UNIVERSITY OF STIRLING

Health & Exercise Sciences Research Group

The Influence of Sex, Training Status, and Fatty Acid Supplementation on T-lymphocyte Populations at Rest and in Response to Acute Exercise.

By

Frankie Fantom Brown

A Thesis Submitted to

The School of Sport

University of Stirling

In partial fulfilment of the Degree of

DOCTOR OF PHILOSOPHY

Health and Exercise Sciences Research Group

School of Sport

University of Stirling

March 2014

DECLARATION

I declare that this thesis was composed by myself and that all the data were collected and analysed by myself. Neither the thesis nor the original work therein has been submitted to this or any other institution for a higher degree.

Frankie Brown 22/03/14

DEDICATION

I dedicate this thesis to my Mum Hilary Ann Fantom (24/07/1958 - 15/09/2013). Your courage and love inspires me every single day.

Science has... bestowed upon [man] powers which may be called almost creative; which have enabled him to change and modify the beings surrounding him, and by his experiments to interrogate nature with power, not simply as a scholar, passive and seeking only to understand her operations, but rather as a master, active with his own instruments... who would not be ambitious of becoming acquainted with the most profound secrets of nature; of ascertaining her hidden operations; and of exhibiting to man that system of knowledge which relates so intimately to their own physical and moral constitution?
'Humphry Davy'

ACKNOWLEDGEMENTS

I would like to thank my Dad Willie, my Brother Jake and Rona. I genuinely couldn't have done it without your constant love and support.

A big thanks to Taylor, Jo, Shannon and all my other friends and family for keeping me sane when the PhD bubble got murky.

A special thanks to my supervisors Stuart Galloway, Richard Simpson and Oliver Witard for putting up with my elusive and often remote learning and working style, your advice throughout the process has been invaluable.

An extra thanks to all the staff and PhD students in the Health and Exercise Sciences Research group, and the School of Sport, at the University of Stirling, for the coffees, cakes and intellectual conversation.

Finally, without my willing participants this thesis wouldn't exist, so thank you all.

THESIS ABSTRACT

This series of studies began with an examination of the effects of training status (Tr vs UTr) and sex on the resting levels and redistribution of senescent ($CD28^-CD57^+$) and naïve ($CD28^+CD57^-$) T-lymphocytes ($CD4^+$, $CD8^+$) following a treadmill test to volitional exhaustion. In this first study exercise elicited a redistribution of senescent $CD4^+$, $CD8^+$ and naïve $CD4^+$,$CD8^+$ T-lymphocytes. UTr had a higher proportion of senescent and a lower proportion of naïve $CD8^+$ T-lymphocytes than Tr. Males had a higher proportion of senescent and lower proportion of naïve T-lymphocytes than females with the highest percentage of senescent and lowest percentage of naïve T-lymphocytes observed in UTr males. CMV was a covariate in the senescent and naïve $CD8^+$ T-lymphocytes. This study highlighted important sex and training status differences in the senescent and naïve T-lymphocyte redistribution in response to exercise.

These findings led on to an investigation of the T-lymphocyte ($CD4^+$, $CD8^+$, $\gamma\delta^+$) response to a period of 2 weeks increased volume training (39% increase in volume) in trained females (Tr, n=13) compared to a period of 2 weeks habitual activity in female controls (UTr, n=13). This second study observed no difference in the resting T-lymphocyte profile from the pre to post increased volume training period. The resting number of $CD3^+$ and proportion of $\gamma\delta^+$ T-lymphocytes was greater in the Tr compared to the UTr. The resting proportion of $CD4^+$T-lymphocytes and the $CD4^+$:$CD8^+$ ratio was greater in the UTr compared to the Tr. CMV was a covariate in the analysis of $CD8^+$, $CD28^+$ $CD8^+$, and naïve $CD8^+$ T-lymphocyte cell numbers but not in the analysis of T-lymphocyte proportions. The increased volume training period had no effect on resting T-lymphocyte populations in Tr females, and T-lymphocyte populations also did not change with 2 weeks of habitual exercise in UTr. The total energy, carbohydrate and protein intake was greater in Tr compared to the UTr during the increased volume training period and was greater than normal in the Tr group. These dietary influences may partly explain the absence of any change in T-lymphocyte proportions pre to post training period in Tr. Differences in the proportions of $\gamma\delta^+$, $CD4^+$ and the ratio of $CD4^+$:$CD8^+$ T-lymphocytes at rest between the Tr and UTr warrants further investigation.

The final study of this series is presented in two parts. The first part focused on the influence of 4 weeks supplementation at 0.1g/kg body mass/day with n-3 polyunsaturated fatty acids (PUFA) as fish oil (FO, n=10), or short-chain saturated fatty acids (SFA) as coconut oil (CO, n=10) on T-lymphocyte ($CD4^+$,$CD8^+$, $\gamma\delta^+$) differentiated populations at rest and in response to exercise in trained males. Changes were examined by Day (Baseline to pre supplementation, Pre Sup (4 week control period), and pre supplementation to post supplementation, Post Sup (4 week supplementation period)). During a 4 week baseline control period no changes were observed in the blood lipid profile in both FO and CO groups. During the control period a main effect of exercise was observed in all the $CD3^+$ and $\gamma\delta^+$ T-lymphocytes subsets. During the control period an interaction of group-by-day was observed in the senescent $CD8^+$ T-lymphocytes from BL to Pre Sup the proportion and number decreased in the FO group and increased in the CO group. Inclusion of CMV as a covariate introduced a main effect of group on the $CD4^+$ naïve proportions and cell counts and the group-by-day interaction observed on the $CD8^+$ senescent T-lymphocyte proportions and cell counts disappeared. During the 4 week supplementation period this study observed an increase in the *n*-3 PUFAs, EPA (20:5n-3), DHA (22:6n-3) and DPA (22:5n-3) in the FO group but not in the CO group (with no changes in blood lipid profile on CO). During the supplementation period a main effect of exercise was observed in all the $CD3^+$ and $\gamma\delta^+$ T-lymphocyte subsets except for the proportion of $CD8^+$ naïve T-lymphocytes. The proportion of $CD8^+$ naïve T-lymphocytes was lower at rest and in response to exercise in FO and CO groups after supplementation. CMV was a significant covariate in senescent $CD4^+$ T-lymphocyte cell counts. At the post exercise time point the $\gamma\delta^+$ T-lymphocyte count increased in the FO group but decreased in the CO group, following the supplementation period. However, this observation did not quite reach statistical significance. Although a difference

between the groups was evident for $\gamma\delta^+$ T-lymphocyte count and proportion there was insufficient evidence to conclude whether the difference was supplement related. It would appear that dose, duration and type of fatty acids ingested could all be important in the overall response but these require further study.

The second part of this final study investigated the influence of 4 week supplementation at 0.1g/kg body mass/day with n-3 polyunsaturated fatty acids (PUFA) as fish oil (FO, n=10) or short-chain saturated fatty acids (SFA) as coconut oil (CO, n=10) on plasma Th1 cytokine: IL-2, TNF- α and IFN-γ, and Th2 cytokine IL-4, IL-6 and IL-10 concentrations, and expression of the T-lymphocyte activation marker CD69 at rest and in response to exercise in trained males. Changes were examined by Day (Baseline to pre supplementation (4 week control period), and pre supplementation to post supplementation (4 week supplementation period)). This study observed an increase in *n*-3 PUFAs, EPA (20:5n-3), DHA (22:6n-3) and DPA (22:5n-3) in the FO group but not in the CO group. There was a significant mobilisation of activated $CD4^+$ $CD69^+$ and $CD8^+$ $CD69^+$ ($P<0.05$) T-lymphocyte numbers in response to exercise in both FO and CO groups. CMV infection was a significant covariate on the number and proportion of $CD4^+CD69^+$ T-lymphocytes ($P<0.05$) but not on the number or proportion of $CD8^+CD69^+$ T-lymphocytes. During the supplementation period there was a significant effect of Day on TNF-α, IL-6, IL-4 and IL-2 with IFN-γ and IL-10 trending towards a difference. The plasma cytokine concentration was greater at post supplementation compared to pre supplementation for both FO and CO groups. Latent CMV infection was a significant covariate for TNF-α, IL-6, IL-4, IL-2, IFN-γ and IL-10. In the current study we observed no evidence of a difference between the CO and FO groups for early T-lymphocyte activation marker or plasma cytokine concentrations despite the membrane lipid composition change over the 4 week supplementation period. It would appear that the plasma Th1 and Th2 cytokine concentration increased from pre supplementation to post supplementation on both PUFA and SFA, highlighting a potential link between fatty acid incorporation and cytokine expression that needs closer examination.

The results of this series of studies highlight that sex and training status impact upon the T-lymphocyte pool at rest and in response to exercise. Increasing the volume of training for 2 weeks without dietary restriction does not alter the resting T-lymphocyte pool in trained females. Alterations to the T-lymphocyte pool at rest and in response to exercise are not related to FO or CO supplementation. Furthermore, the response of Th1, Th2 plasma cytokines, and the early activation marker CD69 at rest and in response to exercise does not differ between a group supplemented with FO compared to a group supplemented with CO it would appear that Th1 and Th2 plasma cytokines increase post supplementation in both groups.

Particular avenues of interest for future research would be, to explore the sex differences in T-lymphocyte subsets at rest and in response to exercise, to determine whether these sex differences are key in susceptibility to disease/infection and to determine the tissue targets of lymphocytes mobilised during exercise.

PUBLICATIONS

1. Training status and sex influence on senescent T-lymphocyte redistribution in response to acute maximal exercise. **Brown FF**, Bigley AB, Sherry C, Neal CM, Witard OC, Simpson RJ, and Galloway SDR. Brain Behaviour and Immunity 2013 Nov 4. pii: S0889-1591(13)00530-8. doi: 10.1016/j.bbi.2013.10.031.
2. The response of T-lymphocyte populations to a period of increased volume training in trained females vs habitual activity in female Controls. **Brown FF**, Bigley AB, Ross JS, LaVoy, EC, Simpson RJ, and Galloway SDR. Brain Behaviour and Immunity (In preparation).

CONFERENCE PRESENTATIONS

1. **Frankie F. Brown**, Richard J. Simpson, Craig M. Neal, J. Austin B. Bigley, Guillaume Spielmann& Stuart D. R. Galloway. Gender And Training Status Effects On Mobilization Of Senescent T-cells Following Acute Exercise. Presented at American College of Sports Medicine conference, Denver, 2011.

2. Craig M. Neal, Richard J. Simpson, **Frankie F. Brown**, J. Austin B. Bigley, Guillaume Spielmann, Angus M. Hunter & Stuart D. R. Galloway. The frequency of highly differentiated or senescent blood T-cells following two different endurance training programmes. Presented at American College of Sports Medicine conference, Denver, 2011.

3. **Frankie F. Brown**, Richard J. Simpson, Craig M. Neal, J. Austin B. Bigley, Guillaume Spielmann& Stuart D. R. Galloway. Cytomegalovirus serostatus increases the proportion of highly differentiated CD8+ T-cells at rest and following acute exercise but the response is influenced by gender and training status. Presented at International Society of Exercise Immunology symposium, Oxford, 2011.

4. Craig M. Neal, Richard J. Simpson, **Frankie F. Brown**, Austin B. Bigely, Emily C.P. LaVoy, Angus M. Hunter & Stuart D. R. Galloway. The impact of an ironman triathlon race on Epstein - Barr virus and Varicella - Zoster virus antibody titres and the frequency of highly differentiated and senescent blood T-cells**.** Presented at International Society of Exercise Immunology symposium, Oxford, 2011.

TABLE OF CONTENTS

ABBREVIATIONS

CD: Cluster of differentiation

MHC: Major histocompatibility complex

APC: Antigen presenting cell

RPE: Rate of perceived exertion

CMV: Cytomegalovirus

IL: Interleukin

TCR: T-cell receptor

FITC: Fluorescein

PerCP: Peridinin Chlorophyll protein

PE: Phycoerythrin

APC: Allophycocyanin

PUFA: Polyunsaturated fatty acids

SFA: Saturated fatty acids

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

EDTA: Ethylenediaminetetraacetic acid

Th1: T-helper 1

Th2: T-helper 2

THESIS INTRODUCTION

The resting T-lymphocyte pool is known to be influenced by many factors, infection (Mallia et al, 2014), age (Simpson et al, 2008), sleep (Dinges et al, 1994), baseline fitness (Spielmann et al, 2011), macronutrient intake (Witard et al, 2013), training volume (Witard et al, 2012), body composition (Tchkonia et al, 2010), sex (Yan et al, 2010) and latent CMV infection (Pawelec et al, 2009; Turner et al, 2013). However the effects of these factors, and the T-lymphocyte populations mobilised, in response to exercise are not fully understood, additionally the effect of fatty acid supplementation on the T-lymphocyte pool and response to exercise is currently unknown.

Thus, the first study of this thesis aimed to characterise the effect of training status and sex on the senescent T-lymphocyte response to an acute bout of intense exercise. In accordance with Spielmann et al. (2011), we hypothesized that there would be less senescent T-lymphocytes in the blood compartment of trained individuals. Given that previous work reported a greater age-related increase in effector memory cells in males vs. females (Yan et al, 2010), we hypothesized that a higher proportion of senescent T-lymphocytes would be observed in males vs. females at rest and in response to exercise.

To further understand the influence of training status and the effect of an increased volume of training on the resting T-lymphocyte pool the aim of the second study in this thesis was to examine the effects of 14-days of increased training volume on the composition of the blood T-lymphocyte pool in highly trained soccer players. Previous work reported a blunted response of effector memory T-lymphocytes in response to a period of increased training volume (Witard et al, 2012). Therefore, we hypothesized that the increased training period in the second study of this thesis would result in marked reductions in the proportions of senescent blood T-lymphocytes in soccer players with no change in untrained controls.

n-3 polyunsaturated fatty acids (PUFA) have been observed to alter T-lymphocyte proliferation which could lead to altered T-lymphocyte differentiation influencing the T-

lymphocyte pool (Kew et al, 2004). Thus, to fully understand the influence of different dietary fatty acids on T-lymphocyte differentiated populations at rest and in response to exercise in the first part of the third study in this thesis we matched participants for baseline blood *n*-3 status and then supplemented the diet of trained males with either short chain saturated fatty acids (coconut oil) or *n*-3 polyunsaturated fatty acids (fish oil). We hypothesized a PUFA related decrease in T-lymphocyte proliferation would result in a decrease in senescent T-lymphocytes at rest in response to PUFA supplementation when investigated in vivo in humans.

In cell culture studies it has been observed that different T-lymphocyte populations secrete different cytokines (LaVoy et al, 2013). In the final study of the thesis we examined the influence of n-3 PUFA and indeed short chain fatty acids as biological mediators of this response by measuring the plasma cytokine concentration. Additionally to investigate if fatty acid supplementation influences the activation of T-lymphocytes we examined the early activation marker CD69 as a measure of T-lymphocyte function at rest and in response to exercise. We hypothesized that PUFA supplementation would potentially increase lymphocyte activation and Th-1 cytokines with a decrease in the Th-2 cytokines.

References

Dinges, D.F., Douglas, S.D., Zaugg, L., Campbell, D.E., McMann, J.M., Whitehouse, W.G., Orne, E.C., Kapoor, S.C., Icaza, E., Orne, M.T., 1994. Leukocytosis and natural killer cell function parallel neurobehavioral fatigue induced by 64 hours of sleep deprivation. J. Clin. Invest 93, 1930-1939.

Kew, S., Mesa, M.D., Tricon, S., Buckley, R., Minihane, A.M., Yaqoob, P., 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am. J. Clin. Nutr. 79, 674-681.

LaVoy, E.C., Bosch, J.A., Lowder, T.W., Simpson, R.J., 2013. Acute aerobic exercise in humans increases cytokine expression in CD27(-) but not CD27(+) CD8(+) T-cells. Brain Behav. Immun. 27, 54-62.

Mallia, P., Message, S.D., Contoli, M., Gray, K., Telcain, A., Laza-Stanca, V., Papi, A., Stanciu, L.A., Elkin, S., Kon, O.M., Johnson, M., Johnstona, S.L., 2014. Lymphocyte subsets in experimental rhinovirus infection in chronic obstructive pulmonary disease. Respir Med. 108(1): 78-85.

Pawelec, G., Derhovanessian, E., Larbi, A., Strindhall, J., Wikby, A., 2009. Cytomegalovirus and human immunosenescence. Rev. Med. Virol. 19, 47-56.

Simpson, R.J., Cosgrove, C., Ingram, L.A., Florida-James, G.D., Whyte, G.P., Pircher, H., Guy, K., 2008. Senescent T-lymphocytes are mobilised into the peripheral blood compartment in young and older humans after exhaustive exercise. Brain Behav. Immun. 22, 544-551.

Spielmann, G., McFarlin, B.K., O'Connor, D.P., Smith, P.J., Pircher, H., Simpson, R.J., 2011. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav. Immun. 25, 1521-1529.

Tchkonia, T., Morbeck, D.E., Von, Z.T., Van, D.J., Lustgarten, J., Scrable, H., Khosla, S., Jensen, M.D., Kirkland, J.L., 2010. Fat tissue, aging, and cellular senescence. Aging Cell 9, 667-684.

