell Count

Participants presented lower red blood cell count and haemoglobin concentration post-training compared with pre-training ($P < 0.05$), but all other variables were unchanged (Table 7.2).

Table 7.2 – Total blood cell count before and after two weeks of training

	Before trainig	After training
RBC	4.9 ± 0.7	4.3 ± 0.7*
HCT (%)	42.6 ± 5.6	37.4 ± 7.1
HB (g/dL)	15.8 ± 0.9	15.1 ± 1.0*
WBC ($x10^9$/L)	5.7 ± 1.4	6.0 ± 2.0
NEU ($x10^9$/L)	2.6 ± 0.8	3.2 ± 1.2
LYM ($x10^9$/L)	2.2 ± 0.6	2.1 ± 0.7
MON ($x10^9$/L)	0.6 ± 0.2	0.6 ± 0.2
EOS ($x10^9$/L)	0.2 ± 0.1	0.2 ± 0.1
BAS ($x10^9$/L)	0.0 ± 0.1	0.1 ± 0.1

(RBC = Red blood cells; HCT = Haematocrit; HB = Haemoglobin; WBC = white blood cell; NEU = neutrophil; LYM = Lymphocyte; MON = Monocyte; EUS = Eosinophil; BAS = Basophil); N= 11. * Statistical difference compared with after ($P < 0.05$)

Monocyte Subpopulation Determination

No significant difference was found in the proportions of the monocyte subpopulations after the exercise training programme ($P > 0.05$), as seen in Table 7.3.

Table 7.3 - Monocyte subpopulation before and after two weeks of training programme

	Before training	After training
$CD14^{++}CD16^{-}$ (%)	77.0 ± 7.8	74.0 ± 9.2
$CD14^{++}CD16^{+}$ (%)	4.8 ± 1.8	5.3 ± 2.1
$CD14^{+}CD16^{++}$ (%)	3.9 ± 2.9	4.9 ± 2.5
Non-gated Monocytes (%)	14.3 ± 7.8	14.8 ± 7.5

No statistical differences ($P > 0.05$)

Monocyte TLR Expression Results

TLR4 expression on CD14 total , $CD14^{++}/CD16^{-}$, $CD14^{++}/CD16^{+}$ and $CD14^{+}/CD16^{++}$ monocyte populations are shown in Figure 7.1. TLR4 expression was two-fold higher after training on total $CD14^{+}$ monocytes (t(10)= -2.90, $P = 0.02$), 2.6-fold higher on $CD14^{++}$ /$CD16^{-}$ (t(10)= -3.05, $P = 0.01$) and 1.5-fold higher on $CD14^{+}$ /$CD16^{++}$ (t(10)= -2.40, $P = 0.04$). There was no significant difference in TLR2 expression before and after the training programme ($P = 0.21$ to 0.48). The GMFI for TLR2 is presented in Table 7.4.

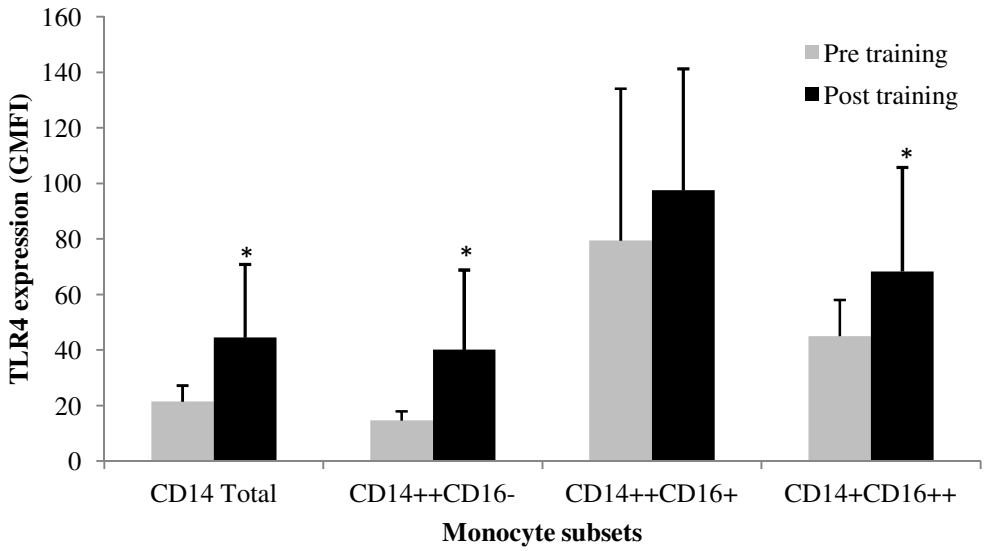

Figure 7.1- TLR4 expression in monocytes before and after the training programme. * Significantly different from pre training ($P = 0.05$).

Table 7.4 - TLR2 expression in monocytes before and after the training programme

	CD14 Total	**$CD14^{++}CD16^{-}$**	**$CD14^{++}CD16^{+}$**	**$CD14^{+}CD16^{++}$**
Pre training (GMFI)	24.7 ± 5.8	14.6 ± 7.2	75.5 ± 30.2	39.7 ± 23.4
Post training (GMFI)	22.4 ± 3.8	16.3 ± 6.7	70.5 ± 24.3	36.9 ± 19.9

There was no significant correlation between TLR expression and VO_{2peak}, BMI and waist-to-hip ratio (all $P > 0.05$).

7.4. Discussion

The aim of this study was to analyse the influence of two weeks of high-intensity intermittent exercise training on $CD14^{+}$ monocyte TLR4 expression in a sedentary, high BMI population. In addition, as a secondary purpose, this study examined the influence of exercise on classical and pro-inflammatory monocytes and the effect of the 2-week exercise training period on TLR4 expression in these monocyte subsets. The novel finding from the present study was that six sessions of high-intensity intermittent exercise increased total monocyte TLR4 expression with no changes in the monocyte

subset population percentages. This increase in TLR4 expression was also seen in all monocyte subsets apart from $CD14^{++}CD16^{+}$. In contrast, TLR2 expression was unchanged following exercise training in all monocytes subsets.

To the author's knowledge, this was the first study to analyse the influence of a short period of high-intensity intermittent exercise training on TLR4 expression in a young, high BMI sedentary population. Several previous studies have examined the influence of a combination of resistance and aerobic training on monocyte TLR4 expression (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Stewart et al. 2005, Timmerman et al. 2008), but the majority of the studies were conducted on elderly women (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Timmerman et al. 2008). In contrast to the findings of the present study, Timmerman et al. (2008) found no difference in TLR4 expression on inflammatory monocytes, classical monocytes or total monocytes after 12 weeks of training in sedentary elderly females (N=15). However, they showed a 64% reduction in the circulating inflammatory monocyte numbers after 12 weeks of training. Reasons for such a difference in the findings may be attributed to differences in sex, age and physical activity status of the participants between studies. Another reason for differences between the present study and other training studies cited previously was that the exercise protocol, including the intensity and type of exercise, was different, although the total duration of the each session was approximately the same (~ 50 min).

In the present study training did not significantly change total body weight, BMI and waist-to-hip circumference. It can therefore be suggested that the changes observed in the monocyte TLR4 expression may occur irrespective of BMI. So far, no studies have been performed on TLR4 expression in young high BMI sedentary males and so no

direct comparisons can be made. However, changes in TLR4 expression (either increases or decreases) have been shown to be independent of BMI values in other training studies (Stewart et al. 2005, Timmerman et al. 2008).

Although the high-intensity exercise training protocol increased VO_{2peak} by 7.6%, the increase in the monocyte TLR4 expression that occurred may not be a positive outcome for a sedentary population. High TLR4 expression may be linked to low-grade chronic inflammation, which is linked to the occurrence of diseases. Booth et al. (2010) reported increased monocyte TLR4 expression immediately and 1 h after both male and female well-trained participants performed a 60 km indoor cycling time trial; however, no follow up was performed to see if these values were higher a few hours after the exercise. In contrast, it has been observed that monocyte TLR4 expression falls but then returns to baseline levels 4 h after long duration exercise (Chapter 5, now published as Oliveira and Gleeson 2010). Therefore, the changes in the monocyte TLR4 expression seen after a single bout of exercise may not reflect similar findings as a training study. The results found in the present study showed an increased TLR4 expression compared with the beginning of the training programme even though the blood sample was taken 48 hours after the end of the last exercise training session.

Nevertheless, acute inflammatory responses may be one of the stimuli that provoke training adaptations in exercised muscle, and people who exercise more have lower circulating levels of inflammatory markers (Nicklas and Brinkley 2009). These support the idea that exercise has anti-inflammatory effects. The rationale for this may be due to the cross-tolerance or desensitisation that physical training may exert on some immune cells such as monocytes (Flynn and McFarlin 2006). Even though the present study showed an increase in the total monocyte $CD14^+$ TLR4 expression and in the majority

of the monocyte subset TLR4 expression, this may be a transitory effect until homeostasis reoccurs in the system. These results may be because participants were not used to any kind of physical exercise, and were even less used to high-intensity efforts. Nevertheless, it is worth noting that the high-intensity training protocol resulted in no difference in plasma IL-6 and TNF-α concentration (data presented elsewhere). Similar findings to this were reported by McFarlin et al. (2004) after an acute bout of resistance exercise performed by trained elderly women. Smart et al. (2011) meta-analysed the effects of different training protocols on IL-6 and TNF- α in heart failure patients. They found that although exercise training increases VO_{2peak} by around 8% this is not correlated to the reduced levels of TNF- α caused by exercise. In addition, IL-6 was unchanged. It can be speculated that even with high TLR4 expression after two weeks of training, other factors may help to reduce, or at least avoid, the increase of cytokine production in a sedentary population. Factors such as changes in the TLR4 pathway activation through augmented endogenous ligands in the blood (e.g. plasma IL-1ra, heat shock proteins) may contribute to that. To support and prove this idea, a control group and analysis of various cytokine production rates would be necessary. It is important to note that depending on the ligand, TLR4 expression elicits different responses, which will influence the type, magnitude and duration of the inflammatory response. For example, the TLR4/MyD88 signalling pathway is used to induce the expression of pro-inflammatory cytokines, while TLR2/MyD88 stimulates the production of Th2 cells (Brown et al. 2010). If TLR4 links with TRAM, TRIF and subsequently associates with TRAF3, this pathway will result in the production of IFN-β (which is involved in the innate immune response activation) and the production of the anti-inflammatory cytokine IL-10 (Brown et al. 2010,). Once again, direct comparisons with other studies are difficult to make when studies examining the effects of high-intensity exercise

training programmes on TLR4 pathway or cytokine production have not yet been published.

To conclude, two weeks of high-intensity intermittent exercise training increased total $CD14^+$ monocyte TLR4 expression in a sedentary, high BMI population. There was no influence of training on the proportions of classical and pro-inflammatory monocyte subsets, but TLR4 expression in the majority of these monocyte subsets was higher after the six training sessions. It is not certain that the effects seen in this study were due to the very short (2 weeks) duration of the training; therefore, a considerably longer duration study with the addition of cytokine production analysis may help to establish if the elevation on monocyte TLR4 expression is only a transitory effect, and help to obtain clearer results on this topic.

8. Effects of a 4-week Period of High-intensity Interval Training (HIIT) on Monocyte TLR4 Expression in Low Activity Level Participants.