Turner, J.E., Campbell, J.P., Edwards, K.M., Howarth, L.J., Pawelec, G., Aldred, S., Moss, P., Drayson, M.T., Burns, V.E., Bosch, J.A., 12-7-2013. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr.)

Witard, O.C., Turner, J.E., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., Bosch, J.A., Tipton, K.D., 10-10-2013. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav. Immun.

Witard, O.C., Turner, J.E., Jackman, S.R., Tipton, K.D., Jeukendrup, A.E., Kies, A.K., Bosch, J.A., 2012. High-intensity training reduces CD8+ T-cell redistribution in response to exercise. Med. Sci. Sports Exerc. 44, 1689-1697.

Yan, J., Greer, J.M., Hull, R., O'Sullivan, J.D., Henderson, R.D., Read, S.J., McCombe, P.A., 2010. The effect of ageing on human lymphocyte subsets: comparison of males and females. Immun. Ageing 7, 4-

CHAPTER 1

CHAPTER 1: General Introduction

Introduction

The human immune system is a vastly complex interplay of many different cells, with one specific aim; protection of the host. The system is constantly evolving to combat the rapidly evolving pathogens faced by the host. Different cells provide protection in different ways. This protection can be roughly split into two branches, the innate and the adaptive immune response. Commonly known as the first line of defence innate immunity is omnipresent, encoded by genes in the hosts germ line. Therefore, the innate response is generic and non-specific. The first defence mechanisms are: the physical barrier of the skin; antimicrobial chemical barriers that rupture some microbial and other pathogenic membranes at mucosal surfaces; the constant movement of epithelial cilia in gastrointestinal and respiratory tracts that remove the bacteria before they have a chance to colonise; and sheer competition between the commensal bacteria in the gut flora to prevent colonisation of a pathogenic bacteria strain (Chaplin, 2003; Chaplin, 2005; Chaplin, 2010; Tosi, 2005). Beyond these barriers are blood resident proteins which trigger a cascade of proteases called the complement system that opsonises the pathogen for lysis by other cells and signals inflammation via C3a and C5a (Dunkelberger and Song, 2010). It is important to distinguish between self and non self to prevent autoimmune damage to healthy cells. The innate immune system achieves this by recognising conserved molecular patterns present on microbes but not on the host and indicators of cell damage. This recognition occurs through various pattern recognition receptors (Toll like receptors (TLRs), NOD like receptors (NLRs), dectin-1, the collectins and the ficolins) observed both on and within leukocytes (monocytes, macrophages, dendritic cells, neutrophils, eosinophils, Langerhans cells) and epithelial cells (Chaplin, 2003; Chaplin, 2006; Chaplin, 2010; Dempsey et al, 2003; Tosi, 2005). Activation of the pattern recognition receptors and the complement system triggers signalling pathways producing chemical signals. These signals are mainly cytokines and chemokines which have local and systemic effects recruiting both innate cells like phagocytic macrophages and neutrophils, along with cells of the adaptive immune response, to the site of infection

triggering an inflammatory response (Dempsey et al, 2003; Dunkelberger and Song, 2010; Esche et al, 2005). Natural Killer cells (NK cells) are also part of the innate response. NK cells are a type of cytotoxic lymphocyte that identify and trigger apoptosis in compromised host cells like tumour and virally infected cells. NK cells do not recognise foreign microbes (Chaplin, 2003; Chaplin, 2006; Chaplin, 2010; Dempsey et al, 2003; Tosi, 2005). Gamma delta (γδ) T cells are a further subset of cell involved in the innate immune response. Large numbers are observed within an hour of infection (Nanno et al, 2007). These are unusual as they possess characteristics of both innate and adaptive immunity and are often thought of as the 'bridge between innate and adaptive immunity'(Chaplin, 2003; Nanno et al, 2007; Nanno and Ishikawa, 2005; Tsuchiya et al, 2003; Uezu et al, 2004).

The adaptive immune response, commonly known as cell mediated immunity, identifies each specific pathogen and subsequently provides an immune response to clear the specific pathogen. This response is encoded by gene elements that somatically rearrange during lymphocyte development in lymphoid organs producing very specific antigen binding molecules, T cell receptors (TCRs) on T lymphocytes that only bind antigen in the context of MHC and immunoglobulin (B Cell antigen receptor) on B lymphocytes (Bonilla and Oettgen, 2010; Chaplin, 2010; Dempsey et al, 2003). This ensures that different receptors are produced to specifically recognise, in a lock and key fashion, the potential pathogens the host could face. Each pathogen recognised elicits a very specific immune response triggering the production of different subsets of T-lymphocytes, B-lymphocytes or both. This response depends on chemical signals in the form of cytokines and chemokines (Bonilla and Oettgen, 2010; Chaplin, 2010; Esche et al, 2005). Each immune response produces a subset of memory T- and B-lymphocytes which are long lived cells remaining in the host and remembering the specific antigen trigger. The result of this immunological memory is a faster more efficient immune response during subsequent infections with the same pathogen (Bonilla and Oettgen, 2010).

Originally it was widely believed that the innate and adaptive immune responses were two very separate entities. The job of the innate immune response was to fight the infection before the cells of the adaptive immune system arrived later to sterilise the area. However there is increasing evidence to show this is not the case. In fact, the systems work synergistically complementing each other to produce the best possible immune response and fight the infection in the quickest most efficient way. This evidence can be seen in the so called cells that bridge the gap of innate and adaptive immunity like the γδ T-lymphocytes (Chaplin, 2010; Nanno et al, 2007) which have characteristics of both innate and adaptive immunity. Complement opsonisation and derived signalling provides an essential prerequisite to prime pathogens for destruction by both innate and adaptive cells (Dunkelberger and Song, 2010). The innate immune response still provides the initial defence against infection during the first few hours of infection, giving the adaptive immune response time to develop during the next few days with the help of innate immunity to produce antigen specific cells and combat an infection.

What becomes apparent when researching the immune system, is the immune response as a whole is a massive systemic reaction to stress. Such a reaction can be triggered by many different stressors produced during the inflammatory response to an infection by a pathogen or tissue damage. Exercise, provides an excellent whole body stress model from which to investigate the way in which the various components of the immune system react to the specific exercise stress (Shek and Shephard, 1998). The well-established ‘J shaped curve’ (Figure1)
has been used to model the relationship between exercise and susceptibility to infection (Nieman, 1994). Moderate activity leads to enhanced immune function compared to a sedentary lifestyle while regular, prolonged high intensity exercise leads to impaired immune function compared to moderate activity.

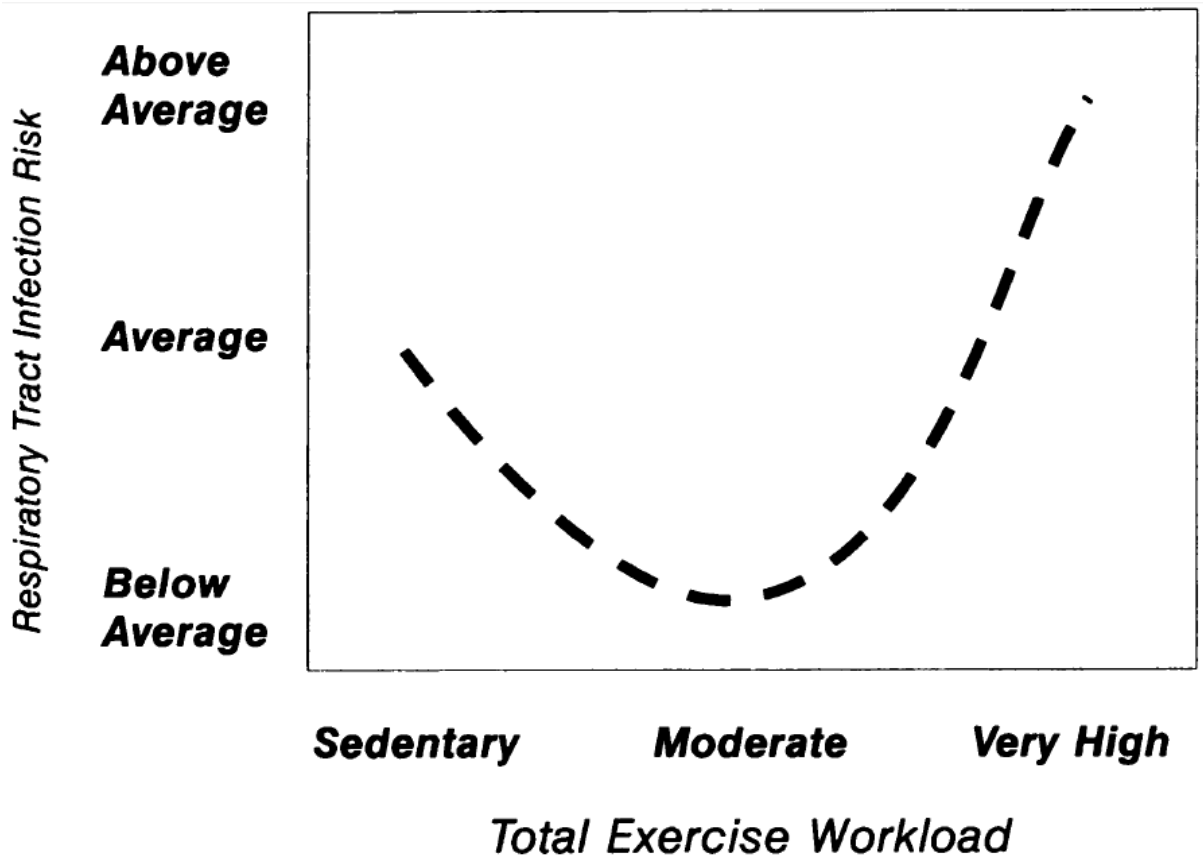

Fig 1. 'J-shaped curve' model of the relationship between exercise and risk of respiratory tract infection, suggests that moderate exercise lowers the risk, but excessive exercise increases the risk taken from Nieman (1994).

Regular moderate exercise of about two hours per day has been reported to decrease the risk of an upper respiratory tract infection (URTI) by 29% compared to a sedentary lifestyle (Matthews et al, 2002). Contrastingly, there is evidence that regular, prolonged, high-intensity exercise impairs immune function. Six days of intensified endurance training in a group of trained cyclists significantly lowered the lymphocyte proliferation at rest (Lancaster et al, 2004). Lymphocyte proliferation increased back to pre-study values after two weeks of reduced training (Lancaster et al, 2004). In addition, a study using professional soccer players observed a decrease in T- lymphocyte proliferation during the competitive season, returning to baseline at the end of the competitive season (Bury et al, 1998). Training volume was increased in a group of elite runners for three weeks, as a result T-lymphocyte proliferation was decreased after a 30 min run at 80% $\dot{V}O_{2max}$ compared to pre training

values (Verde et al, 1992). Various aspects of the innate and adaptive immune response to acute and chronic exercise have been investigated in order to understand the mechanisms responsible for the 'J-shaped curve' model, and to advise athletes during periods of high-intensity exercise training.

Exercise and the innate immune system

Complement

Complement consists of more than 30 proteins located on cell surfaces or free in the plasma. Recognition of a foreign antigen initiates the activation of complement which triggers a proteolytic cascade via one or possibly all three distinct pathways simultaneously. These pathways are known as classical, lectin or alternative depending on the mode of recognition (Dunkelberger and Song, 2010; Sahu and Lambris, 2001). The three pathways trigger different proteolytic cascades with the same aim, termination of the foreign antigen by inflammation. This process which recruits immune cells to the site of infection results in lysis of the bacteria / infected cell or opsonisation (coats the antigen with complement protein which attracts and binds phagocytic cells) of the pathogen.

Various different studies have examined the effect of both acute and chronic exercise on complement proteins. The main complement protein studied as an indication of complement activation is C3 because it is the most abundant and promotes activation of all three pathways (Sahu and Lambris, 2001), C4 promotes activation of the classical and lectin pathways, and C5a is an inflammation trigger produced by all three pathways (Dunkelberger and Song, 2010; Gasque, 2004). Differing results have been observed from acute exercise studies. A series of studies all observed a decrease in C3 and C4 after an acute exercise protocol (Karacabey et al, 2005; Nieman et al, 1989; Saygin et al, 2006). Although all three

studies differed in protocols, subject characteristics, numbers and age which makes them difficult to compare. Wolach et al observed decreased levels of early complement components C1q and C1r in trained and untrained young females following an acute exercise protocol (Wolach et al, 1998). The majority of studies, however, report an increase in complement protein levels following an acute exercise protocol (Camus et al, 1994; Castell et al, 1997; Dufaux et al, 1991; Dufaux and Order, 1989; Romeo et al, 2008; Semple et al, 2006; Smith et al, 1990). Semple et al (2006) observed a significant increase in the C4 levels of pro cyclists 10 days into a three week tour, however, the increase was not as pronounced as those observed in marathon and ultramarathon runners. It was hypothesized that more damage to muscles through weight bearing and footstrike produces a more pronounced inflammatory response (Nieman et al, 2013). This can perhaps explain the few studies that observed no change in complement proteins when using a cycling exercise protocol (Cordova et al, 2010; Thomsen et al, 1992; Ytting et al, 2007). These studies highlight the importance of exercise duration and intensity on the complement response. The effect of chronic exercise on complement proteins is largely general across the literature with most studies observing a decrease in the resting concentrations of complement proteins C3 and C4 in athletes compared to controls (Dufaux et al, 1984; Mattusch et al, 2000; Nieman et al, 1989; Smith et al, 1990; Wolach et al, 1998). The literature suggests acute exercise activates the complement pathways aiding the acute immune response in an exercise duration and intensity dependant manner. Whereas, chronic exercise results in a decrease in the resting complement proteins suggesting a training effect.

Toll like receptors (TLRs)

Toll like receptors (TLRs) are highly conserved transmembrane proteins (Lemaitre et al, 1996) that are important for recognition of foreign antigens and subsequent signalling to both innate and acquired immune systems for appropriate resolution of the pathogen (Takeda et

al, 2003; Takeda and Akira, 2004). To date there have been 11 TLRs identified (Takeda and Akira, 2005) located on and within various different cells of the immune system (monocytes, macrophages, dendritic cells, neutrophils, eosinophils, Langerhans cells) and epithelial cells (Chaplin, 2010; Takeda et al, 2003; Takeda and Akira, 2005). These TLRs recognise different pattern associated molecular patterns (PAMPs) which are specific to foreign pathogens only and not to the host (Chaplin, 2010; Takeda et al, 2003). For example, TLR4 recognises lipopolysaccharide (LPS) in the cell wall of gram negative bacteria (Hoshino et al, 1999; Takeda et al, 2003; Takeda and Akira, 2005). Other PAMPs include viral double stranded RNA and various other features shared by bacterial cells (Takeda et al, 2003; Takeda and Akira, 2004; Takeda and Akira, 2005). Once the TLRs recognise a foreign pathogen an intracellular signalling pathway is triggered inducing the production of inflammatory cytokines and antimicrobial activity by both the innate and adaptive immune systems (Botos et al, 2011; Takeda et al, 2003; Takeda and Akira, 2004; Takeda and Akira, 2005).

Various studies have observed decreased monocyte cell surface expression of TLRs following both acute and chronic exercise. The first to report a change in TLR expression with chronic exercise was Flynn et al (2003) who observed that resistance trained older women had significantly lower whole blood TLR4 mRNA content than sedentary untrained older women (Flynn et al, 2003). A follow up study was conducted by McFarlin et al (2004) to investigate the effect of an acute bout of resistance exercise on the monocyte cell surface expression of TLR4 in trained and untrained women. This study observed lower monocyte cell surface expression of TLR4 in the trained compared to the untrained women, with no effect of the acute exercise bout on TLR4 expression. Concluding that chronic exercise training, but not an acute exercise session, lowers the monocyte cell surface TLR4 expression in older women (McFarlin et al, 2004). However, the exercise protocol carried out by McFarlin et al only lasted 1 hour which may not be long enough to induce a fall in

monocyte cell surface TLR expression (Gleeson et al, 2006). Follow up studies by Stewart et al (2005) and McFarlin et al (2006) investigated chronic exercise training and compared trained to untrained. These studies confirmed the earlier observation that physically active participants have significantly lower monocyte cell surface TLR4 expression than physically inactive subjects irrespective of age (Flynn et al, 2003; McFarlin et al, 2004; McFarlin et al, 2006; Stewart et al, 2005). The only study to examine TLR mRNA in skeletal muscle, as opposed to monocytes, observed decreased levels of TLR4 mRNA in skeletal muscle of obese subjects following 12 weeks of both endurance and resistance exercises (Lambert et al, 2008). In 2005, Lancaster et al were the first to observe a decrease in monocyte TLR1, TLR2 and TLR4 expression following acute prolonged aerobic exercise (Lancaster et al, 2005). The subjects were exercised in the heat (34˚C) to potentiate the exercise induced stress response and thus maximise the observed effect. In 2006, Gleeson et al carried out a follow up experiment to investigate the effect of exercising in the heat on TLR expression. This confirmed that the difference Lancaster observed was due to the acute exercise bout and not the heat stress (Gleeson et al, 2006). The TLR expression observed by Lancaster was on the total monocyte population. However, there are different populations of monocytes and the difference observed post exercise could have been the mobilisation of one of these populations. Therefore, Simpson et al (2009) carried out a study to determine if the change in surface expression of TLRs on total blood monocytes after acute exercise was influenced by altered proportions of monocyte subpopulations. This was the first study to show that TLR4 expression on the total monocyte cell population was lower during the recovery phase of acute exercise in comparison to the pre exercise value. Despite the greater expression of TLR4 in the pro-inflammatory monocyte population the reduced expression of TLR4 on the total monocyte cell population was due to a decrease on the classic monocytes and not the pro-inflammatory monocytes (Simpson et al, 2009). The effects of acute and chronic exercise on TLRs are inconclusive, future understanding of how small changes in surface expression of TLRs impact the intracellular signalling pathways following pathogen recognition remains to be determined.