Abstract: Although there is agreement on the importance of physical exercise for general health benefits, the minimal optimal amount needed to improve health status and to reduce TLR4 expression (and possible chronic inflammation) is still uncertain. The aim of the present study was to analyse the effects of 4-weeks of HIIT on monocyte TLR4 expression, classical and pro-inflammatory monocytes, and cytokine production. In addition, this study analysed the effects of an acute bout of high-intensity exercise on monocyte TLR4 expression immediately and 1 h post-exercise before and after the training period. 8 participants (24 ± 9 years old, 26 ± 5 BMI, 2.8 ± 1.0 VO_{2peak}) completed 4-weeks of HIIT sessions (three sessions per week). Blood samples were taken at three time points (PRE, POST and 1H POST) at the beginning and at the end of the training period for analysis of haematocrit, haemoglobin, total white blood cell (leukocyte), monocyte counts, monocyte $CD14^+$ TLR4 expression and monocyte subsets. 4 participants were selected as a control group (25 ± 3 years old, 24 ± 2 BMI, 2.91 ± 0.7 VO_{2peak}) and had their blood samples taken at the same time points, but without exercise. 4 weeks of training augmented monocyte TLR4 expression in all monocyte subsets at immediately post and 1 h post-exercise, but not at rest. Training also reduced the percentage of $CD14^+CD16^+$ pro-inflammatory monocyte after 4 weeks of training, while classical monocytes $CD14^+CD16^-$ were increased after training. In addition, acute exercise reduced total monocyte TLR4 expression 1 h after exercise, but this change was not found after 4 weeks of training. Control group showed no changes of any variable analysed at any time point. Plasma IL-6 was not different for both groups. This may be a transitory effect until homeostasis reoccurs in the system, since participants were not used to the level and duration of the exercise prescribed.

8.1. Introduction

As seen in Chapter 5, a single bout of 1.5 h of cycling exercise at 75% of VO_{2peak} reduced $CD14^+$ monocyte TLR4 expression immediately and 1 h post-exercise, even though there was an increase in the blood monocyte count after exercise. Similarly, a study by Lancaster et al. (2005) reported that TLR4 expression on $CD14^+$ blood monocytes was reduced following 1.5 h of cycling at 65% VO_{2max} in the heat. Simpson et al. (2009) also showed that TLR4 expression on $CD14^+$ blood monocytes was reduced after running for 45 min at 75% of VO_{2max}. In contrast, Booth et al. (2010) reported increased monocyte TLR4 expression immediately and 1 h after both well-trained male and female participants performed a 60 km indoor cycling time trial. The majority of studies have shown a reduction in monocyte TLR4 expression; however, only well-trained participants have been selected to perform moderate intensity, continuous exercise protocols. The reduction of TLR4 expression might result in an 'open window' to infections such as URTI in a physically active population, but not much is known about the effects of exercise on monocyte TLR4 expression after high-intensity exercise training in a relatively sedentary population.

IL-6 and TNF-α are examples of inflammation biomarkers that have been found to be related to risk of developing chronic metabolic and cardiovascular diseases. It has been suggested that exercise training reduces inflammatory biomarkers (Stewart et al. 2005) through exercise-induced reduction of TLR expression and shifts in monocyte phenotype (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Stewart et al. 2005, Timmerman et al. 2008). Stewart et al. (2009) and Timmerman et al. (2008) demonstrated that 12 weeks of combined aerobic and resistance exercise (3 times per week) significantly reduces TLR4 expression in both old and young people. Moreover,

studies have shown that HIIT (high power output for a few minutes followed by a rest period) results in the augmentation of aerobic capacity and fat oxidation even when total exercise time does not exceed 30 min per session (Cornish et al. 2011, Gibala et al. 2006, Gibala and McGlee 2008, Serpiello et al. 2011, Talanian et al. 2007). These findings suggest that high-intensity intermittent exercise is a time-efficient, powerful training strategy to stimulate adaptations in the skeletal muscle and improve health in both trained and untrained individuals when compared with long-duration, moderate-intensity aerobic exercise (Gibala et al. 2006, Serpiello et al. 2011, Talanian et al. 2007). As HIIT lasting up to one hour has been shown to result in greater physiological adaptations than moderate exercise of the same duration, it would be interesting to know if a high-intensity training protocol can elicit more beneficial immunological changes.

Chapter 7 showed that two weeks of HIIT increased monocyte TLR4 expression in a sedentary, high BMI population, however; it is not certain if the effects were due to the very short duration of the training and so potentially representing a transitory adaptation of the immune system. Therefore, the aim of the present study was to analyse the effects of 4-weeks of HIIT on monocyte TLR4 expression, with the addition of cytokine production analysis. It was hypothesised that 4 weeks of HIIT would reduce monocyte TLR4 expression, especially in the pro-inflammatory monocytes, in a sedentary/low-activity level population. In addition, this study analysed the effects of an acute bout of high-intensity exercise on monocyte TLR4 expression immediately and 1 h post-exercise before and after the training period, with the hypothesis that acute exercise would reduce monocyte TLR4 expression after exercise, but no changes would occur after a 4 week training period.

8.2. Methods

A detailed description of the methods is provided in Chapter 3. Any methods used specifically in this study are fully described in this section, while the methods previously described are briefly summarised. This Chapter describes the influence of 4 weeks of high-intensity intermittent exercise training on IL-6 and monocyte TLR4 expression in a sedentary/low activity level population. In addition, it covers the influence of exercise on classical and pro-inflammatory monocytes and the TLR4 expression before and after the training period in all monocyte subsets. A control study was conducted in parallel with the exercise study in order to check for any changes during 4 weeks of unchanged (low) activity level in the variables analysed, and will also be presented here. This study also analysed the effects of an acute bout of high-intensity exercise on monocyte TLR4 expression immediately and 1 h post-exercise, both before and after the training period.

8.2.1. Participants

A total of 12 participants (8 on the exercise group, 4 on the control group) completed the study. Participants were recruited through word-of-mouth, e-mails and advertisements around the Loughborough University area. They were all non-smokers, were not taking any medication and had remained free of symptoms of respiratory infection for at least 4 weeks prior to participation in the study. The same recommendations and exclusion criteria applied to the resting control group. Potential participants read and signed an informed consent form and completed a health screen questionnaire as described in Chapter 3.

A physical activity questionnaire (IPAQ short version, see appendices) and a VO_{2peak} test (described below) were used to determine physical activity levels. To be included in either the exercise or control group, participants had to report exercising no more than 3 hours per week and had a VO_{2peak} less than 45 $ml.kg^{-1}.min^{-1}$ for men and 40 $ml.kg^{-1}.min^{-1}$ for women. The control participants were asked to maintain and record their habitual activity level throughout the 4-week study by filling a short version of the IPAQ questionnaire. Participants were asked not exercise for more than 3 hours per week outside the training programme while participating in the study. IPAQ results showed that 50% of participants did not exercise vigorously during the 4-week period, while the rest exercised once (25%) or twice (25%) per week for no more than one hour. On average, participants exercised vigorously (8 $METs.min^{-1}$) or moderately (4 $METs.min^{-1}$) for only 9 minutes per day (range 0 – 14 min), and spent no more than 2.9 (MET)-h per day doing physical activity including walking (3.3 $METs.min^{-1}$, range 0.6 – 5.0 METs).

8.2.2. Experimental Procedures

Figure 8.1 shows the study design, which included two VO_{2peak} tests, a Familiarisation trial, two main trials and a 4-week training period. The control group performed two VO_{2peak} tests and two main control trials, as described below:

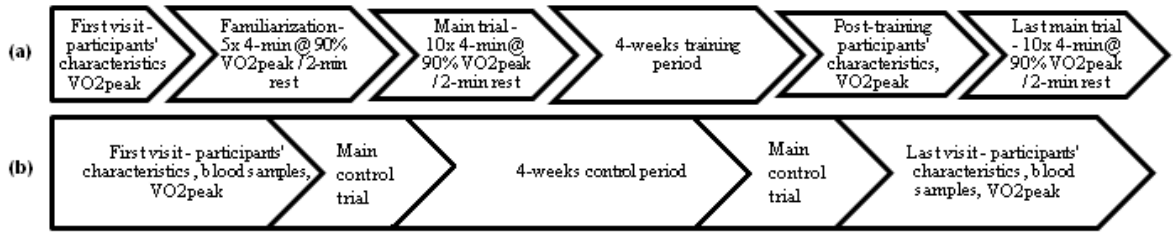

Figure 8.1 – Study Schema (a) Exercise group; (b) Control group

VO_{2peak} Determination

Participants were asked to abstain from alcohol, caffeine and heavy exercise for at least 1 day prior to their visit. Participants completed a continuous incremental exercise test to volitional exhaustion, as described in Chapter 3, using an electrically braked cycle ergometer (Lode Excalibur, Holland). For male participants the test started at 95 W with 35 W increments, while for the female participants, the test started at 75 W with 25 W increments every 3 min until volitional exhaustion. Gas measurements, as well as heart rate and RPE values were recorded throughout the test.

Familiarisation

Participants returned to the lab approximately 1 week after the VO_{2peak} test. They were asked to refrain from any intense physical activity for 24 h prior to the trial. On the day of the test, they were required to complete a daily health questionnaire (see appendices) to ensure they did not present any cold or flu-like symptoms and that they were in good health to participate in the exercise session. They were also asked to fast for at least 10 h and to refrain from caffeine (and food containing caffeine) for 12 h prior to the visit.

The Familiarisation test consisted of five 4-min cycling intervals separated by a 2-min rest at a load corresponding to that which elicited 90% of participants' VO_{2peak}. Water was consumed *ad libitum* during exercise. To confirm that participants were exercising at the right intensity, a 60-s sample of expired gas was collected at the last minute of the second and fourth stages, and measured as described previously. Heart rate and RPE measurements were made 30 s from the end of each 4-min stage. The purpose of this Familiarisation trial was threefold: first, to habituate the participants to the physical stress of the exercise and to the methods involved in the trial; second, to ensure that the

participants were able to maintain the selected intensity for 4 min; and third, to ensure that the selected work rate actually did elicit a relative intensity of 90% VO_{2peak}.

Main Trial

Participants arrived in the laboratory at 08:00 after an overnight fast. They had their body mass, hip and waist circumference measured and then remained seated for 5 min. A blood sample was then taken, after which, participants performed ten 4-min cycling intervals separated by a 2-min rest at a load corresponding to that which elicited 90% of participant's VO_{2peak}. Water was consumed *ad libitum* during exercise. A 60-s sample of expired gas was collected at the last minute of the third, sixth and ninth stages and measured as described previously, to confirm that participants were exercising at the right intensity. Heart rate and RPE measurements were made 30 s from the end of each 4-min stage. Body mass was measured again after exercise. Further venous blood samples were taken immediately post-exercise and at 1 h after exercise. All participants remained seated after exercise until the 1 h post-exercise blood sample had been taken. This session was repeated after the end of the 4-week training period. Ambient temperature was 21 ± 1 and 22 ± 2ºC, barometric pressure was 757 ± 6 and 758 ± 14 mmHg and relative humidity was 40 ± 14 and 46 ± 8% before and after 4 weeks of training respectively.

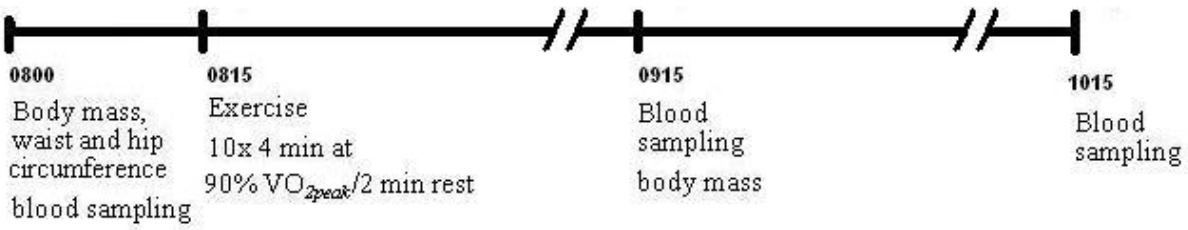

Figure 8.2- Example of exercise schema

Main Trial – Control Group

All participants in the control group visited the lab after an overnight fast. They arrived at the laboratory at 08:00. After 10 min seated rest, a resting blood sample was taken as described in Chapter 3. Participants remained seated for 2 h, and further blood samples were taken after 1 h (equivalent to the post-exercise sample in the exercise protocol) and 2 h (equivalent to the 1 h post-exercise sample in the exercise protocol). Anthropometric measurements were also performed (body mass, hip and waist circumference). This same session was repeated after 4 weeks.

Training Protocol

Participants completed 12 training sessions over a 4-week period (3 sessions per week – mean 11 ± 1 sessions – range 10 – 13). Sessions occurred at any time of the day according to participants' availability. The sessions consisted of the same protocol as described in the main trial, yet they had not fasted and did not have a blood sample taken. The work rate was adjusted (increased by 5 W for females and 10 W for males) during training sessions if participants reported lower RPE and had a lower heart rate value when compared with the values achieved in either the main trial or previous sessions. Since the training sessions proved to increase participants' work load, the last training session before the repetition of their main trial consisted of a new VO_{2peak} test, where a new work load for the post-training main trial was calculated so that the relative intensity of the exercise ($\%VO_{2peak}$) was kept constant.

8.2.3. Biochemical Analysis

Blood Analysis

Haematocrit, haemoglobin, total white blood cell (leukocyte) and monocyte counts were determined from the K_3EDTA blood at each time point during the main trial (PRE, 0, 1 h post-exercise and the equivalent for PRE, 0 and 1 h post at the resting group) using an automated haematology analyser and then centrifuged for 10 min at 1500 *g* at 4ºC. Aliquots of plasma were immediately stored in a -80 °C freezer for future analysis of IL-6. Changes in plasma volume were calculated from measurements of haemoglobin and haematocrit according to the method described by Dill and Costill (1974).

Monocyte Subpopulation Determination and TLR Expression

The same method described in Chapter 7 was also used to analyse the TLR4 expression in this study. In brief, samples were analysed on a flow cytometer equipped with the CellQuest software package. Cells were gated according to side scatter and CD14-FITC expression (which characterises monocytes), and the geometric mean fluorescence intensity (GMFI) of the Isotype control (ISO), and of TLR2 and TLR4 antibodies in the $CD14^+$ cell gated population was obtained to quantify TLR expression (total of 20,000 cells acquired for each sample). Isotype control GMFI was used to correct values for non-specific binding. Additional gates were then used to identify the $CD14^{++}$ /$CD16^-$, $CD14^{++}$/$CD16^+$ and $CD14^+$/$CD16^{++}$ monocyte populations and their expression of both TLR2 and TLR4.

Enzyme Linked Immunosorbent Assay (ELISA) IL-6

For the IL-6 analysis plates were coated with anti-human IL-6 monoclonal capture antibody (OptEIA, BD Biosciences, Oxford, UK) diluted 1:250 in 0.1 M sodium carbonate, then sealed and incubated overnight at 4ºC. After that each plate was washed and then blocked with 5% bovine serum albumin (BSA; Probumin, Millipore, Illinois, USA) in Trisbuffered saline (TBS). Following this, plates were incubated for 1 h at room temperature and then washed. 100 µl of samples or standards were added to the wells and incubated at room temperature for 20 h. Standard IL-6 concentrations varied from 0.780 to 200 $pg \cdot ml^{-1}$. Plates were then washed and 100 µl IL-6 detection antibody (OptEIA, BD Biosciences, Oxford, UK) diluted 1:250 in TBS-Tween 20 (TBS-T) with 1% BSA was added per well. Plates were incubated for further 2 h before washing. The enzyme streptavidin alkaline phosphatase was diluted 1:2,000 in TBS with 1% BSA and 100 µl was added per well. Plates were then incubated for 1 h. After washing, an ELISA amplification system was used (substrate + amplifier, Invitrogen, Paisley, UK). The reaction was stopped with 50 µl of 10% sulphuric acid stop solution, and the absorbance of the wells was read at 490 nm. Samples were analyzed in duplicate with an inter-assay coefficient of 7.1%. The cytokine concentration of each sample was acquired from the standard curve.

8.2.4. Statistical Analysis

Normality tests were carried out on all the data. Data that were not normally distributed were analysed using Friedman's ANOVA. When a statistically significant difference was found, a Wilcoxon signed-rank test (corrected for the time points) was carried out to locate the difference. Pearson's correlation was used to analyse whether there was a correlation between IL-6 and TLR4 expression. Parametric tests were used for normally

distributed data (e.g. t-test, ANOVA). Statistical significance was accepted at $P < 0.05$. All results are presented as mean ± SD.

8.3. Results

8.3.1. Physiological Characteristics

Participants performed their high-intensity intermittent training at ~84% of their VO_{2peak} throughout the 4-week training programme. VO_{2peak} was increased by 8.4% ($P = 0.04$, Table 8.1), and maximum work rate during VO_{2peak} test was 10.8% higher after the training programme compared with pre training ($P = 0.004$). No changes were found in any other variable measured.

Table 8.1 – Participants' anthropometrical and physiological characteristics before and after the 4-week training or resting programme

	Exercise group (N=8)		Control group (N=4)	
	Before training	**After training**	**Before 4 weeks**	**After 4 weeks**
Age (Years)	24 ±9	24 ± 9	25 ± 3	25 ± 3
Body Mass (kg)	79.3 ± 20.7	79.0 ± 20.0	71.3 ± 14.8	71.1 ± 15.0
Height (m)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
BMI ($kg \cdot m^{-2}$)	26 ± 5	26 ± 4	24 ± 2	24 ± 2
Waist circumference (cm)	94.1 ± 10.0	91.7 ± 11.8	88.3 ± 5.8	89.6 ± 5.5**
Hip circumference (cm)	105.0 ± 9.0	103.8 ± 7.8	103.4 ± 5.7	102.4 ± 7.2
Waist-to-hip ratio	0.90 ± 0.05	0.88 ± 0.06	0.85 ± 0.01	0.88 ± 0.01
VO_{2peak} ($l \cdot min^{-1}$)	2.8 ± 1.0	3.1 ± 1.2*	2.9 ± 0.7	2.7 ± 0.4
Work rate at VO_{2peak} (W)	204 ± 72	226 ± 78*	226 ± 34	235 ± 54
% VO_{2peak} during exercise	85 ± 4	83 ± 3	--	--

* Statistically significant compared with pre training values; ** statistically significant compared with pre 4 weeks values. $P = 0.05$

8.3.2. Biochemical Analysis

Monocyte Subpopulation

The percentage of classical monocytes ($CD14^{++}CD16^{-}$) was increased at rest after 4 weeks of training ($P = 0.03$). On the other hand, the percentage of one of the pro-

inflammatory monocyte subsets ($CD14^{++}CD16^{+}$) was reduced after the training period ($P = 0.02$).

Table 8.2 - Monocyte subpopulation before and after training programme

	Exercise group		Control group	
	Before training	**After training**	**Before training**	**After training**
$CD14^{++}CD16^{-}$ (%)	82.2 ±7.8	88.6 ± 5.1*	79.5 ± 5.5	82.9 ± 2.7
$CD14^{++}CD16^{+}$ (%)	4.6 ± 2.9	3.1 ± 1.6*	4.3 ± 1.1	4.6 ± 1.4
$CD14^{+}CD16^{++}$ (%)	3.9 ± 3.3	2.6 ± 2.9	4.9 ± 1.3	3.9 ± 1.0
Non-gated (%)	9.3 ± 5.0	5.7 ± 2.1	11.3 ± 5.3	8.6 ± 3.1

* Significant different from pre-training values

Monocytes were also analysed at post and 1 h post the HIIT was performed (acute exercise). Both before and after 4 weeks training trials showed no difference in monocyte subset at any time point ($P > 0.05$). In addition, no difference was found in the control group ($P > 0.05$).

Monocyte TLR Expression

As can be seen in Figure 8.3, one acute bout of high-intensity exercise reduced total $CD14^{+}$ monocyte TLR4 expression 1 h post exercise ($P = 0.02$, Figure 8.3a). However, no difference was found for TLR4 expression in any monocyte subset 4 weeks after training for the same acute exercise. In contrast, TLR4 expression after 4 weeks of training was higher at post and 1 h post exercise when compared with before training in all monocyte subsets (Figure 8.3b, $P = 0.01$).

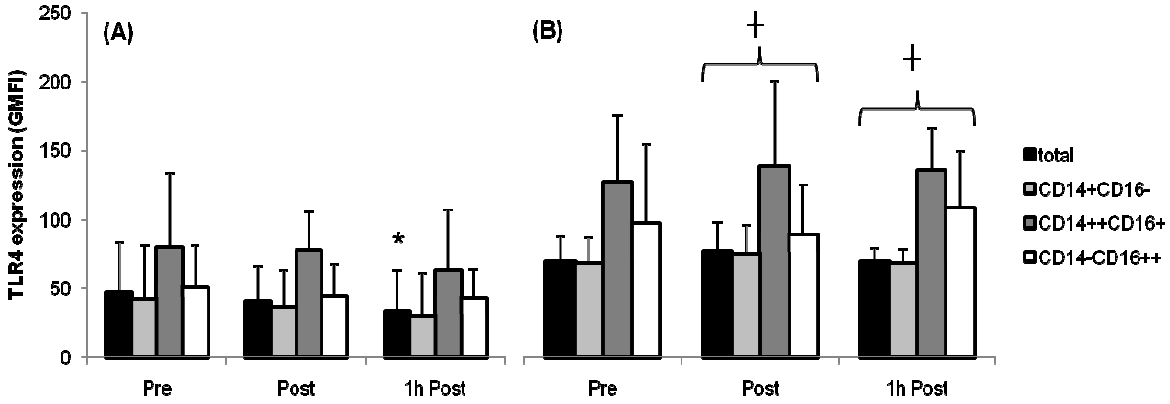

Figure 8.3 – (A) Monocyte TLR4 expression pre, post and 1 h post exercise before and (B) after 4 weeks of high-intensity training.
* Different from Pre. † Different from before training at the same time point.

The control group showed no statistical differences in monocyte TLR4 expression when blood samples were taken at the same time points as the exercise group, neither before nor after 4 weeks ($P > 0.05$). Values for the control group can be seen in Table 8.3.

Table 8.3 - Monocyte TLR4 expression at 0, 1 h and 2h resting before and after 4 weeks for the control group

GMFI	Before			After		
	0 h	**+ 1 h**	**+ 2 h**	**0 h**	**+ 1 h**	**+ 2 h**
Total $CD14^+$	45 ± 28	44 ± 27	49 ± 19	66 ± 26	62 ± 19	54 ± 13
$CD14^+CD16^-$	38 ± 28	37 ± 28	43 ± 20	63 ± 26	59 ± 18	51 ± 13
$CD14^+CD16^+$	64 ± 43	61 ± 39	92 ± 63	124 ± 74	119 ± 48	97 ± 31
$CD14^-CD16^{++}$	59 ± 35	59 ± 32	73 ± 27	95 ± 26	105 ± 40	94 ± 27

No statistical difference at any time point ($P > 0.05$)

As Chapter 4 showed a statistical difference in the TLR4 expression between sex, the training group was divided into male (N=4) and female (N=4) and the effects of an acute bout of exercise and of training on monocyte TLR4 expression were then analysed. The male group presented a lower TLR4 expression in total ($P = 0.03$), $CD14^+CD16^-$ ($P = 0.03$) and $CD14^+CD16^{++}$ ($P = 0.02$) monocytes when compared with the female group, but this difference was only seen before training. Considering males and females together, total monocyte TLR4 expression was the only measurement to show a difference across time (1 h after exercise) before training, therefore male and

female TLR4 expression was compared individually. Results showed that sex did not account for the reduction seen in the total TLR4 expression 1 h post exercise before training ($P = 0.10$).

Plasma IL-6

Plasma IL-6 was analysed in duplicate at rest and immediately post-exercise before and after 4 weeks of training or resting (for the control group). One participant on the exercise group and two participants on the control group showed very high plasma IL-6 levels compared to the other participants. Both the training and control group showed no difference in plasma IL-6 response across time before or after 4 weeks ($P = 0.34$ and $P = 0.14$ respectively, Table 8.4). No correlation was found between plasma IL-6 and TLR4 expression in any monocyte subsets ($P > 0.05$) in any group.