Cytokines (inflammatory response)

Cytokines are small signalling molecules released from activated cells which bind to transmembrane cell surface receptors and include chemokines, interferons, interleukins (IL), lymphokines and tumour necrosis factor. Binding of the cytokine alters the function of the cell (Chaplin, 2010; Tosi, 2005). There are two main cytokine populations, pro-inflammatory and anti-inflammatory cytokines (Gleeson, 2007; Santos et al, 2007; Tosi, 2005). Pro-inflammatory cytokines (IFNγ, IL-1, TNFα, IL-6) mediate an inflammatory response triggering the activation of macrophages, cytotoxic immune cells and other mediators of inflammation. This response triggers subsequent apoptosis of virus infected cells and pathogen phagocytosis (Gleeson, 2007). The anti-inflammatory cytokines (IL-5, IL-13, IL-4, IL-10, IL-6) mediate an anti-inflammatory response by suppressing the pro-inflammatory cytokine production and steering the response towards the less damaging antibody production, they also stimulate Th2 inflammation against asthma and helminth infection (Gleeson, 2007). There are many cytokines, with the interleukin class alone numbering from IL-1 to IL-36. The kinetics of the various cytokines involved during exhaustive exercise are reviewed by Suzuki et al (Suzuki et al, 2002).

Various different studies have been carried out to identify the effect of exercise on the cytokine response (Andersson et al, 2010; Ascensao et al, 2008; Bishop et al, 2002; Bishop et al, 2002; Castellano et al, 2008; Croft et al, 2009; de Salles et al, 2010; Donges et al, 2010; Fischer et al, 2004; Gokhale et al, 2007; Ispirlidis et al, 2008; Izquierdo et al, 2009; Kumae et al, 2009; Nemet et al, 2009; Ostrowski et al, 1999; Pedersen et al, 2000; Phillips et al, 2008; Rosa et al, 2007; Stewart et al, 2007; Zembron-Lacny et al, 2010). The majority of studies have focused on IL-6 for two reasons: firstly, it is produced by exercising skeletal muscle (Steensberg et al, 2000); and secondly it has both pro and anti-inflammatory properties (Petersen and Pedersen, 2006). Cytokine studies are difficult to compare since

there are many variables to consider (King et al, 2003). Age, sex, muscle mass involved, exercise type, duration and intensity can all differ and affect the cytokine response in studies. However, the general conclusion is that acute exercise elicits an inflammatory response similar to a mild form of the septic shock reaction (Camus et al, 1994; Shek and Shephard, 1998), determined by an increase in pro-inflammatory cytokines and the subsequent recruitment of cytotoxic cells to the peripheral blood stream (Gleeson, 2007). Chronic exercise training has the opposite effect by decreasing the resting levels of various inflammatory markers (King et al, 2003) and blunting the magnitude of inflammatory cytokine response to a single bout of exercise (Gokhale et al, 2007; Woods et al, 2000). The nature of this response has led to exercise being recommended as an anti-inflammatory therapy (Bruunsgaard, 2005; Nader and Lundberg, 2009; Pedersen and Bruunsgaard, 2003; Petersen and Pedersen, 2006; Timmons, 2005). The cytokine response to an acute bout of exercise does not appear to vary between trained and untrained individuals (Gokhale et al, 2007). Acute exercise still elicits a pro-inflammatory response and therefore it is only the magnitude of this response that is smaller in trained individuals (Croft et al, 2009; Fischer et al, 2004; Gokhale et al, 2007; Santos et al, 2007). Current literature on the cytokine response to acute and chronic exercise is conflicting. Reasons for confusion is the pleotropic nature of the IL-6 cytokine released from working muscles during exercise, the speed of the influx and removal of cytokines from the peripheral blood stream and the various different cells that produce and are influenced by cytokines.

Cytokine expression in response to a marathon (Nieman et al, 2001), incremental cycling (Moyna et al, 1996) and steady state cycling (Timmons et al, 2005) does not differ markedly between the sexes from the few studies that exist. Potential sex differences in IL-6 have been observed after maximal exercise (Edwards et al, 2006). Genes involved in inflammation are differentially upregulated at different phases of the menstrual cycle phase in response to exercise (Northoff et al, 2008) highlighting the importance of investigating the

response to exercise at different phases of the menstrual cycle phase on different immune markers and controlling for menstrual cycle phase and oral contraceptive.

Dietary supplementation has been briefly investigated in relation to the cytokine response to exercise. Ingestion of a carbohydrate supplement before and/or during prolonged exercise attenuates increases in anti-inflammatory cytokines IL-6, IL10 and IL1ra (Davison and Gleeson, 2005; Nieman, 1998; Nieman and Bishop, 2006). *In vitro* research provides an anti-inflammatory post-exercise rational for *n*-3 polyunsaturated fatty acid supplementation. However, the limited data from athletes is non-supportive and mixed (Nieman et al, 2009) and warrants further investigation in athletic populations.

Macrophages

Macrophages are a vital cell in the day-to-day function of the immune system as a whole. Monocytes are the precursors to macrophages. Produced in the bone marrow, monocytes migrate to tissues where they differentiate to produce tissue specific macrophages, peritoneal in the abdomen, alveolar in the lungs and Langerhans cells in the skin (Robbins and Swirski, 2010). Macrophages remain resident in the specific tissue until they are primed for type 1 activation by a cytokine interferon-γ (IFN-γ) (Boehm et al, 1997). The immune systems ability to react to new challenges relies on these tissue specific macrophages having different functions. Macrophages are ubiquitous and move by way of chemotaxis attracted by cytokines and chemokines produced by various cells like activated T-lymphocytes to sites of infection and tissue damage indicators like histamine (Stow et al, 2009; Watts, 1997). Macrophages constantly survey the environment by way of non-specific pinocytosis, or the more specific endocytosis and phagocytosis, in search of foreign pathogens (micro-organisms, bacteria, proteins and other smaller cells) that could potentially harm the host (Adams and Hamilton, 1984). Phagocytosis is the process by which macrophages engulf the pathogen (Adams and Hamilton, 1984; Djaldetti et al, 2002). Macrophages recognise foreign pathogen and opsonised foreign antigens through TLRs and complement receptors on the antigen surface. They then attach to the antigen using complement and fragment crystallisable region (Fc) receptors on their surface and ingest the antigen, which is subsequently broken down by potent enzymes contained within phagolysosomes inside the macrophage. Peptide fragments from the broken down antigen are then presented on the surface of the macrophage by major histocompatibility complex (MHC) class II molecules for interaction with T cells (Unanue, 1984). During this process the macrophage also secrets various different molecules, pro-inflammatory cytokines TNF-α and IL-6 and anti-inflammatory cytokines IL-10 and IL-4 (Stow et al, 2009), chemokines to regulate lymphocytes, molecules with tumouricidal or microbicidal activity, and molecules to aid tissue remodelling (Hume, 2006). The molecules produced by the macrophage are

dependent on the specific antigen recognised and the best possible way of destroying it while causing the least damage to the host.

An acute bout of exercise induces the mobilisation of various cells including monocytes/macrophages (McCarthy et al, 1991). This mobilisation results in an increase in the monocyte numbers in the peripheral blood (Woods et al, 1999). There are potential exercise intensity or duration dependent changes in monocyte subpopulations, therefore, the dose of exercise in various studies is important. For example, following long duration acute exercise mature monocytes may migrate out of the vasculature (Gabriel et al, 1994), thus altering the blood monocyte profile. Contrastingly, chronic exercise training appears to have no effect on monocyte numbers in the peripheral blood of resting subjects (Woods et al, 1999).

Numerous studies have been carried out to determine the effect of both acute and chronic exercise on macrophage functions. Macrophage function experiments have mostly used animal models due to the difficulty in acquiring differentiated human macrophages. One study published in 1988 observed that acute exercise increased tissue macrophage chemotaxis and phagocytosis in humans (Michna, 1988). Various groups have focused on different aspects of macrophage function, used different animal species, different macrophage subsets and different doses of exercise. However, in general it has been observed that acute exercise has a positive effect on certain aspects of the phagocytosis pathway. Studies using both mice and guinea pigs of various ages have observed that acute swimming to exhaustion results in an increase in peritoneal macrophage chemotaxis, adherence and phagocytosis (De la et al, 1990; De la et al, 1993; Forner et al, 1994; Ortega et al, 1992; Ortega et al, 1993; Ortega et al, 1996; Ortega et al, 1997). In one study, Silveria et al (2007) observed that an acute bout of moderate swimming in rats increased phagocytic

capacity in circulating macrophages. The effect of exercise on macrophage anti-tumour cytotoxicity has been investigated in mouse. Peritoneal macrophage cytotoxicity increased after an acute bout of exercise (Woods et al, 1993; Woods et al, 1994a; Woods et al, 1994b). Anti-tumour cytotoxicity has also been investigated in mouse alveolar macrophages, where it was demonstrated that prolonged exercise could have a protective effect on lung tumour metastases and enhance alveolar macrophage anti-tumour cytotoxicity (Davis et al, 1998). Despite these positive responses to exercise, evidence suggests that acute exhaustive exercise can result in a decrease in alveolar macrophage antiviral resistance to herpes simplex virus (HSV-1). Macrophage resistance to HSV-1 following the exhaustive exercise bout caused a subsequent exercise induced increase in HSV-1 viral replication (Davis et al, 1997; Kohut et al, 1998a; Kohut et al, 1998b). Ceddia et al observed a suppression of antigen presentation in mouse peritoneal macrophages following acute exhaustive exercise (Ceddia and Woods, 1999; Woods et al, 1997). In a follow up study the same group of authors (Ceddia et al, 2000) investigated the intracellular mechanisms responsible for suppression of antigen presentation and concluded that exercise induced suppression was due to an intracellular defect in the macrophage antigen processing pathway.

There are not many studies covering the effects of chronic exercise training on macrophage function. The few studies that exist have investigated age and training concluding aging reduces, and exercise training increases the capacity of resident peritoneal macrophages to respond to IFN-γ and lipopolysaccharide (LPS) with increased tumour cytolysis (Lu et al, 1999; Woods et al, 2000). The effect of chronic exercise training on the acute exercise response in animal models reveals that exercise training can increase induced inflammatory responses of peritoneal macrophages. Exercise training, with or without a low fat diet, reduces visceral adipose tissue and systemic inflammation by reducing macrophage infiltration and pro-inflammatory cytokine gene expression (Vieira et al, 2009a,b). Reductions

in the macrophage inflammatory responses appear to occur in response to chronic training of 30 days, or less, reducing the magnitude of the acute exercise inflammatory response (Fehr et al, 1988; Ortega et al, 1992; Ortega et al, 1993; Ortega et al, 1996).

To conclude, it appears that both moderate and exhaustive exercise have both positive and negative effects on the macrophage response. The overall positive effect of exercise is the innate first line response of macrophages to antigen by enhancing various phagocytic functions. Exercise has been suggested as a possible therapeutic strategy in the progression of cancers following the observed increase in anti-tumour cytotoxicity promoted by exercise. However, further research in this field is required since studies have not shown that an increase in cytotoxicity decreases tumour size, or progression, in response to exercise. Animal studies have highlighted the decreased antiviral responses of alveolar macrophages following strenuous exercise. When related to humans this could partly explain an increase in upper respiratory symptoms (URS) observed in athletes (Gleeson et al, 1999). Innate effector functions of macrophages are increased in response to exercise, however, cell mediated accessory functions like antigen presentation and MHC Class II loading appear to be negatively affected by strenuous exercise. These observations could help explain the increase in infections after strenuous exercise (Cox et al, 2007; Cox et al, 2008) and in overtraining (MacKinnon, 2000).

No difference in total number of blood monocytes were observed between the sexes after submaximal exercise in sedentary participants (Barriga et al, 1993). In an animal inoculation study, the female mice that exercised at moderate intensity had a greater macrophage resistance to herpes simplex virus -1 (HSV-1) than the male mice (Brown et al, 2004). Further investigation into the potential mechanisms for this resistance and future human

studies investigating the macrophage response to exercise between the sexes is warranted before any conclusions can be made.

Various studies have investigated the immune response to exercise and the effect of carbohydrate supplementation (Davison and Gleeson, 2005; Nieman, 1998; Nieman and Bishop, 2006). One of the parameters measured was blood monocyte counts. Carbohydrate supplementation reduced the exercise induced increase in monocytes (Nieman et al, 1998b), highlighting examination of the influence of dietary supplementation on the immune response to exercise needs well controlled future studies.

Neutrophils

Neutrophils are another member of the phagocytic family of cells that have been investigated pre and post exercise. Neutrophils are one of the first cell types to arrive at a site of infection. Like macrophages they engulf foreign antigens with the help of TLRs, complement and Fc receptors on both antigen and neutrophil surface. They then produce reactive oxygen species (ROS) vital for the endogenous destruction of an ingested pathogen. ROS are damaging and toxic to host tissue if overproduced (Sen, 2001). The rapid recruitment, usually within minutes, to the infection site is due to neutrophil prevalence, they make up 60-70% of the circulating white blood cell count (Mackinnon, 2000), and their anatomical location, in the blood stream which gives them easy access to tissues. As a result it is easy to isolate neutrophils from human peripheral blood samples. In vivo experiments to investigate exercise effects on neutrophils have provided conflicting results.

Previous research has focussed on neutrophil count, phagocytic activity and ROS production. Following acute exercise the peripheral blood circulating neutrophil concentration increases (Chinda et al, 2003; Gabriel et al, 1995; Kakanis et al, 2010; Sureda et al, 2009;

Umeda et al, 2008). Kakanis et al observed a 5 fold increase in neutrophil numbers at 2 hours post exercise (Kakanis et al, 2010) suggesting that 80% of neutrophils circulating during the post exercise period were mobilised from the marginal pool and bone marrow (Gabriel et al, 1995; Hack et al, 1992). The observed peak neutrophil count at 2 hours post exercise is suggestive of circulating stress hormones involvement in mobilising neutrophils (Ortega, 2003; Pedersen et al, 1998). Research has provided varying results for neutrophil phagocytic activity. Some studies have observed a decrease in activity (Chinda et al, 2003; Chinda et al, 2003; Kakanis et al, 2010; Muns, 1994; Takahashi et al, 2007) while others have observed that phagocytic activity stayed the same or increased (Ortega et al, 1993; Scharhag et al, 2005) post-exercise. Contrasting results have also been observed in the ROS studies. Some studies have observed an increase in ROS production in response to exercise (Chinda et al, 2003; Mochida et al, 2007; Pedersen and Bruunsgaard, 1995; Takahashi et al, 2007; Umeda et al, 2008). Other studies have observed a decrease in ROS production following exercise (Chinda et al, 2003; Miyazaki et al, 2001; Mochida et al, 2007; Takahashi et al, 2007). The reasons for the differing functional results are unknown. However, there is a lot of variability in the subjects and study protocols used, indicating that neutrophil function is very sensitive to exercise type/dose and subjects individual fitness level or simply very variable (Umeda et al, 2008). Mochida et al argue that the variation observed in phagocytic activity and ROS production are symptomatic of the immune system as a whole, continually adapting and compensating to provide the best possible immune response (Mochida et al, 2007). The increased neutrophil count post-exercise is thought to be the result of muscle damage caused by exercise. Damaged muscle tissue releases intracellular enzymes in to the blood (Flynn et al, 1994; Koutedakis et al, 1993), the increase in such intracellular enzymes has a positive correlation with the increase in neutrophil numbers observed post exercise (Suzuki et al, 1999; Tidball, 2005; Umeda et al, 2008). However, data that uses creatine kinase (CK) as a marker of muscle damage should be interpreted with care due to the massive differences in individual responses to damage (Brancaccio et al, 2007). But speculatively, strenuous exercise causes damage to the

exercising muscle. Following strenuous exercise intracellular enzymes are released along with cytokines and stress hormones resulting in the mobilisation of neutrophils from the marginal pool and bone marrow to the circulation to control and repair muscle damage.

The effect of chronic exercise on neutrophils was reviewed by Mackinnon (Mackinnon, 2000). Neutrophil count does not change with chronic exercise training. Comparing athletes to non-athletes the pre and post exercise neutrophil count and respiratory burst is attenuated in athletes, the same is observed in athletes during periods of high intensity compared to moderate intensity training (Bury et al, 1998; Bury and Pirnay, 1995; Flynn et al, 1994; Hack et al, 1994; Pyne et al, 1995; Saito et al, 2003; Smith et al, 1990). It has been suggested that this attenuated response is a protective mechanism to prevent chronic inflammation and subsequent overproduction of potentially toxic ROS following frequent strenuous exercise in athletes (Smith, 1994).