Table 8.4 - Plasma IL-6 concentration before and after 4-weeks of training or rest

(pg/ml)	Before training	After training
Exercise	30 ± 59	21 ± 11
Control	26 ± 26	23 ± 24

8.4. Discussion

The aim of the present study was to analyse the effects of 4 weeks of HIIT on monocyte TLR4 expression, with the addition of IL-6 analysis. Results found in this study failed to support the hypothesis that 4 weeks of HIIT would reduce monocyte TLR4 expression in a sedentary/low activity population. In fact, 4 weeks of training augmented monocyte TLR4 expression in all monocyte subsets at immediately post and 1 h post-exercise, but not at rest. In addition, this study analysed the effects of an acute bout of high-intensity exercise on monocyte TLR4 expression post and 1 h post exercise before and after training. Acute exercise reduced total monocyte TLR4

expression 1 h after exercise, but this change was not found after 4 weeks of training, supporting the study's alternative hypothesis. The control group showed no difference in any of the variables analysed at any time points.

In relation to the monocyte subsets, classical monocytes ($CD14^{++}CD16^{-}$) numbers increased at resting after 4 weeks of training, and pro-inflammatory monocyte subsets ($CD14^{++}CD16^{+}$) were reduced after the 4-week training period, with no changes seen in the control group. Plasma IL-6 results were similar to resting values after the acute exercise, before and after 4 weeks in both the training and control group. No correlation between TLR4 expression and IL-6 was found at any time points in either group.

Chapter 7 is thought to be the first study to analyse the influence of a short period of high-intensity interval exercise training on TLR4 expression in a young, high BMI, sedentary population. The present Chapter was designed to fill this gap in the field and to answer some questions that arose in Chapter 7. Several previous studies have examined the influence of a combination of resistance and aerobic training on monocyte TLR4 expression (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Stewart et al. 2005, Timmerman et al. 2008), but the majority of the studies were conducted on elderly women (Coen et al. 2010, Flynn et al. 2003, McFarlin et al. 2006, Timmerman et al. 2008). In contrast to the findings of Chapter 7 and the present study, Timmerman et al. (2008) found no difference in TLR4 expression on inflammatory monocytes, classical monocytes or total monocytes after 12 weeks of training in sedentary elderly females (N=15). However, participants showed a 64% reduction in the inflammatory monocyte levels after 12 weeks of training. Similarly, the present study showed a 22% reduction in the $CD14^{+}CD16^{+}$ pro-inflammatory monocyte after 4 weeks of training. In addition, classical monocyte $CD14^{+}CD16^{-}$ showed an 8% increase after training.

Although some findings are similar to Timmerman et al. (2010) study, reasons for differences in the findings can be attributed to differences in sex, age and the physical activity status of the participants. Another reason for differences between the present study and other training studies cited previously was that the exercise protocol, including the intensity and type of exercise was not identical, although the total duration of the each session was approximately the same (~ 50 min).

In the present study, training did not change total body mass, BMI and wait-to-hip circumference. It can therefore be suggested that the changes observed in the monocyte TLR4 expression may occur irrespective of BMI or waist-to-hip ratio. So far, no studies have observed TLR4 expression in young high sedentary males and so no direct comparison on this topic can be made. Likewise, changes in TLR4 expression have been shown to be independent of physiological characteristics (e.g. BMI, waist-to-hip ratio) in other training studies (Stewart et al. 2005, Timmerman et al. 2008).

The high-intensity exercise training protocol resulted in positive physiological outcomes including an increase in VO_{2peak} by 8.4%, and a 10.8% elevation on the maximum work rate during the second VO_{2peak} test compared with the first test. However, the increase in the monocyte TLR4 expression that occurred after the training may or may not be a positive outcome for a sedentary population. High IL-6 values and augmented TLR4 expression may be linked to low-grade chronic inflammation, which is linked to the occurrence of diseases (Nicklas and Brinkley 2009). Even though the present study showed an increase in the classical monocyte $CD14^{+}CD16^{-}$, and in the TLR4 expression in all monocyte subsets after an acute bout of exercise, the high-intensity training protocol resulted in no difference in plasma IL-6 results. Smart et al. (2011) meta-analysed the effects of different training protocols on IL-6 and TNF- α in

heart failure patients. It was found that although exercise training increases VO_{2peak} by around 8%, this is not correlated to the reduced levels of TNF- α caused by exercise. In addition, IL-6 was unchanged. It can be speculated that even with high TLR4 expression after two weeks of training other factors may help to reduce, or at least avoid, the increase of cytokine production in a sedentary population. In addition, augmented classical monocytes TLR4 expression may be related to the increase in pro-inflammatory cytokines, which would contribute to the immunity-enhanced protection induced by exercise. Factors such as changes in the TLR4 pathway activation through augmented endogenous ligands in the blood (e.g. heat shock proteins, TRAF6 pathway activation) may contribute to that. It is important to note that depending on the ligand, TLR4 expression elicits different responses, which will influence the type, magnitude and duration of the inflammatory response. To conclude, 4 weeks of high-intensity intermittent exercise training increased monocyte TLR4 expression in all monocyte subsets at post and 1 h post an acute bout of exercise, but not at rest in a young sedentary population. Training also reduced the percentage of $CD14^{+}CD16^{+}$ pro-inflammatory monocyte after 4 weeks of training, while classical monocyte $CD14^{+}CD16^{-}$ was increased after training. This may be a transitory effect until homeostasis reoccurs in the system, since participants were not used to the level and duration of the exercise prescribed. However more studies in the area are required.

9. General Discussion

This Chapter contains a summary of the main results of the studies comprised within this thesis, followed by a broad discussion that ties the studies together and discusses the relevance of these findings and the practical implications within the field of Sports Sciences and Exercise Immunology. In addition, the limitations of the studies within this thesis are outlined, together with the importance and recommended direction of future studies.

The general aim of this thesis was twofold: 1) to investigate the effect of different exercise protocols on TLR2 and TLR4 expression and 2) to investigate if resting immunological markers were different according to sex and physical activity level. The main findings of the studies comprised within this thesis were:

- The blood measurements and the coefficient of variation of the flow cytometry and cell count analysis were highly reproducible and had a low coefficient of variation.
- Monocyte TLR4 expression was higher in females compared with males during rest.
- Monocyte TLR4 expression was higher in the population with a low compared with a high level of physical activity, although the level of physical activity did not relate to URTI incidence.
- Plasma levels of cytokine were not significantly different neither between groups with different levels of physical activity (after stimulation) nor after 4 weeks of training.

- Prolonged cycling exercise at 75% VO_{2peak} reduced monocyte TLR4 expression in healthy men, with values returning to normal after 4h.
- Monocyte $CD14^{+}$ TLR4 expression did not significantly change after two VO_{2peak} tests separated by 2 hours.
- Two weeks of high-intensity training had no influence on the proportions of classical and pro-inflammatory monocyte subsets.
- TLR4 expression in the majority of the monocyte subsets (apart from $CD14^{++}CD16^{+}$) was higher after six training sessions in high BMI sedentary men.
- 4 weeks of training augmented monocyte TLR4 expression in all monocyte subsets at immediately post and 1 h post-exercise compared to pre training values, but changes were not seen at rest.
- 4 weeks of HIIT reduced the percentage of $CD14^{+}CD16^{+}$ pro-inflammatory monocytes.
- 4 weeks of HIIT increased the percentage of $CD14^{+}CD16^{-}$ classical monocytes.
- Acute exercise reduced total monocyte TLR4 expression 1 h after exercise in low physical activity level participants, but this change was not found after 4 weeks of training.

The results of this thesis suggest that TLR4 expression is different in accordance to sex and physical activity level. As seen in Chapter 4, females have a higher TLR4 expression compared with males. It has been suggested that hormones *per se* (e.g. cortisol and oestrogen) may influence the immune system (Gillum et al. 2011), but the changes in the immune system seen after exercise seem to not be influenced by hormonal changes (Lancaster et al. 2005, Gillum et al. 2011). Moreover, some studies found that the menstrual cycle did not affect some immunological parameters in trained

athletes (Burrows et al. 2002). One of the limitations of Chapter 4 was that the phase of the menstrual cycle was not determined when the blood sample was taken and it was not established whether or not the females were taking oral contraceptives. Nevertheless, as suggested by Gillum et al. (2011) and Burrows et al. (2002), sex differences in immune function among athletes can be taken in consideration independently of females' menstrual phase, and this may also apply when analysing TLR4 expression. However, both male and female levels of TLR4 expression were not related to cytokine production or URTI incidence, and therefore this difference perhaps was not enough to considerably affect susceptibility to URTI. It would, nevertheless, be recommended that males and females are allocated in separate groups when analysing TLR4 expression, or caution is taken when interpreting future data from mixed gender studies.

When participants were divided by their physical activity status, $CD14^+$ monocyte TLR4 expression was higher in the LOW group compared to the HIGH group. These data are in accordance to McFarlin et al. (2004, 2006) and Stewart et al. (2005), where sedentary populations of both sexes presented higher $CD14^+$ monocyte TLR4 expression compared with more active individuals. This may be an indication of why low levels of physical activity are related to chronic inflammation and the development of some diseases (Stewart et al. 2005). However, Chapter 4 in this thesis did not examine the cytokine production and URTI episodes in the LOW group, but instead compared the MED and HIGH groups in relation to URTI and cytokine production. There were found to be no significant differences in the number of URTI episodes between MED and HIGH groups, but the HIGH group produced higher levels of the anti-inflammatory cytokine IL-10 following antibody stimulation. It has been suggested that IL-10 is able to impair the ability of APCs to promote the differentiation and

proliferation of $CD4^+$ T cells, thereby regulating both initiation and perpetuation of adaptive T-cell responses (Murphy et al. 2008).

Further results from this thesis showed that different types of acute exercise result in different responses in the TLR expression. The findings from Chapter 5 is in agreement with Lancaster et al. (2005) showing that strenuous, long duration physical activity reduces TLR4 expression by around 40% immediately post and 1 h post exercise, despite the increase in blood monocyte numbers. This is also in accordance with Simpson et al. (2009), who reported changes in TLR4 but no changes in TLR2 expression after 45 minutes of running as 75% VO_{2max}. These findings were supported in this thesis by the fact that the control participants showed no change in their TLR4 expression at the same time points.

Factors such HLA.DR, HSPs, cortisol, internalisation of the TLR receptors, and the circulating concentration of cytokines such as IL-6 have been suggested to be responsible for the changes in TLR expression (Gleeson et al. 2006, Simpson et al. 2009). The decline in TLR4 expression may influence and be influenced by the changes in the accessory signal molecule expression HLA.DR, as seen by Simpson et al. (2009). HLA.DR is a transmembrane MHC II that activates Th cells and activates the acquired immune system. However, the changes found by Simpson et al. (2009) in both TLR4 and HLA.DR were suggested to be due to a reduction in the total number of classical monocytes in the circulation. Classical monocytes are the major monocyte subset responsible for activating anti-inflammatory cytokine production, which would therefore help in the prevention of a pathogen invasion. Thus, the reduction seen in TLR4 expression after a single bout of acute exercise (as seen Chapter 5 but not in Chapter 6) may lead to a briefly impaired immune function and an increased

susceptibility to infection such as URTI, since TLR4 values returned to resting levels after 4 hours. This thesis did not measure cortisol response after exercise; however, the possibility of cortisol influencing TLR expression was discarded since Lancaster et al. (2005) showed that changes in TLR expression were independent of large diurnal changes in cortisol.

Some endogenous ligands such as HSPs and cytokines may act as inhibitors of TLR expression (Gleeson et al. 2006, Simpson et al. 2009). It is well established that circulating IL-6 levels are elevated after a single bout of exercise, and it has been suggested that IL-6 plays an important role in down regulating TLR expression in different conditions (Lancaster et al. 2005). Pedersen and Febbraio (2008) showed that intramuscular IL-6 production and release into the circulation increase during prolonged exercise. As TLR activation also results in the production of IL-6 through the NF-kB signalling pathway, it is hypothesised that high levels of IL-6 in the blood after exercise down regulate the expression of TLRs. The decline in TLR expression is associated with reductions in accessory signal molecule expression (e.g. CD80 and CD86) and antigen-presenting cell activation after acute exercise. Furthermore, HSPs are proteins present in all cells and are up-regulated during physiological stress and it has been suggested that, in a similar manner to LPS, HSPs act as activators of TLR4. Kilmartin and Reen (2004) showed that repeated monocyte exposure to HSP60 (as seen by athletes' constant training) induces a tolerance to HSP and a 'cross-tolerance' to LPS stimulation, subsequently reducing TLR4 activation.

All potential factors mentioned above (HLA.DR, HSPs, and IL-6) could potentially increase the risk of URTI due to reduction in TLR4 expression; however, it could also support the idea of a controlled immune system working against unnecessary

inflammation, avoiding the development of chronic inflammation and preventing the progression to chronic diseases. This can be seen as a negative feedback and, repeated exposure to similar conditions may result in a 'training' adaptation of the immune system, where by an inhibition of extra pro-inflammatory cytokine production occurs, helping the body to not overreact towards changes in the body homeostasis caused by exercise. This, again, can be a way for the immune system to avoid chronic inflammatory responses. However, this may also augment the chances that the immune system may not be able to react as effectively against common infection such as URTI (Lancaster et al. 2005; Gleeson et al. 2006). Caution in interpreting these findings might be taken since Booth et al. (2010) found that soluble serum factors taken from blood sampled after exercise does not change resting monocyte TLR expression in vitro, suggesting that changes in TLR expression found after exercise are not caused by endogenous ligands. One of the factors that has not been discussed in depth in the literature to date is the possible internalisation or shedding of the TLR receptors after exercise, and clearly more research in this area is needed.

Despite the majority of literature showing findings to suggest a reduction in TLR expression after exercise, the results in this thesis showed that two VO_{2peak} tests separated by two hours did not influence TLR4 expression in healthy men, suggesting that a prolonged and more intense exercise may be necessary to instigate a response in this aspect of the immune system. The same suggestion would be valid for TLR2 expression, since strenuous exercise in Chapter 5 did not instigate statistically significant changes in this receptor, providing the reason why TLR2 was not analysed in further Chapters. Differences in participants' fitness levels, exercise type, duration, intensity and methods used to analyse the variables may contribute to the different results found. Booth et al. (2010) showed an increase in TLR2 and TLR4 expression

after a 60 km cycling time trial (mean completion time 92 ± 7 min, heart rate 83 ± 5% bpm) in both male and female participants. Interestingly, Chapter 8 showed that 10 repetitions of 4 min of intense exercise reduced total $CD14^{+}$ monocyte TLR4 expression 1 h post exercise in sedentary participants, but this change was not seen when the same test was performed after 4 weeks of training. This result suggests some adaptation in the immune system to the stress provoked by exercise training in a population that were not previously accustomed to high-intensity of exercise.

Both Chapters 7 and 8 analysed the effects of a period of HIIT on monocyte subset populations and on TLR4 expression in these monocyte subsets in a sedentary or low level of physical activity population. Based on results reported by Timmerman et al. (2008), it was hypothesised that pro-inflammatory monocytes numbers would reduce after training, together with the TLR4 expression. However, as seen in Chapter 7, although there were no changes in monocyte subsets numbers, TLR4 expression was augmented after 2 weeks of HIIT sessions. In addition, in Chapter 8 it was demonstrated that there was an increase in the numbers of classical monocytes, and a reduction in the pro-inflammatory monocyte subset ($CD14^{++}CD16^{+}$) after the 4-week training period, with no changes seen in the control group. Moreover, TLR4 expression did not change at rest after 4 weeks of training. These results suggest that a transitory increase in the TLR4 expression occurs after 2 weeks due to possible training-induced stress and adaptations, but these changes are not seen after a 4-week protocol. Nevertheless, it is important to mention that participants' aerobic capacity and work load increased considerably in a short period in both groups.

It was interesting to note that after 4 weeks of training a reduction of pro-inflammatory monocyte numbers together with an increase of classical monocytes was observed. As

pro-inflammatory monocytes express more TLR4 compared with classical monocytes (Ziegler-Heitbrock 2007), reduction in the numbers of the former type of monocyte may contribute to the reduction of chronic inflammation, while an increase in the classical monocytes may help the prevention of diseases by augmenting the production of anti-inflammatory cytokines. However, more investigation in this area is required since plasma IL-6 results were similar to resting values after the training period in both the training and control group, and no correlation between monocyte TLR4 expression and plasma IL-6 concentration was found at any time point in either group. One possible explanation for this surprising finding could be related to the experimental protocol used. While Timmerman et al. (2008) used a combination of 20 min of endurance exercise (at 60-70% of heart rate reserve) and some resistive exercise in elderly woman, the studies in Chapters 7 and 8 used healthy young male participants, and a protocol that could be considered very intense (10 x 4 min at ~90% VO_{2peak} with 2 min resting for 1 hour) compared to other studies (McFarlin et al. 2004, Lancaster et al. 2005, Stewart et al. 2005, Timmerman et al. 2008, Simpson et al. 2009).

It is therefore concluded that:

- Resting TLR4 expression is different according to sex and physical activity level. It is recommended that males and females are considered separately when analysing some immunological markers such as TLR4 expression.
- Acute bouts of prolonged strenuous exercise reduce TLR4 expression for a few hours after the completion of the exercise, thus extra caution should be taken in this time period to ensure athletes minimise the risk of contracting an infection, especially commonly infections encountered in our daily lives such as URTI.

- Short duration exhaustive exercise separated by 2 hours does not change TLR4 expression.
- High-intensity intermittent training improves participants' aerobic capacity and modifies monocyte subpopulation concentrations in the blood. Further research is suggested in this area to better understand the changes in TLR4 expression and monocyte subsets after different training protocols.

Although this thesis examined the effects of exercise on TLR2 and TLR4 expression, it is important to note that the immune system is an inter-communicable system and that the clinical significance of small singular changes requires further investigation (Walsh et al. 2011). It is therefore acknowledged that several extra analyses could have been done to try to elucidate the effects of exercise on immune function. However this was not possible due to time and money constrains, and was beyond the agreed scope of this thesis.

References

AKIRA, S., 2003. Mammalian Toll-like receptors. *Current Opinion in Immunology,* **15**(1), 5-11.

AKIRA, S. and HEMMI, H., 2003. Recognition of pathogen-associated molecular patterns by TLR family. *Immunology Letters,* **85**(2), 85-95.

AKIRA, S. and TAKEDA, K., 2004. Toll-like receptor signalling. *Nature Reviews.Immunology,* **4**(7), 499-511.

AKIRA, S., YAMAMOTO, M. and TAKEDA, K., 2003. Role of adapters in Toll-like receptor signalling. *Biochemical Society Transactions,* **31**(Pt 3), 637-642.

BEG, A.A., 2002. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. *Trends in Immunology,* **23**(11), 509-512.

BERMON, S., 2007. Airway inflammation and upper respiratory tract infection in athletes: is there a link? *Exercise Immunol Review,* **13**, 6-14.

BOOTH, S., FLORIDA-JAMES, G.D., MCFARLIN, B.K.S., G., O'CONNOR, D.P. and SIMPSON, R.J., 2010. The impact of acute strenuous exercise on TLR2, TLR4 and HLA.DR expression on human blood monocytes induced by autologous serum. *European Journal of Applied Physiology,* **110**(1259), 1268.

BRINES, R., HOFFMAN-GOETZ, L. and PEDERSEN, B.K., 1996. Can you exercise to make your immune system fitter? *Immunology Today,* **17**(6), 252-254.

BRINKLEY, T.E., LENG, X., MILLER, M.E., KITZMAN, D.W., PAHOR, M., BERRY, M.J., MARSH, A.P., KRITCHEVSKY, S.B. and NICKLAS, B.J., 2009. Chronic inflammation is associated with low physical function in older adults across multiple comorbidities. *The Journals of Gerontology: Series A,* **64A**(4), 455-461.

BRITISH HEART FOUNDATION, January, 13th, 2010-last update. Available: http://www.heartstats.org/ [January, 30th, 2010].

BROWN, J., WANG, H., HAJISHENGALLIS, G.N. and MARTIN, M., 2011. TLR-signaling Networks. *Journal of Dental Research,* **90**(4), 417.

BURROWS, M., BIRD, S. and BISHOP, N., 2002. The menstrual cycle and its effect on the immune status of female endurance runners. *Journal of Sports Sciences,* **20**(4), 339-344.

CESARI, M., PENNINX, B.W.J.H., NEWMAN, A.B., KRITCHEVSKY, S.B., NICKLAS, B.J., SUTTON-TYRRELL, K., RUBIN, S.M., DING, J., SIMONSICK, E.M. and HARRIS, T.B., 2003. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. *Circulation,* **108**(19), 2317.

CHESLEY, A., HEIGENHAUSER, G.J. and SPRIET, L.L., 1996. Regulation of muscle glycogen phosphorylase activity following short-term endurance training. *American Journal of Physiology-Endocrinology and Metabolism,* **270**(2), E328.

COEN, P.M., FLYNN, M.G., MARKOFSKI, M.M., PENCE, B.D. and HANNEMANN R.E., 2010. Adding exercise to rosuvastatin treatment: influence on C-reactive protein, monocyte toll-like receptor 4 expression, and inflammatory monocyte (CD14+CD16+) population. *Metabolism,* **59**(12), 1775-1783.

COLBERT, L.H., VISSER, M., SIMONSICK, E.M., TRACY, R.P. and NEWMAN, A.B., 2004. Physical Activity, Exercise, and Inflammatory Markers in Older Adults: Findings from The Health, Aging and Body Composition Study. *Journal of the American Geriatrics Society,* **52**(7), 1098.

CORNISH, A.K., BROADBENT, S. and CHEEMA, B.S., 2011. Interval training for patients with coronary artery disease: a systematic review. *European Journal of Applied Physiology,* **111**(4), 579-589.

CRAIG, C.L., MARSHALL, A.L., SJOSTROM, M., BAUMAN, A.E., BOOTH, M.L., AINSWORTH, B.E., PRATT, M., EKELUND, U., YNGVE, A., SALLIS, J.F. and OJA, P., 2003. International physical activity questionnaire: 12-country reliability and validity. *Medicine and Science in Sports and Exercise,* **35**(8), 1381-1395.

DILL, D.B. and COSTILL, D.L., 1974. Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *Journal of Applied Physiology,* **37**(2), 247-248.

FAUL, F., ERDFELDER, E., BUCHNER, A. and LANG, A.G., 2009. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. *Behavior Research Methods,* **41**(4), 1149-1160.

FESTA, A., D'AGOSTINO, R.,JR, WILLIAMS, K., KARTER, A.J., MAYER-DAVIS, E.J., TRACY, R.P. and HAFFNER, S.M., 2001. The relation of body fat mass and distribution to markers of chronic inflammation. *International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,* **25**(10), 1407-1415.

FISCHER, C.P., BERNTSEN, A., PERSTRUP, L.B., ESKILDSEN, P. and PEDERSEN, B.K., 2007. Plasma levels of interleukin-6 and C-reactive protein are associated with physical inactivity independent of obesity. *Scandinavian Journal of Medicine & Science in Sports,* **17**(5), 580-587.

FLYNN, M.G. and MCFARLIN, B.K., 2006. Toll like receptor 4: Link to the anti-inflammatory effects of exercise? *Exercise and Sport Sciences Reviews,* **34**(4), 176-181.

FLYNN, M.G., MCFARLIN, B.K., PHILLIPS, M.D., STEWART, L.K. and TIMMERMAN, K.L., 2003. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. *Journal of Applied Physiology,* **95**(5), 1833-1842.

FRANCAUX, M., 2009. Toll-like receptor signalling induced by endurance exercise. *Applied Physiology, Nutrition, and Metabolism,* **34**(3), 454-458.

FRICKER, P.A., PYNE, D.B., SAUNDERS, P.U., COX, A.J., GLEESON, M. and TELFORD, R.D., 2005. Influence of training loads on patterns of illness in elite distance runners. *Clinical Journal of Sport Medicine,* **15**(4), 246-252.

FRISARD, M.I., MCMILLAN, R.P., MARCHAND, J., WAHLBERG, K.A., WU, Y., VOELKER, K.A., HEILBRONN, L., HAYNIE, K., MUOIO, B., LI, L. and HULVER, M.W., 2010. Toll-like receptor 4 modulates skeletal muscle substrate metabolism. *American Journal of Physiology.Endocrinology and Metabolism,* **298**(5), E988-98.