At rest and after 90 mins of steady state cycling higher numbers of circulating neutrophils were observed in females taking oral contraceptives (OC) compared to men and non-users of contraceptives (Timmons et al 2005). No difference in circulating neutrophils was observed between the men and the non-users of contraceptives, highlighting the importance of oral contraceptive use and pregnancy hormones in the immune response to exercise.

Supplementation with high dose vitamins and/or minerals has been investigated in response to exercise. The rationale is that they quench the increased ROS in response to exercise. No conclusive evidence for a decrease in exercise induced ROS production has been observed for vitamin and/or mineral supplementation (Davison and Gleeson, 2005; Davison and Gleeson, 2006; Gleeson et al, 2004; Nieman et al, 2002; Nieman et al, 2004). Carbohydrate supplementation attenuates exercise induced increases in neutrophil counts (Davison and

Gleeson, 2005; Nieman et al, 1998; Nieman and Bishop, 2006), further highlighting the importance of more detailed dietary supplementation studies.

Natural Killer cells

Natural killer cells (NK cells) are a major component of the innate immune system. They are a cytotoxic lymphocyte, important in the defence against viruses and cancer, due to their ability to destroy certain virally infected and tumour cells in an MHC class I independent way. NK and T-lymphocyte subsets can be identified using monoclonal antibodies, which recognise cell surface markers, known as cluster of differentiation (CD). The NK cell phenotype is classified by the presence or absence of certain surface CD receptors. The NK cell phenotype is recognised as $CD3^{-}CD16^{+}CD56^{+}$. Since the NK cell killing mechanism is not MHC class I restricted it does not express the T cell receptor (TCR) therefore it is TCR co-receptor CD3 negative, it does however co-express FcγIII (CD16) and a cell adhesion molecule (CD56). Based on the intensity of CD56 two functionally distinct NK cell subpopulations have been identified. $CD3^{-}CD56^{bright}$ cells, which express low or no levels of CD16 and $CD3^{-}CD56^{dim}$ cells, which express high levels of CD16 and are the more cytotoxic subtype (Lanier et al, 1986). A meta-analysis carried out by (Shephard and Shek, 1999) examined 94 studies for the acute and chronic exercise effects on NK cells. The general conclusion suggested that acute exercise elicits an increase in the numbers of circulating NK cells, followed by a suppression of the circulating NK cell count during the recovery period. This suppression persists for a few hours, combined with a smaller decrease in total cytolytic activity and a suggestion of decreased cytolytic activity per NK cell (NKCA). Gleeson and Bishop (2005) published a review and suggested that NKCA is depressed for several hours following a prolonged, intense and stressful bout of exercise. (Timmons and Cieslak, 2008) reviewed the effect of exercise on NK cell subtypes reporting that both $CD56^{bright}$ and $CD56^{dim}$ cells are mobilised into peripheral blood circulation with acute exercise. The more

cytotoxic $CD56^{dim}$ cell subset was shown to be more responsive to acute exercise. The importance of further research, investigating the individual functional responses of the subsets, sensitive to acute exercise, was highlighted by observations of the same group (Timmons et al, 2004; Timmons et al, 2005; Timmons et al, 2006b; Timmons et al, 2006a; Timmons and Bar-Or, 2007). These studies demonstrated that carbohydrate intake, sex, puberty, exercise duration and intensity could all influence subset mobilisation.

Gamma-Delta T-lymphocytes

Gamma-delta T-lymphocytes (γδ T cells) are a versatile lymphocyte often referred to as 'the bridge between the innate and adaptive immune response' (Carding and Egan, 2002; Girardi, 2006; Kalyan and Chow, 2009) referring to the shared characteristics of both innate and adaptive immunity which these cells display. For example, γδ T-lymphocytes use pattern recognition receptors to recognise and respond to various different antigens just like cells of the innate immune system, but γδ T-lymphocytes can also develop a memory phenotype similar to T-lymphocytes (Girardi, 2006). In humans γδ T-lymphocytes account for about 5% of the whole blood lymphocytes, but up to 50% of T-lymphocytes in the epithelial tissues, such as the skin and lining of the gastro-intestinal, respiratory and genito-urinary tracts (Carding and Egan, 2002). γδ T-lymphocytes are important in the process of eliminating bacterial infection (Nakasone et al, 2007), in delayed-type hypersensitivity reactions (Askenase, 2001), and in wound repair (Girardi, 2006; Jameson et al, 2002). γδ T-lymphocytes also have immunosurveillance and antigen presenting properties (Brandes et al, 2005; Girardi, 2006).

With regards to exercise the γδ T-lymphocyte literature is sparse. The first study (Anane et al, 2009) to investigate the effect of exercise on γδ T-lymphocytes observed they are stress

responsive, mobilized by acute exercise, psychological stress and β-agonist infusion. The same group (Anane et al, 2010) further investigated the phenotypes of the γδ T-lymphocyte subtypes mobilised by psychological stress. They observed that the subset mobilised by stress was mostly γδ memory lymphocytes with high cytotoxic capability, tissue homing potential and capacity for rapid, innate like target recognition. Following this research the group concluded that the selective mobilisation observed following acute exercise, psychological stress and β-agonist infusion provides protection in situations where tissue damage and antigen exposure are more likely to occur. These conclusions support the idea of γδ cell involvement in innate responses (Anane et al, 2009; Anane et al, 2010).

No work, to my knowledge, has examined dietary supplementation or any differences between sexes for γδ T-lymphocytes in response to exercise.

The majority of early exercise immunology work focussed on the innate immune response to exercise. Understandably, it is the first line of defence against infection, either dealing with the antigen itself, or signalling for help from the adaptive immune response. Therefore understanding the innate arm of the immune response was initially logistically easier because it is the first to respond to exercise stress. Early exercise immunology studies used the self-reported URTI as a marker of infection incidence with the hypothesis that changes in innate immune cell numbers and markers were indicative of infection risk. An examination of this work demonstrates the importance of exercise stress on immune response and also possibly age and training status, but little evidence exists investigating the effect of sex or dietary intervention on the immune response to exercise. If countermeasures such as nutritional supplements targeted the innate arm of the immune response the risk of infection would be more effectively countered than targeting the slower moving adaptive immune response.

Exercise and the adaptive immune response

In recent years, due to developments in fluorescent antibodies various T-lymphocyte sub-types and receptors can be identified, allowing for a more detailed understanding of the specific sub-populations of T-lymphocytes involved in the response. As a result the adaptive immune response has come under scrutiny. The adaptive arm of the immune response is of more interest to study, not only due to relatively less work having been done on the adaptive response but in relation to the roles the innate and adaptive immune responses play. The innate response is non-specific while the adaptive is very specific, and as a result specific T-lymphocytes are recruited, with specific properties to eliminate a pathogen. The T-lymphocyte pool alters throughout life as a result of chronological aging and latent virus infection, but very little work exists on the influence of training status, sex and dietary supplementation on the T-lymphocyte pool at rest and in response to acute exercise.

T-lymphocytes

During T-lymphocyte development in the thymus, T-lymphocytes acquire a T-cell receptor (TCR) that fits the major histocompatibility complex (MHC) unique to the individual, before entering the periphery as a fully functional naïve T-lymphocyte (Simpson, 2011). Therefore, all T-lymphocyte recognition and killing is MHC restricted. Unlike NK cells, T-lymphocytes are TCR co-receptor CD3 positive, helper T-lymphocytes express CD4, and most cytotoxic T-lymphocytes express CD8. In resting blood and secondary lymphoid organs, 60% to 70% of T-lymphocytes are helper $CD4^+$ T-lymphocytes and 30% to 40% are cytotoxic $CD8^+$ T-lymphocytes (Bruunsgaard et al, 1999). When the MHC class II molecule displays the antigen on the cell surface of antigen presenting cells (APCs), $CD4^+$ T-lymphocytes bind to the antigen and stimulate the APC to release the cytokine interleukin 1 (IL-1). IL-1 stimulates T-lymphocyte growth and division. $CD4^+$ and $CD8^+$ T-lymphocytes are stimulated to undergo

further proliferation and growth from IL-2, in an autocrine fashion (Gleeson et al, 2006). In the resolution phase of an immune response, most of the excess clones of effector T-lymphocytes die by apoptosis, but some recirculate to tissues to protect the host against further attacks from the same infectious agent, providing an immunological memory of previously encountered antigens (Simpson, 2011).

Previous studies have observed no difference in lymphocyte count between the sexes in response to submaximal exercise (Barriga et al, 1993) and incremental cycling (Moyna et al, 1996). However, in a study that investigated oral contraceptive (OC) use on the immune response to exercise females taking OC had a greater post exercise increase in lymphocytes compared to men and non-OC users (Timmons et al, 2005). A greater post exercise increase in lymphocytes was also observed in the non-OC users compared to men after steady state moderate cycling (Timmons et al, 2005). Differences in immune markers as a result of oral contraceptive use, highlights the importance of investigating the effect of sex hormones on the immune response to exercise.

Carbohydrate supplementation before and/or during prolonged exercise observed no effect on the T-lymphocyte response (Davison and Gleeson, 2005; Nieman et al, 1998; Nieman and Bishop, 2006), highlighting the importance of investigating other dietary supplements on the T-lymphocyte response to exercise.

Senescent T-lymphocytes

Cellular senescence is a state whereby cells are unable to further proliferate in response to stimuli. Senescence can be caused by excess rounds of cell division in response to repeated

antigenic stimuli (Effros, 2007). During culture conditions cellular proliferation is known to stop after a fixed number of cell divisions (the 'Hayflick limit') (Effros, 2004a; Effros, 2004b). However, this limit is dependent upon the particular cell type (von, 2002). Cellular senescence is therefore affected by the replicative history of cells, not necessarily by chronological time (von, 2002). Human blood lymphocytes in vitro, have been reported to undergo around 33 cellular divisions for $CD4^+$ T-lymphocytes and around 23 in $CD8^+$ T-lymphocytes in culture before cell-cycle arrest (Pawelec et al, 1996; Perillo et al, 1993).

At the terminal ends of linear chromosomes there are repeated DNA sequences called telomeres (Effros, 2007). Short telomeres have been suggested as a marker of replicative senescence (Effros, 2004; Effros, 2007a; Effros, 2007c; van et al, 2005a). Telomeres shorten by 50-100 base pairs with each cell division as a result of DNA replication, ultimately resulting in critically short telomeres after repeated rounds of cell division. When telomeres reach a critical length cell cycle arrest is triggered. The signal for the arrest has been suggested to be either the cell detecting DNA damage, a reduction in telomere-binding proteins, or the shortened telomeres themselves (Effros, 2007). In order to prevent further cell division and potential tumourigenesis, signalling mechanisms for senescence are triggered (Effros, 2007).

CD57 is a cell surface glycoprotein expressed on the surface of T-lymphocytes with a senescent phenotype (Brenchley et al, 2003). In healthy participants, the CD57 antigen is expressed by a minority of $CD8^+$ and $CD4^+$ T-lymphocytes in peripheral blood (Focosi et al, 2010). $CD8^+$ $CD57^+$ T-lymphocytes have a higher cytotoxic effector potential including perforin, granzymes and granulysin, and express more adhesion molecules and chemokine receptors than $CD8^+$ $CD57^-$ T-lymphocytes (Focosi et al, 2010). In response to TCR, IL-2, IL-7 and IL-15 stimulation $CD8^+$ $CD57^+$ T-lymphocytes express a lower level of genes involved

in cell-cycle regulation (Focosi et al, 2010). CD28 is an important co-stimulatory cell surface glycoprotein receptor on the surface of T-lymphocytes (Linsley and Ledbetter, 1993). T-lymphocytes expressing CD57 on the cell surface have short telomeres, a long history of proliferation, lack the ability to proliferate in response to mitogenic stimuli (Brenchley et al, 2003), and do not express the co-stimulatory receptor molecule CD28 on the cell surface (Bruunsgaard et al, 1999). When T-lymphocytes encounter an APC expressing either of the CD28 ligands, B7-1 or B7-2 a co-stimulatory signal is transduced through CD28 (Boise et al, 1995). $CD4^+$ Helper T-lymphocyte cytokine production is enhanced by CD28 co-stimulation, through transcriptional and post-transcriptional regulation of gene expression (Thompson et al, 1989). The cytolytic potential of cytotoxic $CD8^+$ T-lymphocytes is activated by CD28 co-stimulation (Boise et al, 1995). CD28 is clearly important for the activation and proliferation of naïve T-cells (Labalette et al, 1999; Linsley and Ledbetter, 1993; Ouyang et al, 2003). T-lymphocytes lacking the expression of CD28 have a lower proliferative capacity (Nociari et al, 1999), have shorter telomere lengths (Effros et al, 1996), and in cell culture these senescent T-lymphocytes lose expression of CD28 altogether (Effros, 2004). CD28 is even important in the activity of the telomere protective enzyme, telomerase (Valenzuela and Effros, 2002; Weng et al, 1997). Telomere analysis and proliferation assays confirm the surface expression of $CD57^+$ and $CD28^-$ T-lymphocytes define the end-stage cell in the senescent pathway (Brenchley et al, 2003). On antigen recognition, $CD8^+$ T- lymphocytes proliferate more than $CD4^+$ (Foulds et al, 2002), have shorter telomeres (Bruunsgaard et al, 1999), and lack the ability to upregulate telomerase (Effros, 2007). Therefore a higher proportion of senescent T-lymphocytes are observed in the $CD8^+$ T-lymphocyte population (Simpson et al, 2008).

The proportion of naïve and activated T- lymphocyte populations in the peripheral T-lymphocyte pool is tightly controlled (Freitas et al, 1996; Rocha et al, 1989; Tanchot and Rocha, 1995). It has been suggested that T-lymphocyte senescence may involve a central

block in apoptosis signalling pathways (Spaulding et al, 1999). Apoptosis and proliferative status are linked, so the inability of senescent cultures to enter cell cycle is a factor in the reduced apoptosis (Spaulding et al, 1999). Due to the lack of apoptosis the proportion of T-lymphocytes with a senescent phenotype may increase (Spaulding et al, 1999) replacing the naïve T-lymphocytes, causing the naïve T-lymphocyte repertoire to shrink (Koch et al, 2006). Naïve T-lymphocytes are vital in the response to novel antigen therefore the shrinking naïve T-lymphocyte repertoire may leave an individual at a greater risk of infection or accumulation of $CD8^+$ T-lymphocytes (Koch et al, 2007; Ouyang et al, 2003; Pawelec et al, 2006). The proportion of senescent T-lymphocytes in older adults positively correlates with disease status (Effros, 2007), the pathogenesis of autoimmune disorders (Effros, 2007), a blunted response to influenza (Goronzy et al, 2001), and increased mortality risk (Wikby et al, 2002).

Senescent $CD4^+$ and $CD8^+$ T-lymphocytes are preferentially mobilised to the peripheral blood circulation, followed by a subsequent egress from the circulation, in response to exercise (Campbell et al, 2008; Simpson et al, 2007; Simpson et al, 2008; Turner et al, 2010). This preferential influx of cytotoxic senescent $CD8^+$ T-lymphocytes to the circulation and subsequently into peripheral tissues is considered important for immunosurveillance. A model proposed by Dhabhar et al (2012) suggests that in response to a stressor naïve T-lymphocytes traffic to lymph nodes where they come into contact with novel antigens (Dhabhar et al, 2012). Concomitantly antigen experienced effector and memory T-lymphocytes traffic to peripheral tissues like the skin, lung or mucosal lining of the gut where they encounter familiar antigens (Dhabhar et al, 2012). In agreement with this model, Bosch et al (2003) observed a selective mobilisation of T-lymphocytes primed for inflammation in response to an acute stressor (Bosch et al, 2003).

The percentage of circulating T-lymphocytes with a senescent phenotype at rest is known to be influenced by body composition (Tchkonia et al, 2010), age (Tchkonia et al, 2010; Yan et al, 2010), maximal aerobic capacity (Spielmann et al, 2011), sex (Yan et al, 2010), and latent CMV infection (Pawelec et al, 2009; Turner et al, 2014) but responses to training status, increased volume of training or dietary manipulation are unknown.

Latent Herpes viruses

Prevalent herpes viruses in humans are herpes simplex virus (HSV-1, HSV-2), cytomegalovirus (CMV), Epstein-Barr Virus (EBV) and Varicella Zoster Virus (VZV) (Simpson, 2011). These viruses are symptomatic on primary infection for example chickenpox on primary infection with VZV. The viruses then remain in the host in a latent state. Further reactivations of the latent virus can occur during periods of stress, for example reactivation of VZV causes shingles. This reactivation of latent viruses as a result of stress induces $CD8^+$ T-lymphocyte proliferation (Koch et al, 2006; Koch et al, 2007; Pawelec et al, 2009). Thus, frequent latent viral reactivation is associated with a greater frequency of senescent T-lymphocytes in the periphery (Koch et al, 2007). As a result of persistent viral reactivation, excess viral specific T-lymphocytes are not apoptosed post infection because they are needed by the host for further reactivation of the latent virus. Alternatively, the excess viral specific T-lymphocytes join the memory T-lymphocyte pool taking up 'immune space' and shrinking the naïve T-lymphocyte repertoire by replacing the antigen inexperienced naïve T-lymphocyte population (Simpson, 2011). The mobilisation of senescent T-lymphocytes to the peripheral blood circulation and subsequent egress is amplified in latent CMV infection (Turner et al, 2010). Acute psychological stress mobilises CMV- and EBV-specific effector T-lymphocytes into the blood (Atanackovic et al, 2006). This observation suggests that acute exercise may initiate a beneficial feedback loop by mobilising senescent T-lymphocytes to the periphery thus freeing 'immune space' for

occupation by antigen inexperienced naïve T-lymphocytes thus restoring the naïve T-lymphocyte repertoire that was preoccupied by the viral specific T-lymphocytes (Simpson, 2011).