FULOP, T., LARBI, A., DOUZIECH, N., LEVESQUE, I., VARIN, A. and HERBEIN, G., 2006. Cytokine receptor signalling and aging. *Mechanisms of Ageing and Development,* **127**(6), 526-537.

GALLOWAY, S.D. and MAUGHAN, R.J., 1997. Effects of ambient temperature on the capacity to perform prolonged cycle exercise in man. *Medicine and Science in Sports and Exercise,* **29**(9), 1240-1249.

GIBALA, M.J., LITTLE, J.P., VAN ESSEN, M., WILKIN, G.P., BURGOMASTER, K.A., SAFDAR, A., RAHA, S. and TARNOPOLSKY, M.A., 2006. Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *Journal of Physiology,* **575**(3), 901-911.

GIBALA, M.J. and MCGEE, S.L., 2008. Metabolic Adaptations to Short-term High-Intensity Interval Training: A Little Pain for a Lot of Gain? *Exercise and Sports Science Review,* **36**(2), 58-63.

GILLUM, T.L., KUENNEN, M.R., SCHNEIDER, S. and MOSELEY, P., 2011. A review of sex differences in immune function after aerobic exercise. *Exercise immunology Review,* **17**, 104-121.

GLEESON, M., 2006. *Immune function in sport and exercise.* Edinburgh: Churchill Livingstone.

GLEESON, M., 2007. Immune function in sport and exercise. *Journal of Applied Physiology,* **103**(2), 693.

GLEESON, M., 2006. Immune system adaptation in elite athletes. *Current Opinion in Clinical Nutrition and Metabolic Care,* **9**(6), 659-665.

GLEESON, M. and BISHOP, N.C., 2005. The T cell and NK cell immune response to exercise. *Annals of Transplantation : Quarterly of the Polish Transplantation Society,* **10**(4), 43-48.

GLEESON, M., BISHOP, N., OLIVEIRA, M., MCCAULEY, T. and TAULER, P., 2011a. Sex differences in immune variables and respiratory infection incidence in an athletic population. *Exercise Immunology Review,* **17**, 122-135.

GLEESON, M., BISHOP, N., OLIVEIRA, M., MCCAULEY, T., TAULER, P. and MUHAMAD, A.S., 2011b. Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion. *Scandinavian Journal of Medicine & Science in Sports,* doi: 10.1111/j.1600-0838.2010.01272.x

GLEESON, M., BISHOP, N.C., STENSEL, D.J., LINDLEY, M.R., MASTANA, S.S. and NIMMO, M.A., 2011c. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nature reviews.Immunology,* **11**, 1-10.

GLEESON, M., MCFARLIN, B. and FLYNN, M., 2006. Exercise and Toll-like receptors. *Exercise Immunology Review,* **12**, 34-53.

GORMLEY, S.E., SWAIN, D.P., HIGH, R., SPINA, R.J., DOWLING, E.A., KOTIPALLI, U.S. and GANDRAKOTA, R., 2008. Effect of Intensity of Aerobic Training on VO2max. *Medicine & Science in Sports & Exercise,* **40**(7), 1336-1343.

GUAN, Y., RANOA, D.R., JIANG, S., MUTHA, S.K., LI, X., BAUDRY, J. and TAPPING, R.I., 2010. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *Journal of Immunology,* **184**(9), 5094-5103.

HARDMAN, A.E. and STENSEL, D.J., 2009. *Physical Activity and Health, The evidence explained.* Second edn. London and New York: Routledge.

HOEBE, K., JANSSEN, E. and BEUTLER, B., 2004. The interface between innate and adaptive immunity. *Nature Immunology,* **5**(10), 971-974.

HORRIGAN, L.A., KELLY, J.P. and CONNOR, T.J., 2006. Immunomodulatory effects of caffeine: friend or foe? *Pharmacology & Therapeutics,* **111**(3), 877-892.

KILMARTIN, B. and REEN, D.J., 2004. HSP60 induces self-tolerance to repeated HSP60 stimulation and cross-tolerance to other pro-inflammatory stimuli. *European Journal of Immunology,* **34**(7), 2041-2051.

LANCASTER, G.I., KHAN, Q., DRYSDALE, P., WALLACE, F., JEUKENDRUP, A.E., DRAYSON, M.T. and GLEESON, M., 2005. The physiological regulation of toll-like receptor expression and function in humans. *Journal of Physiology,* **563**(3), 945.

MARTIN, S.A., PENCE, B.D. and WOODS, J.A., 2009. Exercise and respiratory tract viral infections. *Exercise and Sport Sciences Reviews,* **37**(4), 157.

MATTHEWS, C.E., OCKENE, I.S., FREEDSON, P.S., ROSAL, M.C., MERRIAM, P.A. and HEBERT, J.R., 2002. Moderate to vigorous physical activity and risk of upper-respiratory tract infection. *Medicine and Science in Sports and Exercise,* **34**(8), 1242-1248.

MAYNARD, C.L. and WEAVER, C.T., 2008. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunological reviews,* **226**, 219-233.

MCFARLIN, B.K., FLYNN, M.G., CAMPBELL, W.W., CRAIG, B.A., ROBINSON, J.P., STEWART, L.K., TIMMERMAN, K.L. and COEN, P.M., 2006. Physical activity status, but not age, influences inflammatory biomarkers and toll-like receptor 4. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences,* **61**(4), 388-393.

MCFARLIN, B.K., FLYNN, M.G., CAMPBELL, W.W., STEWART, L.K. and TIMMERMAN, K.L., 2004. TLR4 is lower in resistance-trained older women and related to inflammatory cytokines. *Medicine and Science in Sports and Exercise,* **36**(11), 1876-1883.

MEDZHITOV, R., PRESTON-HURLBURT, P. and JANEWAY JR, C.A., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature,* **388**, 394-397.

MONICK, M.M., YAROVINSKY, T.O., POWERS, L.S., BUTLER, N.S., CARTER, A.B., GUDMUNDSSON, G. and HUNNINGHAKE, G.W., 2003. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *The Journal of Biological Chemistry,* **278**(52), 53035-53044.

MOREIRA, A., DELGADO, L., MOREIRA, P. and HAAHTELA, T., 2009. Does exercise increase the risk of upper respiratory tract infections? *British Medical Bulletin,* **90**, 111-131.

MURPHY, E.A., DAVIS, J.M., CARMICHAEL, M.D., GANGEMI, J.D., GHAFFAR, A. and MAYER, E.P., 2008. Exercise stress increases susceptibility to influenza infection. *Brain, Behavior, and Immunity,* **22**(8), 1152-1155.

MYERS, J., KAYKHA, A., GEORGE, S., ABELLA, J., ZAHEER, N., LEAR, S., YAMAZAKI, T. and FROELICHER, V., 2004. Fitness versus physical activity patterns in predicting mortality in men. *The American Journal of Medicine,* **117**(12), 912-918.

NICKLAS, B.J. and BRINKLEY, T.E., 2009. Exercise Training as a Treatment for Chronic Inflammation in the Elderly. *Exercise and Sport Sciences Reviews,* **37**(4), 165.

NICKLAS, B.J., YOU, T. and PAHOR, M., 2005. Behavioural treatments for chronic systemic inflammation: effects of dietary weight loss and exercise training. *Canadian Medical Association Journal,* **172**(9), 1199.

NIEMAN, D.C., 1997. Immune response to heavy exertion. *Journal of applied physiology,* **82**(5), 1385-1394.

NIEMAN, D.C., 1995. Upper respiratory tract infections and exercise. *Thorax,* **50**(12), 1229-1231.

NIEMAN, D.C., 1994. Exercise, infection, and immunity. *International Journal of Sports Medicine,* **15 Suppl 3**, S131-41.

NIEMAN, D.C., HENSON, D.A., AUSTIN, M.D. and SHA, W., 2010. Upper respiratory tract infection is reduced in physically fit and active adults. *British Journal of Sports Medicine,* **45**(12):987-92 .

OLIVEIRA, M. and GLEESON, M., 2010. The influence of prolonged cycling on monocyte Toll-like receptor 2 and 4 expression in healthy men. *European Journal of Applied Physiology,* **109**(2), 251-257.

OSTROWSKI, K., ROHDE, T., ASP, S., SCHJERLING, P. and PEDERSEN, B.K., 1999. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *The Journal of Physiology,* **515 (Pt 1)**, 287-291.

PARKER, L.C., PRINCE, L.R. and SABROE, I., 2007. Translational mini-review series on Toll-like receptors: networks regulated by Toll-like receptors mediate innate and adaptive immunity. *Clinical and Experimental Immunology,* **147**(2), 199.

PEDERSEN, B.K., 2009. Edward F. Adolph Distinguished Lecture: Muscle as an Endocrine Organ: IL-6 and other myokines. *Journal of Applied Physiology,* **107**, 1006-1014.

PEDERSEN, B.K. and FEBBRAIO, M.A., 2008. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiological Reviews,* **88**(4), 1379-1406.

PETERSEN, A.M. and PEDERSEN, B.K., 2005. The anti-inflammatory effect of exercise. *Journal of Applied Physiology,* **98**(4), 1154-1162.

PETERSEN, A.M. and PEDERSEN, B.K., 2006. The role of IL-6 in mediating the anti-inflammatory effects of exercise. *Journal of Physiology and Pharmacology,* **57 Suppl 10**, 43-51.

RANDOLPH, G.J., SANCHEZ-SCHMITZ, G., LIEBMAN, R.M. and SCHAKEL, K., 2002. The CD16 (Fc {gamma} RIII) Subset of Human Monocytes Preferentially Becomes Migratory Dendritic Cells in a Model Tissue Setting. *Journal of Experimental Medicine,* **196**(4), 517.

ROGACEV, K.S., ULRICH, C., BLOMER, L., HORNOF, F., OSTER, K., ZIEGELIN, M., CREMERS, B., GRENNER, Y., GEISEL, J., SCHLITT, A., KOHLER, H., FLISER, D., GIRNDT, M. and HEINE, G.H., 2010. Monocyte heterogeneity in obesity and subclinical atherosclerosis. *European Heart Journal,* **31**(3), 369-376.

ROST, N.S., WOLF, P.A., KASE, C.S., KELLY-HAYES, M., SILBERSHATZ, H., MASSARO, J.M., D'AGOSTINO, R.B., FRANZBLAU, C. and WILSON, P.W.F., 2001. Plasma concentration of C-reactive protein and risk of ischemic stroke and transient ischemic attack: the Framingham study. *Stroke,* **32**(11), 2575.

SERPIELLO, F.R., MCKENNA, M.J., STEPTO, N.K., BISHOP, D.J. and AUGHEY, R.J., 2011. Performance and physiological responses to repeated-sprint exercise: a novel multiple-set approach. *European Journal of Applied Physiology,* **111**(4), 669-678.

SIMPSON, R.J., MCFARLIN, B.K., MCSPORRAN, C., SPIELMANN, G., O HARTAIGH, B. and GUY, K., 2009. Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. *Brain, Behavior, and Immunity,* **23**(2), 232-239.

SMART, N.A., LARSEN, A.I., LE MAITRE, J.P. and FERRAZ, A.S., 2011. Effect of exercise training on interleukin-6, tumour necrosis factor alpha and functional capacity in heart failure. *Cardiology Research and Practice,* **2011**, 532620.

STEWART, L.K., FLYNN, M.G., CAMPBELL, W.W., CRAIG, B.A., ROBINSON, J.P., MCFARLIN, B.K., TIMMERMAN, K.L., COEN, P.M., FELKER, J. and TALBERT, E., 2005. Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. *Brain Behavior and Immunity,* **19**, 389-397.

TALANIAN, J.L., GALLOWAY, S.D.R., HEIGENHAUSER, G.J.F., BONEN, A. and SPRIET, L.L., 2007. Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women. *Journal of Applied Physiology,* **102**, 1439-1447.

TESTRO, A.G. and VISVANATHAN, K., 2009. Toll-like receptors and their role in gastrointestinal disease. *Journal of Gastroenterology and Hepatology,* **24**(6), 943-954.

TIMMERMAN, K.L., FLYNN, M.G., COEN, P.M., MARKOFSKI, M.M. and PENCE, B.D., 2008. Exercise training-induced lowering of inflammatory (CD14+CD16+) monocytes: a role in the anti-inflammatory influence of exercise? *Journal of Leukocyte Biology,* **84**(5):1271-8..

VITSEVA, O.I., TANRIVERDI, K., TCHKONIA, T.T., KIRKLAND, J.L., MCDONNELL, M.E., APOVIAN, C.M., FREEDMAN, J. and GOKCE, N., 2008. Inducible Toll-like receptor and NF-kappaB regulatory pathway expression in human adipose tissue. *Obesity,* **16**(5), 932-937.

WALSH, N.P., GLEESON, M., SHEPHARD, R.J., GLEESON, M., WOODS, J.A., BISHOP, N.C., FLESHNER, M., GREEN, C., PEDERSEN, B.K. and HOFFMAN-GOETZ, L., 2011. Position statement. Part one: Immune function and exercise. *Exercise Immunology Review,* **17**, 6-63.

WALSH, N.P. and WHITHAM, M., 2006. Exercising in environmental extremes : a greater threat to immune function? *Sports Medicine,* **36**(11), 941-976.

WEST, N.P., PYNE, D.B., PEAKE, J.M. and CRIPPS, A.W., 2009. Probiotics, immunity and exercise: a review. *Exercise Immunology Review,* **15**, 107-126.

WHALEY, M., ARMSTRONG, L.E., BRUBAKER, P.H. and OTTO, R.M., eds, 2005. *ACSM's guidelines for exercise testing and prescription* . 7th Revised edition edition edn. Philadelphia, Pa: Lippincott Williams and Wilkins.

WORLD HEALTH ORGANISATION (WHO) September 2011-last updated. Available: http://www.who.int/en/ [February, 11th 2010]

ZANCHI, N.E., LIRA, F.S., DE SIQUEIRA FILHO, M.A., ROSA, J.C., DE OLIVEIRA CARVALHO, C.R., SEELAENDER, M., SANTOS, R.V. and LANCHA, A.H.,JR, 2010. Chronic low frequency/low volume resistance training reduces pro-inflammatory cytokine protein levels and TLR4 mRNA in rat skeletal muscle. *European Journal of Applied Physiology,* **109**(6), 1095-1102.

ZIEGLER-HEITBROCK, L., 2007. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *Journal of Leukocyte Biology,* **81**(3), 584-592.

Appendices

Example of informed consent form

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.

I agree to participate in this study.

Your name _____

Telephone contact _____

E-mail: _____

Your signature _____

Signature of investigator _____

Date _____

Example of Health Screen questionnaire

HEALTH SCREEN QUESTIONNAIRE FOR STUDY VOLUNTEERS Name/Number .....…….

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research [*include for projects involving invasive procedures*].

Please complete this brief questionnaire to confirm your fitness to participate:

1.	At present, do you have any health problem for which you are:					
	(a) on medication, prescribed or otherwise	Yes		No		
	(b) attending your general practitioner	Yes		No		
	(c) on a hospital waiting list	Yes		No		
2.	In the past two years, have you had any illness which required you to:					
	(a) consult your GP	Yes		No		
	(b) attend a hospital outpatient department	Yes		No		
	(c) be admitted to hospital	Yes		No		
3.	Have you ever had any of the following:					
	(a) Convulsions/epilepsy	Yes		No		
	(b) Asthma	Yes		No		
	(c) Eczema	Yes		No		
	(d) Diabetes	Yes		No		
	(e) A blood disorder	Yes		No		
	(f) Head injury	Yes		No		
	(g) Digestive problems	Yes		No		
	(h) Heart problems	Yes		No		
	(i) Problems with bones or joints	Yes		No		
	(j) Disturbance of balance/coordination	Yes		No		
	(k) Numbness in hands or feet	Yes		No		
	(l) Disturbance of vision	Yes		No		
	(m) Ear / hearing problems	Yes		No		
	(n) Thyroid problems	Yes		No		
	(o) Kidney or liver problems	Yes		No		
	(p) Allergy to nuts	Yes		No		
4.	Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?	Yes		No		

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....

.....

5	Additional questions for female participants					
	(a) are your periods normal/regular?	Yes		No		
	(b) are you on "the pill"?	Yes		No		
	(c) could you be pregnant?	Yes		No		
	(d) are you taking hormone replacement therapy (HRT)?	Yes		No		

In case of emergency, please provide details of your next of kin.

Name: ……Telephone Number:……..

Are you currently involved in any other research studies at the University?

Yes ☐ No ☐

Example of a daily Health screen questionnaire

Health Questionnaire

Please complete the following brief questions to confirm your fitness to participate in today's session:

1) At present do you have any health problems for which you are:

a) On medication, prescribed or otherwise YES ☐ NO ☐ .

b) Attending your general practitioner YES ☐ NO ☐ .

2) Do you have any symptoms of ill health, such as those associated with a cold or other common infection?

YES ☐ NO ☐ .

3) Are you presently acting as a subject for any other experiment or research study?

YES ☐ NO ☐ .

If you have answered yes to any of the above questions please give more details below:

Do you want to take part in today's experiments? YES ☐ NO ☐ .

Signature:_____ Date:

Participant information sheet – resting study

The effects of habitual level of physical activity on blood markers of inflammation and leukocyte TLR expression

Miss Marta Oliveira, Loughborough University – M.Oliveira@lboro.ac.uk

Professor Mike Gleeson, Loughborough University – M.Gleeson@lboro.ac.uk

Participant Information Sheet

Background

The health benefits of regular physical exercise have been reported for several years. Moderate to vigorous exercise training brings about physiological adaptations that can reduce the risk of chronic diseases. In contrast, sedentary behaviour is associated with an increased risk of chronic diseases such as coronary heart disease, diabetes, hypertension and rheumatoid arthritis.

It has been suggested that increased levels of inflammatory markers in the plasma are related to the development of some diseases. However, it has been suggested that reduced levels of Toll-like receptor (TLR) expression over the long term may be beneficial to health due to associated reductions in chronic inflammation, consequently reducing the risk of developing chronic diseases. Several studies have attempted to explain the association between ageing and/or sedentary lifestyle and health risks, but no solid conclusions about the mechanisms have been found yet, justifying the need for more studies in this area. The proposal of this study is to investigate the effects of habitual level of physical activity on blood markers of inflammation and leukocyte (white blood cell) TLR expression.

This study is part of a Student research project funded by Loughborough University and it will be conducted in the Exercise Immunology Laboratory of the School of Sport, Exercise and Health Sciences. All the data will be treated in strict confidence in and will be kept anonymous and confidential to the researchers, in accordance with the University regulations for data security, storage and analysis.

Participant requirements

You will be asked to come to the Exercise Immunology Laboratory for one visit where we will explain you in details the aim and procedures of the research. You will then be asked to fill in a health questionnaire and an informed consent form. After that you will have your body mass and height measured and will be asked to give us a venous blood sample (12ml).

The last thing you will be asked to do is to answer a questionnaire about your physical activity habits. In total this will take about 30 minutes.

A proportion of participants will be randomly selected to come to the lab for an identical second visit, to assess the intra-individual variability of the measurements. You can choose not to participate in that if you wish.

We will provide you with some results of the blood analysis (e.g. your red and white blood cell counts, haemoglobin concentration) at the end of the whole study if you wish. If you change your mind after taking part, you can withdraw from this study at any time, for any reason and you will not be asked to explain your reasons for withdrawing. Please just contact the main investigator.

If you have any more questions related to this study please contact:

Marta Oliveira (M.Oliveira@lboro.ac.uk – 01509 226371 / 07813195537)

If you are not happy with how the research was conducted the University has a policy relating to Research Misconduct and Whistle Blowing which is available online at http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm.

Participant information sheet – Acute study

Study Title: Effects of acute exercise on Toll-like receptor expression on human monocytes

Location: Exercise physiology teaching laboratory and Exercise Immunology Laboratory, Loughborough University

Investigators: Marta Oliveira

Supervisor: Professor Mike Gleeson

Background: Several studies have indicated that physical activity may play a role in modifying inflammatory and immune responses. Furthermore, it has been suggested that a large number of immune system aspects can be influenced by strenuous and/or chronic exercise, which may impair immune function and increases susceptibility to infection such as upper respiratory tract infections.

Although the number of studies which analyse the immune response after exercise has increased, the mechanisms which modulate it are not fully understood, and more studies examining the clinical importance of changes and function in the immune system are required.

Toll-like receptors (TLRs) are receptors located in the immune cell surfaces (e.g. monocytes) responsible for recognizing pathogens. TLRs expression may change after exercise, which may influence the changes in the immune function. Therefore, the intention of this research is to investigate the effects of 1.5-h of exercise at 75% of the VO_2max on the monocyte TLR2 and 4 expression.

Volunteers: **Physically active males (18 - 35 yr)**

Study Procedures: You will be required to attend the lab on 3 occasions in total.

During your **first visit** you will undertake a maximal oxygen uptake **(VO_{2max})** test in a cycle ergometer. The total duration of the visit is around 1 hour (~30 min for the test).

On your **second visit** baseline measurements (weight and height) will be recorded. You will also perform a Familiarisation, which consist in 1.5-h cycling at 75% VO_2max. Basically, this session is for you to get used to the protocol that is going to be used in the main trial. This visit will take around 2 hours in total.

On the **third visit** we will ask you to come to the lab 2-h after having a normal breakfast, and we will weigh you. A blood sample (7ml) will be taken before exercise. As soon as possible after that you will be cycling for 1.5-h at 75% of your VO_2max. Four more blood samples will be required: One immediately after cycling, one 1 hour after, one 4 hours after exercising and the last one 24-h after finishing the trial (total of 35 ml of blood during the whole trial, using repeated veinpuncture). 200ml of water will be given every 20 min of exercise. Expired air samples will be obtained every 30min using the Douglas bags. Heart Rate and RPE will be collected every 20 min throughout the trial. Body mass measurement will be repeated after the trial.

Requirements: You will be asked to refrain from caffeine and any strenuous exercise 24h before coming to the laboratory and the following 24 hours after the end of the trial. A food diary will be given to be filled the day before and the 24 hours after the last blood sample.

Possible risk and discomforts: The risks involved in this study are minimal. Prior to the study you will complete a University Health Questionnaire to assess your suitability for the study. Each session will be attended by suitable qualified personnel.

Benefits of the study: From the measurements made we will be able to give you information regarding your aerobic fitness and will be able to give you some indication of your immune response to exercise.

Data protection: All samples will be used to examine Toll-like receptor response and will be disposed of immediately after analysis. All information obtained in this study will be kept confidential, however, we will be pleased to discuss the results with you at a later date if you wish.

Further Questions please contact any of the following:

Marta Oliveira E-mail: M.Oliveira@lboro.ac.uk Tel: 01509 22 6371

Professor Mike Gleeson E-mail: M.Gleeson@lboro.ac.uk Tel: 01509 22 6345

Participant Information Sheet – Exercise training study (4-week)

Effects of acute exercise and a period of exercise training on Toll-like receptor expression on human monocytes

Investigators: Professor Mike Gleeson – M.Gleeson@lboro.ac.uk

Miss Marta Oliveira – M.Oliveira@lboro.ac.uk

Mr Kurt Rumley – K.Rumley-09@student.lboro.ac.uk

Background

Toll-Like Receptors (TLRs) are recognition receptors located on the surface of immune cells (such as monocytes and neutrophils), responsible for recognizing pathogens. TLRs play an important role in the activation of both the innate and the acquired immune system.

Several studies have indicated that physical activity may play a role in modifying inflammatory and immune responses. Furthermore, it has been suggested that a large number of immune system aspects can be influenced by strenuous and/or chronic exercise, including the TLR expression on monocytes. This may reduce inflammation markers and may prevent the development of some diseases such as diabetes and rheumathoid arthritis.

The mechanisms which modulate TLR expression after exercise are not fully understood, and more studies examining the clinical importance of changes in TLR expression and function on the generation of immune responses are required. Therefore, the intention of this research is to determine the effects of an acute bout of aerobic exercise and a short period of exercise training on monocyte TLR expression in the general population.