The sex difference in herpes viruses has only been investigated in animal inoculation models. Male and female mice inoculated with herpes simplex virus 1 (HSV-1) were equally susceptible to an infection at rest and post exercise. However, more females survived the infection (Brown et al, 2007). The female mice that exercised at moderate intensity had a greater number of macrophages resistant to HSV-1 than the male mice (Brown et al, 2004). This greater macrophage resistance in the females may be responsible for the difference in mortality between the sexes. Although these studies were only carried out in animal models, it highlights not only a sex difference, but a sex difference in response to exercise worth investigating.

To summarise, the current exercise immunology literature has produced confusing and often contrasting findings. Perhaps more so in the innate immune field due to the vast redundancies that occur when responding to a pathogen or a stressor, the speed of the response and the pleotropic nature of cytokines for example IL-6. The T-lymphocyte pool at rest is known to be influenced by many factors, infection (Mallia et al, 2014), age (Simpson et al, 2008), sleep (Dinges et al, 1994), baseline fitness (Spielmann et al, 2011), macronutrient intake (Witard et al, 2013), training volume (Witard et al, 2012), body composition (Tchkonia et al, 2010), sex (Yan et al, 2010) and latent CMV infection (Pawelec et al, 2009; Turner et al, 2013). However the effects of these factors, and the T-lymphocyte populations mobilised, in response to exercise are not fully understood, additionally the effect of fatty acid supplementation on the T-lymphocyte pool and response to exercise is currently unknown.

References

Adams, D.O. , Hamilton, T.A., 1984. The cell biology of macrophage activation. Annu. Rev. Immunol. 2, 283-318.

Anane, L.H., Edwards, K.M., Burns, V.E., Drayson, M.T., Riddell, N.E., van Zanten, J.J., Wallace, G.R., Mills, P.J., Bosch, J.A., 2009. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. Brain Behav. Immun. 23, 823-829.

Anane, L.H., Edwards, K.M., Burns, V.E., Zanten, J.J., Drayson, M.T., Bosch, J.A., 2010. Phenotypic characterization of gammadelta T cells mobilized in response to acute psychological stress. Brain Behav. Immun. 24, 608-614.

Andersson, J., Jansson, J.H., Hellsten, G., Nilsson, T.K., Hallmans, G., Boman, K., 2010. Effects of heavy endurance physical exercise on inflammatory markers in non-athletes. Atherosclerosis 209, 601-605.

Ascensao, A., Rebelo, A., Oliveira, E., Marques, F., Pereira, L., Magalhaes, J., 2008. Biochemical impact of a soccer match - analysis of oxidative stress and muscle damage markers throughout recovery. Clin. Biochem. 41, 841-851.

Askenase, P.W., 2001. Yes T cells, but three different T cells (alphabeta, gammadelta and NK T cells), and also B-1 cells mediate contact sensitivity. Clin. Exp. Immunol. 125, 345-350.

Atanackovic, D., Schnee, B., Schuch, G., Faltz, C., Schulze, J., Weber, C.S., Schafhausen, P., Bartels, K., Bokemeyer, C., Brunner-Weinzierl, M.C., Deter, H.C., 2006. Acute psychological stress alerts the adaptive immune response: stress-induced mobilization of effector T cells. J. Neuroimmunol. 176, 141-152.

Barriga, C., Pedrera, M.I., Maynar, M., Maynar, J., Ortega, E., 1993. Effect of submaximal physical exercise performed by sedentary men and women on some parameters of the immune system. Rev. Esp. Fisiol. 49, 79-85.

Bishop, N.C., Gleeson, M., Nicholas, C.W., Ali, A., 2002. Influence of carbohydrate supplementation on plasma cytokine and neutrophil degranulation responses to high intensity intermittent exercise. Int. J. Sport Nutr. Exerc. Metab 12, 145-156.

Boehm, U., Klamp, T., Groot, M., Howard, J.C., 1997. Cellular responses to interferon-gamma. Annu. Rev. Immunol. 15, 749-795.

Boise, L.H., Noel, P.J., Thompson, C.B., 1995. CD28 and apoptosis. Curr. Opin. Immunol. 7, 620-625.

Bonilla, F.A. , Oettgen, H.C., 2010. Adaptive immunity. J. Allergy Clin. Immunol. 125, S33-S40.

Bosch, J.A., Berntson, G.G., Cacioppo, J.T., Dhabhar, F.S., Marucha, P.T., 2003. Acute stress evokes selective mobilization of T cells that differ in chemokine receptor expression: a potential pathway linking immunologic reactivity to cardiovascular disease. Brain Behav. Immun. 17, 251-259.

Botos, I., Segal, D.M., Davies, D.R., 13-4-2011. The structural biology of Toll-like receptors. Structure. 19, 447-459.

Brancaccio, P., Maffulli, N., Limongelli, F.M., 2007. Creatine kinase monitoring in sport medicine. Br. Med. Bull. 81-82, 209-230.

Brandes, M., Willimann, K., Moser, B., 8-7-2005. Professional antigen-presentation function by human gammadelta T Cells. Science 309, 264-268.

Brenchley, J.M., Karandikar, N.J., Betts, M.R., Ambrozak, D.R., Hill, B.J., Crotty, L.E., Casazza, J.P., Kuruppu, J., Migueles, S.A., Connors, M., Roederer, M., Douek, D.C., Koup, R.A., 1-4-2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 101, 2711-2720.

Brown, A.S., Davis, J.M., Murphy, E.A., Carmichael, M.D., Carson, J.A., Ghaffar, A., Mayer, E.P., 2007. Susceptibility to HSV-1 infection and exercise stress in female mice: role of estrogen. J. Appl. Physiol (1985.) 103, 1592-1597.

Brown, A.S., Davis, J.M., Murphy, E.A., Carmichael, M.D., Ghaffar, A., Mayer, E.P., 2004. Gender differences in viral infection after repeated exercise stress. Med. Sci. Sports Exerc. 36, 1290-1295.

Bruunsgaard, H., 2005. Physical activity and modulation of systemic low-level inflammation. J. Leukoc. Biol. 78, 819-835.

Bruunsgaard, H., Jensen, M.S., Schjerling, P., Halkjaer-Kristensen, J., Ogawa, K., Skinhoj, P., Pedersen, B.K., 1999. Exercise induces recruitment of lymphocytes with an activated phenotype and short telomeres in young and elderly humans. Life Sci. 65, 2623-2633.

Bury, T., Marechal, R., Mahieu, P., Pirnay, F., 1998. Immunological status of competitive football players during the training season. Int. J. Sports Med. 19, 364-368.

Bury, T.B. , Pirnay, F., 1995. Effect of prolonged exercise on neutrophil myeloperoxidase secretion. Int. J. Sports Med. 16, 410-412.

Campbell, J.P., Guy, K., Cosgrove, C., Florida-James, G.D., Simpson, R.J., 2008. Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human

peripheral blood following an acute bout of high-intensity exercise. Brain Behav. Immun. 22, 375-380.

Camus, G., by-Dupont, G., Duchateau, J., Deby, C., Pincemail, J., Lamy, M., 1994. Are similar inflammatory factors involved in strenuous exercise and sepsis? Intensive Care Med. 20, 602-610.

Camus, G., Duchateau, J., by-Dupont, G., Pincemail, J., Deby, C., Juchmes-Ferir, A., Feron, F., Lamy, M., 1994. Anaphylatoxin C5a production during short-term submaximal dynamic exercise in man. Int. J. Sports Med. 15, 32-35.

Carding, S.R. , Egan, P.J., 2002. Gammadelta T cells: functional plasticity and heterogeneity. Nat. Rev. Immunol. 2, 336-345.

Castell, L.M., Poortmans, J.R., Leclercq, R., Brasseur, M., Duchateau, J., Newsholme, E.A., 1997. Some aspects of the acute phase response after a marathon race, and the effects of glutamine supplementation. Eur. J. Appl. Physiol Occup. Physiol 75, 47-53.

Castellano, V., Patel, D.I., White, L.J., 2008. Cytokine responses to acute and chronic exercise in multiple sclerosis. J. Appl. Physiol 104, 1697-1702.

Ceddia, M.A., Voss, E.W., Jr., Woods, J.A., 2000. Intracellular mechanisms responsible for exercise-induced suppression of macrophage antigen presentation. J. Appl. Physiol 88, 804-810.

Ceddia, M.A. , Woods, J.A., 1999. Exercise suppresses macrophage antigen presentation. J. Appl. Physiol 87, 2253-2258.

Chaplin, D.D., 2003. 1. Overview of the immune response. J. Allergy Clin. Immunol. 111, S442-S459.

Chinda, D., Nakaji, S., Umeda, T., Shimoyama, T., Kurakake, S., Okamura, N., Kumae, T., Sugawara, K., 2003. A competitive marathon race decreases neutrophil functions in athletes. Luminescence. 18, 324-329.

Chinda, D., Umeda, T., Shimoyama, T., Kojima, A., Tanabe, M., Nakaji, S., Sugawara, K., 2003. The acute response of neutrophil function to a bout of judo training. Luminescence. 18, 278-282.

Cordova, A., Sureda, A., Tur, J.A., Pons, A., 2010. Immune response to exercise in elite sportsmen during the competitive season. J. Physiol Biochem. 66, 1-6.

Cox, A.J., Gleeson, M., Pyne, D.B., Callister, R., Hopkins, W.G., Fricker, P.A., 2008. Clinical and laboratory evaluation of upper respiratory symptoms in elite athletes. Clin. J. Sport Med. 18, 438-445.

Cox, A.J., Pyne, D.B., Saunders, P.U., Callister, R., Gleeson, M., 2007. Cytokine responses to treadmill running in healthy and illness-prone athletes. Med. Sci. Sports Exerc. 39, 1918-1926.

Croft, L., Bartlett, J.D., MacLaren, D.P., Reilly, T., Evans, L., Mattey, D.L., Nixon, N.B., Drust, B., Morton, J.P., 2009. High-intensity interval training attenuates the exercise-induced increase in plasma IL-6 in response to acute exercise. Appl. Physiol Nutr. Metab 34, 1098-1107.

Davis, J.M., Kohut, M.L., Colbert, L.H., Jackson, D.A., Ghaffar, A., Mayer, E.P., 1997. Exercise, alveolar macrophage function, and susceptibility to respiratory infection. J. Appl. Physiol 83, 1461-1466.

Davis, J.M., Kohut, M.L., Jackson, D.A., Colbert, L.H., Mayer, E.P., Ghaffar, A., 1998. Exercise effects on lung tumor metastases and in vitro alveolar macrophage antitumor cytotoxicity. Am. J. Physiol 274, R1454-R1459.

Davison, G. , Gleeson, M., 2005. Influence of acute vitamin C and/or carbohydrate ingestion on hormonal, cytokine, and immune responses to prolonged exercise. Int. J. Sport Nutr. Exerc. Metab 15, 465-479.

Davison, G. , Gleeson, M., 2006. The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man. Eur. J. Appl. Physiol 97, 454-461.

De la, F.M., Martin, I., Ortega, E., 1993. Effect of physical exercise on the phagocytic function of peritoneal macrophages from Swiss mice. Comp Immunol. Microbiol. Infect. Dis. 16, 29-37.

De la, F.M., Martin, M.I., Ortega, E., 1990. Changes in the phagocytic function of peritoneal macrophages from old mice after strenuous physical exercise. Comp Immunol. Microbiol. Infect. Dis. 13, 189-198.

de Salles, B.F., Simao, R., Fleck, S.J., Dias, I., Kraemer-Aguiar, L.G., Bouskela, E., 2010. Effects of resistance training on cytokines. Int. J. Sports Med. 31, 441-450.

Dempsey, P.W., Vaidya, S.A., Cheng, G., 2003. The art of war: Innate and adaptive immune responses. Cell Mol. Life Sci. 60, 2604-2621.

Dhabhar, F.S., Malarkey, W.B., Neri, E., McEwen, B.S., 2012. Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. Psychoneuroendocrinology 37, 1345-1368.

Djaldetti, M., Salman, H., Bergman, M., Djaldetti, R., Bessler, H., 15-6-2002. Phagocytosis--the mighty weapon of the silent warriors. Microsc. Res. Tech. 57, 421-431.

Donges, C.E., Duffield, R., Drinkwater, E.J., 2010. Effects of resistance or aerobic exercise training on interleukin-6, C-reactive protein, and body composition. Med. Sci. Sports Exerc. 42, 304-313.

Dufaux, B. , Order, U., 13-1-1989. Complement activation after prolonged exercise. Clin. Chim. Acta 179, 45-49.

Dufaux, B., Order, U., Geyer, H., Hollmann, W., 1984. C-reactive protein serum concentrations in well-trained athletes. Int. J. Sports Med. 5, 102-106.

Dufaux, B., Order, U., Liesen, H., 1991. Effect of a short maximal physical exercise on coagulation, fibrinolysis, and complement system. Int. J. Sports Med. 12 Suppl 1, S38-S42.

Dunkelberger, J.R. , Song, W.C., 2010. Complement and its role in innate and adaptive immune responses. Cell Res. 20, 34-50.

Edwards, K.M., Burns, V.E., Ring, C., Carroll, D., 2006. Sex differences in the interleukin-6 response to acute psychological stress. Biol. Psychol. 71, 236-239.

Effros, R.B., 2004. From Hayflick to Walford: the role of T cell replicative senescence in human aging. Exp. Gerontol. 39, 885-890.

Effros, R.B., 2004. Impact of the Hayflick Limit on T cell responses to infection: lessons from aging and HIV disease. Mech. Ageing Dev. 125, 103-106.

Effros, R.B., 2004. T cell replicative senescence: pleiotropic effects on human aging. Ann. N. Y. Acad. Sci. 1019, 123-126.

Effros, R.B., 8-1-2007. Role of T lymphocyte replicative senescence in vaccine efficacy. Vaccine 25, 599-604.

Effros, R.B., 2007. Telomerase induction in T cells: a cure for aging and disease? Exp. Gerontol. 42, 416-420.

Effros, R.B., Allsopp, R., Chiu, C.P., Hausner, M.A., Hirji, K., Wang, L., Harley, C.B., Villeponteau, B., West, M.D., Giorgi, J.V., 1996. Shortened telomeres in the expanded CD28-CD8+ cell subset in HIV disease implicate replicative senescence in HIV pathogenesis. AIDS 10, F17-F22.

Esche, C., Stellato, C., Beck, L.A., 2005. Chemokines: key players in innate and adaptive immunity. J. Invest Dermatol. 125, 615-628.

Fehr, H.G., Lotzerich, H., Michna, H., 1988. The influence of physical exercise on peritoneal macrophage functions: histochemical and phagocytic studies. Int. J. Sports Med. 9, 77-81.

Fischer, C.P., Plomgaard, P., Hansen, A.K., Pilegaard, H., Saltin, B., Pedersen, B.K., 2004. Endurance training reduces the contraction-induced interleukin-6 mRNA expression in human skeletal muscle. Am. J. Physiol Endocrinol. Metab 287, E1189-E1194.

Flynn, M.G., McFarlin, B.K., Phillips, M.D., Stewart, L.K., Timmerman, K.L., 2003. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. J. Appl. Physiol 95, 1833-1842.

Flynn, M.G., Pizza, F.X., Boone, J.B., Jr., Andres, F.F., Michaud, T.A., Rodriguez-Zayas, J.R., 1994. Indices of training stress during competitive running and swimming seasons. Int. J. Sports Med. 15, 21-26.

Focosi, D., Bestagno, M., Burrone, O., Petrini, M., 2010. CD57+ T lymphocytes and functional immune deficiency. J. Leukoc. Biol. 87, 107-116.

Forner, M.A., Collazos, M.E., Barriga, C., De la, F.M., Rodriguez, A.B., Ortega, E., 1994. Effect of age on adherence and chemotaxis capacities of peritoneal macrophages. Influence of physical activity stress. Mech. Ageing Dev. 75, 179-189.

Foulds, K.E., Zenewicz, L.A., Shedlock, D.J., Jiang, J., Troy, A.E., Shen, H., 15-2-2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. J. Immunol. 168, 1528-1532.

Freitas, A.A., Agenes, F., Coutinho, G.C., 1996. Cellular competition modulates survival and selection of CD8+ T cells. Eur. J. Immunol. 26, 2640-2649.

Gabriel, H., Muller, H.J., Kettler, K., Brechtel, L., Urhausen, A., Kindermann, W., 1995. Increased phagocytic capacity of the blood, but decreased phagocytic activity per individual circulating neutrophil after an ultradistance run. Eur. J. Appl. Physiol Occup. Physiol 71, 281-284.