Study procedures

You will be asked to come to the lab at the following times:

Visit 1 –Introductory visit: one of the researchers will explain the aim and procedures of the research for you. You will also fill in a health questionnaire, a physical activity questionnaire and an informed consent form at this visit. In addition, you will then be allocated to a training or control treatment for 4 weeks.

Visit 2 - You will be asked to perform an incremental cycling to volitional exhaustion (VO2max test). At this visit you will also have their body weight, height and waist and hip circumference measured.

Visit 3 - Familiarisation trial for exercise group: If you are allocated to this group you will be asked to arrive in the lab at around 8.30am. During the familiarisation you will complete five 4-minute intervals at a workload corresponding to approximately 85%-90% of you VO2max separated by 2-min rest. Heart rate and rating of perceived exertion will be monitored throughout the trial.

Visit 4 (a) - First main trial exercise group: you will arrive at the lab at 7.30am after an overnight fast. After resting for 5-10 min, you will have your blood taken. You will then complete ten 4-min bouts of cycle ergometer exercise at 90% of their VO2max separated by 2-min rest. On cessation of exercise another blood sample will be taken. You will then be asked to remain seated for one more hour, and a final blood sample will be taken. A light meal will be given upon completion of this trial.

Visit 4 (b) - Main trial for control group: If you are allocated to the control group you will be asked to come to the lab at 7.30am after an overnight fast. After resting for 5-10 min, you will have your blood taken. You will then be asked to remain seated for two more hours, and blood samples will be taken after 1 hour and 2 hours. A light meal will be given upon completion of the trial.

Training sessions: for the training sessions you will be asked to complete the training session mentioned on visit 4a (without the blood sampling) 3 times per week for 4 weeks. Participants allocated to the control group will be asked not to exercise for a 4-week period (i.e. continue with their normal routine).

Visit 5 - Last main trial after the 4 weeks of training or control: You will be asked to repeat the same procedures as on visit 4a or 4b.

The blood samples will be analysed for total blood cell count (e.g. Red blood cells, Haemoglobin concentration, White blood cell count), and for immunological aspects such as monocyte TLRs expression. All will be done immediately after the last blood collection has been taken each day. In addition, some plasma samples will be stored for future analyses, which will be done as soon as the study is finished. All the remaining samples would be appropriately disposed according to the Departmental H&S guidelines.

Possible risk and discomforts

The risks involved in this study are minimal. Prior to the study you will have completed a University Health Questionnaire to assess your suitability for the study.

Physical challenge (the VO2max test and the exercise protocol) - The VO2max test requires you to exercise to volitional fatigue. The discomfort is, therefore, by definition tolerable and when it becomes intolerable you will simply stop exercising. The exercise protocol is considered as high-intensity exercise however the discomfort is tolerable as well. If you cannot continue any further you will simply stop exercising. The risks of injury for both procedures are minimal, as it is a well-controlled procedure with the investigator standing alongside you throughout the test to ensure your safety at all times.

Blood sampling – taking blood via the vein puncture may cause minor bruising, but good practice minimizes the risk.

All this information will be kept confidential; however, we will be pleased to discuss the results with you at a later date.

Benefits of participating

At the end of the study you will be given a fitness result, and from the measurements made we will be able to give you information regarding your resting blood status i.e. red and white blood cell numbers and haemoglobin concentration if you wish.

Additional information

You can withdraw from the study at any time, for any reason and you will not be asked to explain your reasons for withdrawing.

If you are not happy with the way the research is conducted, please visit the University policy relating to Research Misconduct and Whistle Blowing which is available online at http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm.

If you have any more questions or would you like to discuss the project a bit further, please contact one of the investigators:

Professor Mike Gleeson - email: m.gleeson@lboro.ac.uk Tel: 01509 226345

Miss Marta Oliveira - PhD student in the SSES, email: M.Oliveira@lboro.ac.uk tel: 01509 226371, (mobile): 07813195537

Participant Information Sheet Probiotic 1

Study Title: Effects of a probiotic (Lactobacillus salivarius) intervention on infection, cold symptom duration/severity and mucosal immunity in endurance athletes

Investigator: Professor Michael Gleeson

Background

Your immune system protects your body against infections. It is now widely accepted that single bouts of prolonged strenuous exercise are associated with a temporary depression of immune function. This is said to provide a 'window of opportunity' for infections to enter the body. It is thought that these effects may be cumulative over a period of time, so that during periods of heavy training and competition, athletes' immune systems may become chronically depressed, i.e. demonstrating lower values in measures of immune function integrity at rest. In this study we intend to examine the efficacy of a probiotic supplement containing "friendly" bacteria (Lactobacillus salivarius strain) on infection incidence and resting immune function in sportsmen and women over a 4-month period.

Study Procedures

You will complete health-history questionnaires concerning your past and present health status and this will be followed by a small (5 mL) blood sample being taken to check that your red and white cell counts are normal. These will be used to confirm that you are suitable subject for the study. You will be randomly allocated to the supplement group or a placebo group and be required to take one sachet containing 2 grams of powder (which you add to a cup of water) with your breakfast every day for 4 months. You will be provided with the sachets which should be kept unopened in your fridge until the day of use. For the duration of the study you should not consume any other fermented milk products (i.e. other probiotics, yoghurts, soured milk or cream, crème fraiche).

You will be required to provide a resting blood sample (no more than 20 mL) and a saliva sample at the start of the study, after 2 months of taking the product and again after 4 months of taking the product (which is the end of the study) for assessment of your immune status. On these occasions you will need to arrive at the Exercise Immunology Laboratory after an overnight fast (though you are free to drink water at any time). At weekly intervals during the study you are requested to complete 3 short questionnaires to evaluate your current mood state, physical activity levels, and record any symptoms of colds/flu or digestive discomfort that you may have experienced in the past week.

Measurements

Height and weight will be determined in normal clothing.

A resting saliva sample will be taken at the start and end of the study. This will be collected by dribbling into a plastic tube.

A blood sample will be taken at the start of the study, after 2 months of taking the product and again after 4 months of taking the product (which is the end of the study). This will be no more than 20 ml of blood (less than 1% of your total blood volume). You are requested to return your used sachets (and any unused ones) at the end of the study period and you will be asked few questions about the palatability and ease of administration of the powder.

Possible risk and discomforts

The risks involved in this study are minimal. The supplement has been previously tested in human trials without any adverse effects being reported. The supplement was formulated by a company specialising in the manufacture of probiotics for human consumption and has been tested to confirm that it is free from contamination with an extensive list of banned steroids and stimulants. Should you experience any ill effects you are asked to contact the investigators immediately and stop taking the supplement. Prior to the

study you will have completed a University Health Questionnaire and a Bowel Disease History Questionnaire to assess your suitability for the study. All information regarding the blood and saliva tests will be used for immunological purposes and then disposed of immediately. This information will be kept confidential; however, we will be pleased to discuss the results with you at a later date.

You are free to withdraw from the study at any time

Benefits of the study

From the measurements made we will be able to give you information regarding your resting blood status i.e. red and white blood cell numbers, haemoglobin concentration and saliva IgA concentration.

Confidentiality

Personal information will be treated in confidence, and will be stored on a computer, identified by a number rather than by your name – in line with the Data Protection Act.

Further questions?

Please contact any of the following investigators:

Principal Investigator: Professor Mike Gleeson email: m.gleeson@lboro.ac.uk

Tel: 01509 226345

Other Investigators:

Miss Catherine Coyle, BSc final year student in the School of Sport and Exercise Sciences, email: C.M.Coyle-08@student.lboro.ac.uk Tel (mobile): 07730565690

Miss Marta Oliveira MSc; Technician and Exercise Physiology PhD student in the School of Sport and Exercise Sciences, email: M.Oliveira@lboro.ac.uk

Tel: 01509 226371 Tel (mobile): 07813195537

Participant Information Sheet – Probiotic 2

Study Title: Effects of a probiotic (Yakult) intervention on infection, cold symptom duration/severity and mucosal immunity in endurance athletes

Investigator: Professor Michael Gleeson

Background

Your immune system protects your body against infections. It is now widely accepted that single bouts of prolonged strenuous exercise are associated with a temporary depression of immune function. This is said to provide a 'window of opportunity' for infections to enter the body. It is thought that these effects may be cumulative over a period of time, so that during periods of heavy training and competition, athletes' immune systems may become chronically depressed, i.e. demonstrating lower values in measures of immune function integrity at rest. In this study we intend to examine the efficacy of a probiotic supplement (Yakult) containing "friendly" bacteria (*Lactobacillus casei* Shirota strain) on infection incidence and resting immune function in sportsmen and women over a 4-month period.

Study Procedures

You will complete health-history questionnaires concerning your past and present health status and this will be followed by a small (5 mL) blood sample being taken to check that your red and white cell counts are normal. These will be used to confirm that you are suitable subject for the study. You will be randomly allocated to the supplement group or a placebo group and be required to take two 65 ml bottles of the product every day (one to be taken with your breakfast and one with your evening meal) for 4 months. You will be provided with the bottles which should be kept unopened in your fridge until the day of use. These products have a shelf life of 2 weeks, so you will need to come to the laboratory for further supplies every 2 weeks. For the duration of the study you should not consume any other fermented milk products (i.e. other probiotics, yoghurts, soured milk or cream, crème fraiche).

You will be required to provide a resting blood sample (no more than 20 mL) and a saliva sample at the start of the study, after 2 months of taking the product and again after 4 months of taking the product (which is the end of the study) for assessment of your immune status. On these occasions you will need to arrive at the Exercise Immunology Laboratory after an overnight fast (though you are free to drink water at any time). At weekly intervals during the study you are requested to complete 3 short questionnaires to evaluate your current mood state, physical activity levels, and record any symptoms of colds/flu or gastrointestinal problems that you may have experienced in the past week.

Measurements

Height and weight will be determined in normal clothing.

A resting saliva sample will be taken at the start of the study, after 2 months of taking the product and again after 4 months of taking the product (which is the end of the study). This will be collected by dribbling into a plastic tube.

A blood sample will be taken at the start of the study, after 2 months of taking the product and again after 4 months of taking the product (which is the end of the study). This will be no more than 20 ml of blood (less than 1% of your total blood volume). You are requested to return your used bottles (and any unused ones) when you return to the lab to collect fresh supplies of product every 2 weeks. At the end of the study period you will be asked few questions about the palatability and ease of administration of the product.

Possible risk and discomforts

The risks involved in this study are minimal. The product is commercially available as a fermented milk drink (sold as a food in all major supermarkets) and has been drunk extensively by humans without adverse effects for over 70 years (currently by 25 million people in 31 different countries). The supplement was formulated and manufactured by a company specialising in the manufacture of probiotics

for human consumption. Some people taking probiotics for the first time may notice a slight change in bowel function in the first few days. This does not always happen, and will settle down. Should you experience any ill effects you are asked to contact the investigators immediately and stop taking the product. Prior to the study you will have completed a University Health Questionnaire and a Bowel Disease History Questionnaire to assess your suitability for the study. All information regarding the blood and saliva tests will be used for immunological purposes and then disposed of immediately. This information will be kept confidential; however, we will be pleased to discuss the results with you at a later date.

You are free to withdraw from the study at any time

Benefits of the study

From the measurements made we will be able to give you information regarding your resting blood status i.e. red and white blood cell numbers, haemoglobin concentration and saliva IgA concentration.

Confidentiality

Personal information will be treated in confidence, and will be stored on a computer, identified by a number rather than by your name – in line with the Data Protection Act.

Further questions?

Please contact any of the following investigators:

Principal Investigator: Professor Mike Gleeson email: m.gleeson@lboro.ac.uk

Tel: 01509 226345

Other Investigators:

Miss Marta Oliveira MSc; Technician and Exercise Physiology PhD student in the School of Sport and Exercise Sciences, email: M.Oliveira@lboro.ac.uk

Tel: 01509 226371 Tel (mobile): 07813195537

Appendix (H) IPAQ questionnaires - long and short versions.