Gabriel, H., Urhausen, A., Brechtel, L., Muller, H.J., Kindermann, W., 1994. Alterations of regular and mature monocytes are distinct, and dependent of intensity and duration of exercise. Eur. J. Appl. Physiol Occup. Physiol 69, 179-181.

Gasque, P., 2004. Complement: a unique innate immune sensor for danger signals. Mol. Immunol. 41, 1089-1098.

Girardi, M., 2006. Immunosurveillance and immunoregulation by gammadelta T cells. J. Invest Dermatol. 126, 25-31.

Gleeson, M., 2007. Immune function in sport and exercise. J. Appl. Physiol 103, 693-699.

Gleeson, M., McDonald, W.A., Pyne, D.B., Cripps, A.W., Francis, J.L., Fricker, P.A., Clancy, R.L., 1999. Salivary IgA levels and infection risk in elite swimmers. Med. Sci. Sports Exerc. 31, 67-73.

Gleeson, M., McFarlin, B., Flynn, M., 2006. Exercise and Toll-like receptors. Exerc. Immunol. Rev. 12, 34-53.

Gleeson, M., Nieman, D.C., Pedersen, B.K., 2004. Exercise, nutrition and immune function. J. Sports Sci. 22, 115-125.

Gokhale, R., Chandrashekara, S., Vasanthakumar, K.C., 2007. Cytokine response to strenuous exercise in athletes and non-athletes--an adaptive response. Cytokine 40, 123-127.

Goronzy, J.J., Fulbright, J.W., Crowson, C.S., Poland, G.A., O'Fallon, W.M., Weyand, C.M., 2001. Value of immunological markers in predicting responsiveness to influenza vaccination in elderly individuals. J. Virol. 75, 12182-12187.

Hack, V., Strobel, G., Rau, J.P., Weicker, H., 1992. The effect of maximal exercise on the activity of neutrophil granulocytes in highly trained athletes in a moderate training period. Eur. J. Appl. Physiol Occup. Physiol 65, 520-524.

Hack, V., Strobel, G., Weiss, M., Weicker, H., 1994. PMN cell counts and phagocytic activity of highly trained athletes depend on training period. J. Appl. Physiol 77, 1731-1735.

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., Akira, S., 1-4-1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. 162, 3749-3752.

Hume, D.A., 2006. The mononuclear phagocyte system. Curr. Opin. Immunol. 18, 49-53.

Ispirlidis, I., Fatouros, I.G., Jamurtas, A.Z., Nikolaidis, M.G., Michailidis, I., Douroudos, I., Margonis, K., Chatzinikolaou, A., Kalistratos, E., Katrabasas, I., Alexiou, V., Taxildaris, K., 2008. Time-course of changes in inflammatory and performance responses following a soccer game. Clin. J. Sport Med. 18, 423-431.

Izquierdo, M., Ibanez, J., Calbet, J.A., Navarro-Amezqueta, I., Gonzalez-Izal, M., Idoate, F., Hakkinen, K., Kraemer, W.J., Palacios-Sarrasqueta, M., Almar, M., Gorostiaga, E.M., 2009. Cytokine and hormone responses to resistance training. Eur. J. Appl. Physiol 107, 397-409.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., Havran, W.L., 26-4-2002. A role for skin gammadelta T cells in wound repair. Science 296, 747-749.

Kakanis, M.W., Peake, J., Brenu, E.W., Simmonds, M., Gray, B., Hooper, S.L., Marshall-Gradisnik, S.M., 2010. The open window of susceptibility to infection after acute exercise in healthy young male elite athletes. Exerc. Immunol. Rev. 16, 119-137.

Kalyan, S. , Chow, A.W., 2009. Linking innate and adaptive immunity: human Vgamma9Vdelta2 T cells enhance CD40 expression and HMGB-1 secretion. Mediators. Inflamm. 2009, 819408-

Karacabey, K., Saygin, O., Ozmerdivenli, R., Zorba, E., Godekmerdan, A., Bulut, V., 2005. The effects of exercise on the immune system and stress hormones in sportswomen. Neuro. Endocrinol. Lett. 26, 361-366.

King, D.E., Carek, P., Mainous, A.G., III, Pearson, W.S., 2003. Inflammatory markers and exercise: differences related to exercise type. Med. Sci. Sports Exerc. 35, 575-581.

Koch, S., Larbi, A., Ozcelik, D., Solana, R., Gouttefangeas, C., Attig, S., Wikby, A., Strindhall, J., Franceschi, C., Pawelec, G., 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. Ann. N. Y. Acad. Sci. 1114, 23-35.

Koch, S., Solana, R., Dela, R.O., Pawelec, G., 2006. Human cytomegalovirus infection and T cell immunosenescence: a mini review. Mech. Ageing Dev. 127, 538-543.

Kohut, M.L., Davis, J.M., Jackson, D.A., Colbert, L.H., Strasner, A., Essig, D.A., Pate, R.R., Ghaffar, A., Mayer, E.P., 1998a. The role of stress hormones in exercise-induced suppression of alveolar macrophage antiviral function. J. Neuroimmunol. 81, 193-200.

Kohut, M.L., Davis, J.M., Jackson, D.A., Jani, P., Ghaffar, A., Mayer, E.P., Essig, D.A., 1998b. Exercise effects on IFN-beta expression and viral replication in lung macrophages after HSV-1 infection. Am. J. Physiol 275, L1089-L1094.

Koutedakis, Y., Raafat, A., Sharp, N.C., Rosmarin, M.N., Beard, M.J., Robbins, S.W., 1993. Serum enzyme activities in individuals with different levels of physical fitness. J. Sports Med. Phys. Fitness 33, 252-257.

Kumae, T., Ishii, T., Osanai, H., Ito, T., 2009. Effects of summer training camp on serum opsonic activity and plasma cytokine levels in female long-distance runners. Luminescence. 24, 438-443.

Labalette, M., Leteurtre, E., Thumerelle, C., Grutzmacher, C., Tourvieille, B., Dessaint, J.P., 1999. Peripheral human CD8(+)CD28(+)T lymphocytes give rise to CD28(-)progeny, but IL-4 prevents loss of CD28 expression. Int. Immunol. 11, 1327-1336.

Lambert, C.P., Wright, N.R., Finck, B.N., Villareal, D.T., 2008. Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. J. Appl. Physiol 105, 473-478.

Lancaster, G.I., Halson, S.L., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A.E., Drayson, M.T., Gleeson, M., 2004. Effects of acute exhaustive exercise and chronic exercise training on type 1 and type 2 T lymphocytes. Exerc. Immunol. Rev. 10, 91-106.

Lancaster, G.I., Khan, Q., Drysdale, P., Wallace, F., Jeukendrup, A.E., Drayson, M.T., Gleeson, M., 15-3-2005. The physiological regulation of toll-like receptor expression and function in humans. J. Physiol 563, 945-955.

Lanier, L.L., Le, A.M., Civin, C.I., Loken, M.R., Phillips, J.H., 15-6-1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J. Immunol. 136, 4480-4486.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 20-9-1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973-983.

Linsley, P.S. , Ledbetter, J.A., 1993. The role of the CD28 receptor during T cell responses to antigen. Annu. Rev. Immunol. 11, 191-212.

Lu, Q., Ceddia, M.A., Price, E.A., Ye, S.M., Woods, J.A., 1999. Chronic exercise increases macrophage-mediated tumor cytolysis in young and old mice. Am. J. Physiol 276, R482-R489.

Mackinnon, L.T., 2000. Chronic exercise training effects on immune function. Med. Sci. Sports Exerc. 32, S369-S376.

MacKinnon, L.T., 2000. Special feature for the Olympics: effects of exercise on the immune system: overtraining effects on immunity and performance in athletes. Immunol. Cell Biol. 78, 502-509.

Matthews, C.E., Ockene, I.S., Freedson, P.S., Rosal, M.C., Merriam, P.A., Hebert, J.R., 2002. Moderate to vigorous physical activity and risk of upper-respiratory tract infection. Med. Sci. Sports Exerc. 34, 1242-1248.

Mattusch, F., Dufaux, B., Heine, O., Mertens, I., Rost, R., 2000. Reduction of the plasma concentration of C-reactive protein following nine months of endurance training. Int. J. Sports Med. 21, 21-24.

Mallia, P., Message, S.D., Contoli, M., Gray, K., Telcain, A., Laza-Stanca, V., Papi, A., Stanciu, L.A., Elkin, S., Kon, O.M., Johnson, M., Johnstona, S.L., 2014. Lymphocyte subsets in experimental rhinovirus infection in chronic obstructive pulmonary disease. Respir Med. 108(1): 78-85.

McCarthy, D.A., Grant, M., Marbut, M., Watling, M., Wade, A.J., Macdonald, I., Nicholson, S., Melsom, R.D., Perry, J.D., 1991. Brief exercise induces an immediate and a delayed leucocytosis. Br. J. Sports Med. 25, 191-195.

McFarlin, B.K., Flynn, M.G., Campbell, W.W., Craig, B.A., Robinson, J.P., Stewart, L.K., Timmerman, K.L., Coen, P.M., 2006. Physical activity status, but not age, influences inflammatory biomarkers and toll-like receptor 4. J. Gerontol. A Biol. Sci. Med. Sci. 61, 388-393.

McFarlin, B.K., Flynn, M.G., Campbell, W.W., Stewart, L.K., Timmerman, K.L., 2004. TLR4 is lower in resistance-trained older women and related to inflammatory cytokines. Med. Sci. Sports Exerc. 36, 1876-1883.

Michna, H., 1988. The human macrophage system: activity and functional morphology. Bibl. Anat.1-84.

Miyazaki, H., Oh-ishi, S., Ookawara, T., Kizaki, T., Toshinai, K., Ha, S., Haga, S., Ji, L.L., Ohno, H., 2001. Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. Eur. J. Appl. Physiol 84, 1-6.

Mochida, N., Umeda, T., Yamamoto, Y., Tanabe, M., Kojima, A., Sugawara, K., Nakaji, S., 2007. The main neutrophil and neutrophil-related functions may compensate for each other following exercise-a finding from training in university judoists. Luminescence. 22, 20-28.

Moyna, N.M., Acker, G.R., Fulton, J.R., Weber, K., Goss, F.L., Robertson, R.J., Tollerud, D.J., Rabin, B.S., 1996. Lymphocyte function and cytokine production during incremental exercise in active and sedentary males and females. Int. J. Sports Med. 17, 585-591.

Muns, G., 1994. Effect of long-distance running on polymorphonuclear neutrophil phagocytic function of the upper airways. Int. J. Sports Med. 15, 96-99.

Nader, G.A. , Lundberg, I.E., 2009. Exercise as an anti-inflammatory intervention to combat inflammatory diseases of muscle. Curr. Opin. Rheumatol. 21, 599-603.

Nakasone, C., Yamamoto, N., Nakamatsu, M., Kinjo, T., Miyagi, K., Uezu, K., Nakamura, K., Higa, F., Ishikawa, H., O'brien, R.L., Ikuta, K., Kaku, M., Fujita, J., Kawakami, K., 2007. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. Microbes. Infect. 9, 251-258.

Nanno, M. , Ishikawa, H., 2005. [Physiological function of intestinal intraepithelial gamma-delta T lymphocytes]. Nippon Rinsho 63 Suppl 4, 454-458.

Nanno, M., Shiohara, T., Yamamoto, H., Kawakami, K., Ishikawa, H., 2007. gammadelta T cells: firefighters or fire boosters in the front lines of inflammatory responses. Immunol. Rev. 215, 103-113.

Nemet, D., Eliakim, A., Mills, P.J., Meckal, Y., Cooper, D.M., 2009. Immunological and growth mediator response to cross-country training in adolescent females. J. Pediatr. Endocrinol. Metab 22, 995-1007.

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

Nieman, D.C., 1998. Influence of carbohydrate on the immune response to intensive, prolonged exercise. Exerc. Immunol. Rev. 4, 64-76.

Nieman, D.C. , Bishop, N.C., 2006. Nutritional strategies to counter stress to the immune system in athletes, with special reference to football. J. Sports Sci. 24, 763-772.

Nieman, D.C., Henson, D.A., McAnulty, S.R., Jin, F., Maxwell, K.R., 2009. n-3 polyunsaturated fatty acids do not alter immune and inflammation measures in endurance athletes. Int. J. Sport Nutr. Exerc. Metab 19, 536-546.

Nieman, D.C., Henson, D.A., McAnulty, S.R., McAnulty, L., Swick, N.S., Utter, A.C., Vinci, D.M., Opiela, S.J., Morrow, J.D., 2002. Influence of vitamin C supplementation on oxidative and immune changes after an ultramarathon. J. Appl. Physiol (1985.) 92, 1970-1977.

Nieman, D.C., Henson, D.A., McAnulty, S.R., McAnulty, L.S., Morrow, J.D., Ahmed, A., Heward, C.B., 2004. Vitamin E and immunity after the Kona Triathlon World Championship. Med. Sci. Sports Exerc. 36, 1328-1335.

Nieman, D.C., Henson, D.A., Smith, L.L., Utter, A.C., Vinci, D.M., Davis, J.M., Kaminsky, D.E., Shute, M., 2001. Cytokine changes after a marathon race. J. Appl. Physiol 91, 109-114.

Nieman, D.C., Luo, B., Dreau, D., Henson, D.A., Shanely, R.A., Dew, D., Meaney, M.P., 19-9-2013. Immune and inflammation responses to a 3-day period of intensified running versus cycling. Brain Behav. Immun.

Nieman, D.C., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Henson, D.A., Utter, A., Davis, J.M., Williams, F., Butterworth, D.E., 1998. Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. J. Appl. Physiol 84, 1252-1259.

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

Nociari, M.M., Telford, W., Russo, C., 15-3-1999. Postthymic development of CD28-CD8+ T cell subset: age-associated expansion and shift from memory to naive phenotype. J. Immunol. 162, 3327-3335.

Northoff, H., Symons, S., Zieker, D., Schaible, E.V., Schafer, K., Thoma, S., Loffler, M., Abbasi, A., Simon, P., Niess, A.M., Fehrenbach, E., 2008. Gender- and menstrual phase dependent regulation of inflammatory gene expression in response to aerobic exercise. Exerc. Immunol. Rev. 14, 86-103.

Ortega, E., 2003. Neuroendocrine mediators in the modulation of phagocytosis by exercise: physiological implications. Exerc. Immunol. Rev. 9, 70-93.

Ortega, E., Collazos, M.E., Barriga, C., De la, F.M., 1992. Stimulation of the phagocytic function in guinea pig peritoneal macrophages by physical activity stress. Eur. J. Appl. Physiol Occup. Physiol 64, 323-327.

Ortega, E., Collazos, M.E., Maynar, M., Barriga, C., De la, F.M., 1993. Stimulation of the phagocytic function of neutrophils in sedentary men after acute moderate exercise. Eur. J. Appl. Physiol Occup. Physiol 66, 60-64.

Ortega, E., Forner, M.A., Barriga, C., 1-2-1997. Exercise-induced stimulation of murine macrophage chemotaxis: role of corticosterone and prolactin as mediators. J. Physiol 498 (Pt 3), 729-734.

Ortega, E., Forner, M.A., Barriga, C., De la, F.M., 1-8-1993. Effect of age and of swimming-induced stress on the phagocytic capacity of peritoneal macrophages from mice. Mech. Ageing Dev. 70, 53-63.

Ortega, E., Rodriguez, M.J., Barriga, C., Forner, M.A., 1996. Corticosterone, prolactin and thyroid hormones as hormonal mediators of the stimulated phagocytic capacity of peritoneal macrophages after high-intensity exercise. Int. J. Sports Med. 17, 149-155.

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., Pedersen, B.K., 15-2-1999. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J. Physiol 515 (Pt 1), 287-291.

Ouyang, Q., Wagner, W.M., Wikby, A., Walter, S., Aubert, G., Dodi, A.I., Travers, P., Pawelec, G., 2003. Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. J. Clin. Immunol. 23, 247-257.

Pawelec, G., Derhovanessian, E., Larbi, A., Strindhall, J., Wikby, A., 2009. Cytomegalovirus and human immunosenescence. Rev. Med. Virol. 19, 47-56.

Pawelec, G., Koch, S., Franceschi, C., Wikby, A., 2006. Human immunosenescence: does it have an infectious component? Ann. N. Y. Acad. Sci. 1067, 56-65.

Pawelec, G., Sansom, D., Rehbein, A., Adibzadeh, M., Beckman, I., 1996. Decreased proliferative capacity and increased susceptibility to activation-induced cell death in late-passage human CD4+ TCR2+ cultured T cell clones. Exp. Gerontol. 31, 655-668.

Pedersen, B.K. , Bruunsgaard, H., 1995. How physical exercise influences the establishment of infections. Sports Med. 19, 393-400.

Pedersen, B.K. , Bruunsgaard, H., 2003. Possible beneficial role of exercise in modulating low-grade inflammation in the elderly. Scand. J. Med. Sci. Sports 13, 56-62.

Pedersen, B.K., Bruunsgaard, H., Ostrowski, K., Krabbe, K., Hansen, H., Krzywkowski, K., Toft, A., Sondergaard, S.R., Petersen, E.W., Ibfelt, T., Schjerling, P., 2000. Cytokines in aging and exercise. Int. J. Sports Med. 21 Suppl 1, S4-S9.

Pedersen, B.K., Rohde, T., Ostrowski, K., 1998. Recovery of the immune system after exercise. Acta Physiol Scand. 162, 325-332.

Perillo, N.L., Naeim, F., Walford, R.L., Effros, R.B., 1993. The in vitro senescence of human T lymphocytes: failure to divide is not associated with a loss of cytolytic activity or memory T cell phenotype. Mech. Ageing Dev. 67, 173-185.

Petersen, A.M. , Pedersen, B.K., 2006. The role of IL-6 in mediating the anti-inflammatory effects of exercise. J. Physiol Pharmacol. 57 Suppl 10, 43-51.

Phillips, M.D., Flynn, M.G., McFarlin, B.K., Stewart, L.K., Timmerman, K.L., Ji, H., 2008. Resistive exercise blunts LPS-stimulated TNF-alpha and Il-1 beta. Int. J. Sports Med. 29, 102-109.

Pyne, D.B., Baker, M.S., Fricker, P.A., McDonald, W.A., Telford, R.D., Weidemann, M.J., 1995. Effects of an intensive 12-wk training program by elite swimmers on neutrophil oxidative activity. Med. Sci. Sports Exerc. 27, 536-542.

Robbins, C.S. , Swirski, F.K., 2010. The multiple roles of monocyte subsets in steady state and inflammation. Cell Mol. Life Sci. 67, 2685-2693.

Rocha, B., Dautigny, N., Pereira, P., 1989. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. Eur. J. Immunol. 19, 905-911.

Romeo, J., Jimenez-Pavon, D., Cervantes-Borunda, M., Warnberg, J., Gomez-Martinez, S., Castillo, M.J., Marcos, A., 2008. Immunological changes after a single bout of moderate-intensity exercise in a hot environment. J. Physiol Biochem. 64, 197-204.

Rosa, J.S., Oliver, S.R., Flores, R.L., Graf, S.C., Pontello, A.M., Lbardolaza, M., Zaldivar, F.P., Galassetti, P.R., 2007. Kinetic profiles of 18 systemic pro- and anti-inflammatory mediators during and following exercise in children. J. Pediatr. Endocrinol. Metab 20, 1293-1305.

Sahu, A. , Lambris, J.D., 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol. Rev. 180, 35-48.

Saito, D., Nakaji, S., Umeda, T., Kurakake, S., Danjo, K., Shimoyama, T., Sugawara, K., 2003. Effects of long-distance running on serum opsonic activity measured by chemiluminescence. Luminescence. 18, 122-124.

Santos, R.V., Tufik, S., De Mello, M.T., 2007. Exercise, sleep and cytokines: is there a relation? Sleep Med. Rev. 11, 231-239.

Saygin, O., Karacabey, K., Ozmerdivenli, R., Zorba, E., Ilhan, F., Bulut, V., 2006. Effect of chronic exercise on immunoglobin, complement and leukocyte types in volleyball players and athletes. Neuro. Endocrinol. Lett. 27, 271-276.

Scharhag, J., Meyer, T., Gabriel, H.H., Schlick, B., Faude, O., Kindermann, W., 2005. Does prolonged cycling of moderate intensity affect immune cell function? Br. J. Sports Med. 39, 171-177.

Semple, S.J., Smith, L.L., McKune, A.J., Hoyos, J., Mokgethwa, B., San Juan, A.F., Lucia, A., Wadee, A.A., 2006. Serum concentrations of C reactive protein, alpha1 antitrypsin, and complement (C3, C4, C1 esterase inhibitor) before and during the Vuelta a Espana. Br. J. Sports Med. 40, 124-127.

Sen, C.K., 2001. Antioxidants in exercise nutrition. Sports Med. 31, 891-908.

Shek, P.N. , Shephard, R.J., 1998. Physical exercise as a human model of limited inflammatory response. Can. J. Physiol Pharmacol. 76, 589-597.

Shephard, R.J. , Shek, P.N., 1999. Effects of exercise and training on natural killer cell counts and cytolytic activity: a meta-analysis. Sports Med. 28, 177-195.

Simpson, R.J., 2011. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc. Sport Sci. Rev. 39, 23-33.

Simpson, R.J., Cosgrove, C., Ingram, L.A., Florida-James, G.D., Whyte, G.P., Pircher, H., Guy, K., 2008. Senescent T-lymphocytes are mobilised into the peripheral blood compartment in young and older humans after exhaustive exercise. Brain Behav. Immun. 22, 544-551.

Simpson, R.J., Florida-James, G.D., Cosgrove, C., Whyte, G.P., Macrae, S., Pircher, H., Guy, K., 2007. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. J. Appl. Physiol 103, 396-401.

Simpson, R.J., McFarlin, B.K., McSporran, C., Spielmann, G., Hartaigh, B., Guy, K., 2009. Toll-like receptor expression on classic and pro-inflammatory blood monocytes after acute exercise in humans. Brain Behav. Immun. 23, 232-239.

Smith, J.A., 1994. Neutrophils, host defense, and inflammation: a double-edged sword. J. Leukoc. Biol. 56, 672-686.

Smith, J.A., Telford, R.D., Mason, I.B., Weidemann, M.J., 1990. Exercise, training and neutrophil microbicidal activity. Int. J. Sports Med. 11, 179-187.

Smith, J.K., Chi, D.S., Krish, G., Reynolds, S., Cambron, G., 1990. Effect of exercise on complement activity. Ann. Allergy 65, 304-310.

Spaulding, C., Guo, W., Effros, R.B., 1999. Resistance to apoptosis in human CD8+ T cells that reach replicative senescence after multiple rounds of antigen-specific proliferation. Exp. Gerontol. 34, 633-644.

Spielmann, G., McFarlin, B.K., O'Connor, D.P., Smith, P.J., Pircher, H., Simpson, R.J., 2011. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav. Immun. 25, 1521-1529.

Steensberg, A., van, H.G., Osada, T., Sacchetti, M., Saltin, B., Klarlund, P.B., 15-11-2000. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. J. Physiol 529 Pt 1, 237-242.

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., Talbert, E., 2005. Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. Brain Behav. Immun. 19, 389-397.

Stewart, L.K., Flynn, M.G., Campbell, W.W., Craig, B.A., Robinson, J.P., Timmerman, K.L., McFarlin, B.K., Coen, P.M., Talbert, E., 2007. The influence of exercise training on inflammatory cytokines and C-reactive protein. Med. Sci. Sports Exerc. 39, 1714-1719.

Stow, J.L., Ching, L.P., Offenhauser, C., Sangermani, D., 2009. Cytokine secretion in macrophages and other cells: pathways and mediators. Immunobiology 214, 601-612.

Sureda, A., Cordova, A., Ferrer, M.D., Tauler, P., Perez, G., Tur, J.A., Pons, A., 2009. Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. Free Radic. Res. 43, 828-835.

Suzuki, K., Nakaji, S., Yamada, M., Totsuka, M., Sato, K., Sugawara, K., 2002. Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. Exerc. Immunol. Rev. 8, 6-48.

Suzuki, K., Totsuka, M., Nakaji, S., Yamada, M., Kudoh, S., Liu, Q., Sugawara, K., Yamaya, K., Sato, K., 1999. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. J. Appl. Physiol 87, 1360-1367.

Takahashi, I., Umeda, T., Mashiko, T., Chinda, D., Oyama, T., Sugawara, K., Nakaji, S., 2007. Effects of rugby sevens matches on human neutrophil-related non-specific immunity. Br. J. Sports Med. 41, 13-18.

Takeda, K. , Akira, S., 2004. TLR signaling pathways. Semin. Immunol. 16, 3-9.

Takeda, K. , Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17, 1-14.

Tanchot, C. , Rocha, B., 1995. The peripheral T cell repertoire: independent homeostatic regulation of virgin and activated CD8+ T cell pools. Eur. J. Immunol. 25, 2127-2136.

Tchkonia, T., Morbeck, D.E., Von, Z.T., Van, D.J., Lustgarten, J., Scrable, H., Khosla, S., Jensen, M.D., Kirkland, J.L., 2010. Fat tissue, aging, and cellular senescence. Aging Cell 9, 667-684.

Thompson, C.B., Lindsten, T., Ledbetter, J.A., Kunkel, S.L., Young, H.A., Emerson, S.G., Leiden, J.M., June, C.H., 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. Proc. Natl. Acad. Sci. U. S. A 86, 1333-1337.

Thomsen, B.S., Rodgaard, A., Tvede, N., Hansen, F.R., Steensberg, J., Halkjaer, K.J., Pedersen, B.K., 1992. Levels of complement receptor type one (CR1, CD35) on erythrocytes, circulating immune complexes and complement C3 split products C3d and C3c are not changed by short-term physical exercise or training. Int. J. Sports Med. 13, 172-175.

Tidball, J.G., 2005. Inflammatory processes in muscle injury and repair. Am. J. Physiol Regul. Integr. Comp Physiol 288, R345-R353.

Timmons, B.W., 2005. Paediatric exercise immunology: health and clinical applications. Exerc. Immunol. Rev. 11, 108-144.

Timmons, B.W. , Bar-Or, O., 2007. Evidence of sex-based differences in natural killer cell responses to exercise and carbohydrate intake in children. Eur. J. Appl. Physiol 101, 233-240.

Timmons, B.W. , Cieslak, T., 2008. Human natural killer cell subsets and acute exercise: a brief review. Exerc. Immunol. Rev. 14, 8-23.

Timmons, B.W., Hamadeh, M.J., Devries, M.C., Tarnopolsky, M.A., 2005a. Influence of gender, menstrual phase, and oral contraceptive use on immunological changes in response to prolonged cycling. J. Appl. Physiol 99, 979-985.

Timmons, B.W., Tarnopolsky, M.A., Bar-Or, O., 2004. Immune responses to strenuous exercise and carbohydrate intake in boys and men. Pediatr. Res. 56, 227-234.

Timmons, B.W., Tarnopolsky, M.A., Bar-Or, O., 2006. Sex-based effects on the distribution of NK cell subsets in response to exercise and carbohydrate intake in adolescents. J. Appl. Physiol 100, 1513-1519.

Timmons, B.W., Tarnopolsky, M.A., Snider, D.P., Bar-Or, O., 2006. Puberty effects on NK cell responses to exercise and carbohydrate intake in boys. Med. Sci. Sports Exerc. 38, 864-874.

Tosi, M.F., 2005. Innate immune responses to infection. J. Allergy Clin. Immunol. 116, 241-249.

Tsuchiya, T., Fukuda, S., Hamada, H., Nakamura, A., Kohama, Y., Ishikawa, H., Tsujikawa, K., Yamamoto, H., 15-11-2003. Role of gamma delta T cells in the inflammatory response of experimental colitis mice. J. Immunol. 171, 5507-5513.

Turner, J.E., Aldred, S., Witard, O.C., Drayson, M.T., Moss, P.M., Bosch, J.A., 2010. Latent cytomegalovirus infection amplifies CD8 T-lymphocyte mobilisation and egress in response to exercise. Brain Behav. Immun. 24, 1362-1370.

Turner, J.E., Campbell, J.P., Edwards, K.M., Howarth, L.J., Pawelec, G., Aldred, S., Moss, P., Drayson, M.T., Burns, V.E., Bosch, J.A., 2014. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr.) 36, 287-297.

Uezu, K., Kawakami, K., Miyagi, K., Kinjo, Y., Kinjo, T., Ishikawa, H., Saito, A., 15-6-2004. Accumulation of gammadelta T cells in the lungs and their regulatory roles in Th1 response and host defense against pulmonary infection with Cryptococcus neoformans. J. Immunol. 172, 7629-7634.

Umeda, T., Saito, K., Matsuzaka, M., Nakaji, S., Totsuka, M., Okumura, T., Tsukamoto, T., Yaegaki, M., Kudoh, U., Takahashi, I., 2008. Effects of a bout of traditional and original sumo training on neutrophil immune function in amateur university sumo wrestlers. Luminescence. 23, 115-120.

Umeda, T., Yamai, K., Takahashi, I., Kojima, A., Yamamoto, Y., Tanabe, M., Totsuka, M., Nakaji, S., Sugawara, N., Matsuzaka, M., 2008. The effects of a two-hour judo training session on the neutrophil immune functions in university judoists. Luminescence. 23, 49-53.

Unanue, E.R., 1984. Antigen-presenting function of the macrophage. Annu. Rev. Immunol. 2, 395-428.

Valenzuela, H.F. , Effros, R.B., 2002. Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus. Clin. Immunol. 105, 117-125.

van, B.D., Tsegaye, A., Miedema, F., Akbar, A., 15-2-2005. Significance of senescence for virus-specific memory T cell responses: rapid ageing during chronic stimulation of the immune system. Immunol. Lett. 97, 19-29.

Verde, T.J., Thomas, S.G., Moore, R.W., Shek, P., Shephard, R.J., 1992. Immune responses and increased training of the elite athlete. J. Appl. Physiol (1985.) 73, 1494-1499.

Vieira VJ, Valentine RJ, Wilund KR, Antao N, Baynard T and Woods JA., 2009. Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice. Am J Physiol Endocrinol Metab 296: E1164-E1171.

Vieira VJ, Valentine RJ, Wilund KR and Woods JA., 2009. Effects of diet and exercise on metabolic disturbances in high-fat diet-fed mice. Cytokine 46: 339-345.

von, Z.T., 2002. Oxidative stress shortens telomeres. Trends Biochem. Sci. 27, 339-344.

Wang, L., Kamath, A., Das, H., Li, L., Bukowski, J.F., 2001. Antibacterial effect of human V gamma 2V delta 2 T cells in vivo. J. Clin. Invest 108, 1349-1357.

Watts, C., 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu. Rev. Immunol. 15, 821-850.

Weng, N.P., Palmer, L.D., Levine, B.L., Lane, H.C., June, C.H., Hodes, R.J., 1997. Tales of tails: regulation of telomere length and telomerase activity during lymphocyte development, differentiation, activation, and aging. Immunol. Rev. 160, 43-54.

Wikby, A., Johansson, B., Olsson, J., Lofgren, S., Nilsson, B.O., Ferguson, F., 2002. Expansions of peripheral blood CD8 T-lymphocyte subpopulations and an association with cytomegalovirus seropositivity in the elderly: the Swedish NONA immune study. Exp. Gerontol. 37, 445-453.

Wolach, B., Eliakim, A., Gavrieli, R., Kodesh, E., Yarom, Y., Schlesinger, M., Falk, B., 1998. Aspects of leukocyte function and the complement system following aerobic exercise in young female gymnasts. Scand. J. Med. Sci. Sports 8, 91-97.

Woods, J., Lu, Q., Ceddia, M.A., Lowder, T., 2000. Special feature for the Olympics: effects of exercise on the immune system: exercise-induced modulation of macrophage function. Immunol. Cell Biol. 78, 545-553.

Woods, J.A., Ceddia, M.A., Kozak, C., Wolters, B.W., 1997. Effects of exercise on the macrophage MHC II response to inflammation. Int. J. Sports Med. 18, 483-488.

Woods, J.A., Davis, J.M., Kohut, M.L., Ghaffar, A., Mayer, E.P., Pate, R.R., 1994a. Effects of exercise on the immune response to cancer. Med. Sci. Sports Exerc. 26, 1109-1115.

Woods, J.A., Davis, J.M., Mayer, E.P., Ghaffar, A., Pate, R.R., 1993. Exercise increases inflammatory macrophage antitumor cytotoxicity. J. Appl. Physiol 75, 879-886.

Woods, J.A., Davis, J.M., Mayer, E.P., Ghaffar, A., Pate, R.R., 1994b. Effects of exercise on macrophage activation for antitumor cytotoxicity. J. Appl. Physiol 76, 2177-2185.

Woods, J.A., Davis, J.M., Smith, J.A., Nieman, D.C., 1999. Exercise and cellular innate immune function. Med. Sci. Sports Exerc. 31, 57-66.

Yan, J., Greer, J.M., Hull, R., O'Sullivan, J.D., Henderson, R.D., Read, S.J., McCombe, P.A., 2010. The effect of ageing on human lymphocyte subsets: comparison of males and females. Immun. Ageing 7, 4-

Ytting, H., Christensen, I.J., Thiel, S., Jensenius, J.C., Svendsen, M.N., Nielsen, L., Lottenburger, T., Nielsen, H.J., 2007. Biological variation in circulating levels of mannan-binding lectin (MBL) and MBL-associated serine protease-2 and the influence of age, gender and physical exercise. Scand. J. Immunol. 66, 458-464.

Zembron-Lacny, A., Slowinska-Lisowska, M., Ziemba, A., 2010. Integration of the thiol redox status with cytokine response to physical training in professional basketball players. Physiol Res. 59, 239-245.

CHAPTER 2

CHAPTER 2: Training status and sex influence on senescent T-lymphocyte redistribution in response to acute maximal exercise.

2.1 Abstract

PURPOSE: Investigate training status and sex effects on the redistribution of senescent and naïve T-lymphocytes following acute exercise. METHODS: Sixteen (8 male, 8 female) trained (18.3±1.7yrs) soccer players (Tr) and sixteen (8 male, 8 female) untrained (19.3±2.0yrs) controls (UTr) performed a treadmill running test to volitional exhaustion. Blood lymphocytes were isolated before (Pre), immediately post, and 1-hour post-exercise for assessment of cell surface expression of CD28 and CD57 on $CD4^+$ and $CD8^+$ T-lymphocyte subsets. Plasma was used to determine Cytomegalovirus (CMV) serostatus. RESULTS: Exercise elicited a redistribution of senescent $CD4^+(F_{(1,31)}=3.53,P<0.05)$, $CD8^+(F_{(1,31)}=13.4,P<0.05)$ and naïve $CD4^+(F_{(1,31)}=7.48,P<0.05)$, $CD8^+(F_{(2,83)}=18.94,P<0.05)$ T-lymphocytes. A main effect of training status was observed for senescent $CD4^+(F_{(1,15)}=5.66,P<0.05)$, $CD8^+(F_{(1,15)}=8.0,P<0.05)$ and naïve $CD8^+(F_{(1,15)}=4.31,P<0.05)$ T-lymphocytes: UTr had a higher proportion of senescent and a lower proportion of naïve $CD8^+$ T-lymphocytes than Tr. A main effect of sex was observed in senescent $CD4^+(F_{(1,15)}=10.20,P<0.05)$, $CD8^+(F_{(1,15)}=20.18,P<0.05)$ and naïve $CD4^+(F_{(1,15)}=7.56,P<0.05)$, $CD8^+(F_{(1,15)}=14.70,P<0.05)$ T-lymphocytes. Males had a higher proportion of senescent and lower proportion of naïve T-lymphocytes than females. A sex-by-training status interaction was observed for the senescent ($F_{(1,15)}=11.0,P<0.05$) and naïve ($F_{(1,15)}=8.83,P<0.05$) $CD4^+$ T-lymphocytes (but not $CD8^+$) with the highest percentage of senescent and lowest percentage of naïve T-lymphocytes observed in UTr males. CMV exerted a significant main covariate effect in the senescent ($F_{(1,15)}=17.76,P<0.05$) and naïve ($F_{(1,15)}=18.88,P<0.05$) $CD8^+$ T-lymphocytes but not in the senescent ($F_{(1,15)}=0.67,P>0.05$) and naïve ($F_{(1,15)}=1.65,P>0.05$) $CD4^+$ T-lymphocytes. CONCLUSION: This study highlights important sex and training status differences in the senescent and naïve T-lymphocyte redistribution in response to exercise that warrants further investigation.

Keywords: Immunosurveillance; $CD4^+$ T-lymphocytes; $CD8^+$ T-lymphocytes; Trained;

Untrained.

2.2 Introduction

T-lymphocytes that fail to divide in response to antigenic stimulation are considered senescent (Brenchley et al, 2003). Despite this loss of novel antigen defence, senescent T-lymphocytes retain immediate effector functions, such as recognition and killing of virally infected cells (Effros, 2004). Antigen-experienced $CD4^+$ and $CD8^+$ effector and memory T-lymphocytes with a senescent phenotype (Table 1) undergo preferential mobilisation into the peripheral blood circulation followed by a subsequent egress from the circulation in response to exercise (Campbell et al, 2008; Simpson et al, 2007; Simpson et al, 2008; Turner et al, 2010). This preferential influx of $CD4^+$ and cytotoxic senescent $CD8^+$ T-lymphocytes to the peripheral tissues with retained effector killing functions following exercise is considered important for immunosurveillance. A model proposed by Dhabhar et al (2012) suggests that in response to a stressor naïve T-lymphocytes traffic to lymph nodes where they come into contact with novel antigens. Concomitantly antigen-experienced effector and memory T-lymphocytes traffic to peripheral tissues like the skin, lung or mucosal lining of the gut where they encounter familiar antigens. In agreement with this model Bosch et al (2003) observed a selective mobilization of T-lymphocytes primed for inflammation in response to an acute stressor (Bosch et al, 2003).

The percentage of circulating T-lymphocytes with a senescent phenotype at rest is known to be influenced by body composition (Tchkonia et al, 2010), age (Tchkonia et al, 2010; Yan et al, 2010), maximal aerobic capacity (Spielmann et al, 2011), sex (Yan et al, 2010) and latent CMV infection (Pawelec et al, 2009; Turner et al, 2013) and infection (Mallia et al, 2014). It was recently demonstrated that one week of high-intensity exercise training resulted in a blunted mobilisation of effector memory $CD8^+$ T-lymphocytes compared with normal-intensity training in trained cyclists (Witard et al, 2012). Given that the trafficking pattern of lymphocytes is very sensitive to acute exercise training, characterising how training status,

as opposed to an acute bout of high-intensity exercise impacts exercise-induced changes in senescent T-lymphocyte redistribution warrants investigation. Given that senescent T-lymphocytes accumulate at rest in CMV^+ individuals (Pawelec et al, 2009; Turner et al, 2013), and the response of lymphocytes, particularly $CD8^+$ T-lymphocytes, to exercise is influenced by CMV infection history (Turner et al, 2010), it is important to consider CMV serostatus when investigating differences between groups for senescent T-lymphocyte responses to exercise. Although total lymphocyte counts at rest are similar between sexes (Giltay et al, 2000), the percentage of T cells is lower in males (Bouman et al, 2004). However, it remains unknown whether sex alone or in combination with training status influences the redistribution of senescent T-lymphocytes in young adults.

Thus, the primary aim of this study was to characterise the effect of training status and sex on the senescent T-lymphocyte response to an acute bout of intense exercise. In addition, we simultaneously characterised the redistribution of naïve T-lymphocytes into or out of the blood compartment. In accordance with Spielmann et al. (2010), we hypothesized that the redistribution of senescent T-lymphocytes into or out of the blood compartment would be blunted in trained individuals. Given that previous work reported a greater age-related increase in effector memory cells in males vs. females (Yan et al, 2010), we hypothesized that a higher proportion of senescent T-lymphocytes would be observed in males vs. females at rest and in response to exercise.

2.3 Methods

Participants

Sixteen well-trained soccer players (eight male [mean ± SD age: 17.8 ± 0.7 yr., height: 180.4 ± 5.3 cm, mass: 74.5 ± 6.0 kg, body fat: 10.7 ± 1.5 %] and eight female [age: 18.9 ± 2.3 yr., height: 166.0 ± 8.3 cm, mass: 61.8 ± 7.8 kg, body fat: 18.2 ± 3.4 %]) were compared to a group of 16 age-matched untrained controls (eight male [age: 19.4 ± 2.3 yr., height: 180.1 ± 4.2 cm, mass: 74.4 ± 11.7 kg, body fat: 10.7 ± 4.3 %] and eight female [age: 19.1 ± 1.8 yr., height: 169.0 ± 5.3 cm, mass: 59.9 ± 4.2 kg, body fat: 17.5 ± 3.8 %]). All participants were non-smokers who were not taking any medication and were free from infectious illness for 6 weeks. prior to the study. Each participant completed a pre-participation health screen questionnaire and provided their written informed consent. Ethical approval was granted by the University of Stirling Research Ethics Committee. To be included in the study, it was important that untrained active controls were not exercising any more than the UK Department of Health recommended healthy living guidelines of 2-3 hours per week (Department of Health, Reducing Obesity and Improving Diet, 2011).

Experimental protocol

Participants arrived at the laboratory in the morning (between 7:30-9:30am) after a >10h fast and after 24 h of rest and were instructed to rest supine on a treatment couch for approximately 5 min. Next, a cannula was inserted into a forearm vein connected with cannula extension tube (Becton Dickson, Oxford, UK). After returning to a seated position, a baseline 10mL blood sample was collected in a K_2EDTA blood collection tube (Becton Dickson, Oxford, UK) that was placed horizontal on a mixer at room temperature for later separation of cells.

Participants then completed an incremental exercise test to volitional exhaustion on a motorised treadmill (Powerjog G100). Starting speed was 10km/h for trained males and

8km/h for trained females, untrained males and untrained females. These starting speeds elicited similar relative exercise intensities for all participants in an attempt to match total exercise duration between groups. The incline of the treadmill was set at 1% to best simulate outdoor running conditions (Jones and Doust, 1997). Treadmill speed was increased by 1km/h at 3 min increments. Heart rate (HR) during the test was recorded using Polar RS200sd HR monitors (Finland). Rating of perceived exertion (RPE) also was recorded at the end of each increment using the category-ratio Borg (CR10) scale (Borg, 1982a; Borg, 1982b). Participants were verbally encouraged to run to volitional exhaustion. At exhaustion, participants straddled the running belt and treadmill speed was decreased to 3.5km/hr for a short recovery walk (2 min) whilst a second 10mL blood sample was collected. A final 10mL blood sample was collected 1-hour post-exercise. Participants were allowed to consume water ad libitum during the test.

Peripheral blood mononuclear cells (PBMC) isolation

The methods used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood have been described elsewhere (Simpson et al, 2006). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Lymphoprep®, Axis-Shield, Dundee, UK). Whole blood was diluted with an equal volume of 0.9% NaCl (B. Braun, Melsungen AG), and 6 ml of the diluted blood was layered over 3 ml of density-gradient media. Samples were then centrifuged for 30 min at 2000rpm at 20°C. Following centrifugation, the distinct band formed by the PBMCs was carefully removed and washed twice for 10 min at 1100rpm at 20°C, firstly with 0.9% NaCl then with RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK). Staining the cells with trypan blue revealed ~98% viability for all samples.

The isolated PBMCs were then stored in 70% (700µl) foetal bovine serum (Sigma-Aldrich, Ltd, UK), 20% (200µl) RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK) and 10% (100µl)

DMSO (Sigma-Aldrich, Ltd, UK) that was added to the samples on ice. Samples were snap frozen in liquid nitrogen and stored until later analysis. Plasma was also obtained from whole blood centrifugation for later analysis of latent CMV infection serostatus.

Labelling of cell-surface antigens

Overlapping phenotypes exist for senescent T-lymphocyte identification as shown in Table 1. Hence, prior studies have used several different markers for characterisation of the same distinct T-lymphocyte subsets. In this study we used the $CD57^{+}$ $CD28^{-}$ phenotype to identify senescent T-lymphocytes and the $CD57^{-}$ $CD28^{+}$ phenotype to identify naïve T-lymphocytes.

Table 1: Surface phenotypic identification of T-lymphocyte subsets.

T-lymphocyte subset	Surface phenotype	Reference
Naïve T-lymphocytes	$CD57^-/CD28^+$	(Appay et al, 2002; Koch
	$KLRG1^-/CD28^+$	et al, 2007)
Senescent T-	$KLRG1^+/CD27^-$	(Voehringer et al, 2002)
lymphocytes[a]	$KLRG1^+/CD28^-$	(Brenchley et al, 2003)
	$CD57^+/CD28^-$	(Appay et al, 2002;
		Appay et al, 2008),
Memory T-lymphocytes	$CD45RA^+/CD28^+$	(Ibegbu et al, 2005)
	$KLRG1^+/CD57^-/CD28^+$	
Central memory T-	$CD45RA^-/CD27^-$	(Koch et al, 2007)
lymphocytes	$KLRG1^+/CD57^-/CD28^+$	(Romero et al, 2007)
Early effector-memory T-lymphocytes	$KLRG1^+/CD57^-/CD28^+$	(Koch et al, 2007)
Effector-memory T-	$CD45RO^+/CD27^-$	(Appay et al, 2002)
lymphocytes	$CD45RA^-/CD27^-$	(Romero et al, 2007)
'Reverent' effector	$CD45RA^+/CD28^-$	(Appay et al, 2002)
memory T-lymphocytes	$CD45RA^+/CD27^-$	(Romero et al, 2007)
(EMRA)		

[a] Phenotype $KLRG1^+/CD28^-$ has been used to identify senescent T-lymphocytes. However under certain circumstances proliferative capabilities may be restored (Akbar and Henson, 2011). Therefore in the present study $CD57^+/CD28^-$ was chosen to identify senescent T-lymphocytes.

PMBC's were labelled with an APC conjugated anti-CD3 (Clone SK7) or anti-CD4 (Clone SK3) monoclonal antibody (mAb), a PerCP Cy-5.5 conjugated anti-CD8 (Clone RPA-T8) mAb, a PE conjugated anti-CD28 (Clone CD28.2) mAb and an FITC conjugated anti-CD3 (Clone MEM-57) or anti-CD57 (Clone NK-1) mAb in preparation for a four-colour direct immunofluorescence procedure. Cells were incubated with 50µl of each pre-diluted mAb for 1-hour at room temperature, protected from the light. The anti-CD28 mAb was purchased

from BD Pharmingen (San Jose, CA, USA). The anti-CD3, anti-CD4 and anti-CD8 mAbs were purchased from Ebioscience (San Diego, CA, USA) and the anti-CD57 mAb from Abcam (Cambridge, UK). Cells also were labelled with each mAb in a one-color immunofluorescence procedure to account for spectral overlap and adjust compensation settings during flow cytometry analysis. Appropriately conjugated isotype controls also were used in each assay to account for background binding of immunoglobulin (Ig). After incubation the cell and mAb mixture was vortexed to resuspend the cells in solution before analysis using flow cytometry.

Flow cytometry

Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA), equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter mode using Accuri C6 (CFlow ® v1) software. Side scatter was used to identify and gate the $CD3^+$ cells. The $CD4^+$ or $CD8^+$ populations were then identified in the $CD3^+$ cell population. For each sample, 40,000 $CD3^+/CD4^+$ or $CD3^+/CD8^+$ events were collected for analysis. The expression of CD57 and CD28 was assessed on the $CD4^+$ and $CD8^+$ T-lymphocytes by four colour flow cytometry. The percentage of all $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T-lymphocyte subsets expressing the markers of interest was tabulated for statistical analysis.

CMV serostatus

CMV serostatus was defined for all participants. Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) and a SpectromaxM2 plate reader (Molecular Devices, CA, USA).

Data presentation and statistical analysis

All data are presented as means ± standard deviation (SD), unless otherwise stated. Data were analysed using Mini-tab v-16 software. Physical characteristics and exercise capacity were compared between groups by independent sample t-tests. Lymphocyte data were analysed using a 3-factor ANCOVA with cell type as the dependent variable and time, training status and gender as independent factors with CMV serostatus representing the covariate factor. Post-hoc analysis used the Tukey test and 95% confidence interval. Main effects and interactions were accepted as statistically significant at the $P < 0.05$ level.

2.4 Results

No differences were observed between trained (n=16) and untrained (n=16) participants within each gender group for any physical characteristics. Body fat percentage was significantly greater in females compared to males. Females were also shorter and had a lower body mass than males. Training load was approximately four-fold greater in trained (mean for both males and females: 10.5 ± 1.5 hr/wk) vs. untrained groups (mean for both males and females: 2.5 ± 0.9 h/wk, $P < 0.05$, Table 2). Total exercise duration was unmatched between groups. Exercise test duration was significantly shorter in untrained females (15.8 ± 4.5 min) compared with all other groups (Table 2). However, no difference was observed for the max RPE, max heart rate or final lactate concentration obtained at volitional exhaustion between groups.

Table 2: Number, training volume, exercise capacity, maximal rating of perceived exertion (RPE), max heart rate and final lactate concentration of the trained and untrained male and female participants

	Trained		**Untrained**	
Parameter	Male	Female	Male	Female
Participants (n)	8	8	8	8
Training volume (hr/wk)	12.0 ± 0.0	9.0 ± 0.0	3.3 ± 0.8*	2.0 ± 0.5*
Exercise capacity test duration (min)	22.9 ± 1.6	21.8 ± 3.8	21.4 ± 4.4	15.8 ± 4.5*
Max RPE (0-10 scale)	9.1 ± 0.8	8.4 ± 1.9	8.8 ± 1.7	8.6 ± 1.8
Max HR (beats/min)	200 ± 8	191 ± 11	190 ± 15	194 ± 9
Lactate (mmol/L^{-1})	9.2 ± 1.7	8.7 ± 3.0	7.8 ± 2.9	9.6 ± 0.7

Values are means ± standard deviation. * Indicates significant difference vs. trained ($P<0.05$) in corresponding gender groups

Senescent $CD4^+$ and $CD8^+$ T-lymphocyte redistribution in response to acute exercise

A significant main effect of exercise was detected for both senescent $CD4^+$ ($P < 0.05$) and $CD8^+$ ($P < 0.05$) T-lymphocyte subsets. The increase ($P < 0.05$) in percentage of senescent $CD8^+$ T-lymphocytes immediately post exercise subsequently decreased ($P < 0.05$) 1-hour post exercise (Figure 1A). A similar, albeit less pronounced, pattern was observed for the senescent $CD4^+$ T-lymphocyte subset, although the increased proportion with exercise did not reach statistical significance ($P > 0.05$). However, the reduction from post-exercise to the 1-hour post exercise period was significant ($P < 0.05$) (Figure 1A).

Naïve $CD4^+$ and $CD8^+$ T-lymphocyte redistribution in response to acute exercise

A significant main effect of exercise was detected for both naïve $CD4^+$ ($P < 0.05$), and $CD8^+$ ($P < 0.05$), T-lymphocyte subsets. The significant decrease ($P < 0.05$) in the percentage of naïve $CD8^+$ T-lymphocytes immediately post exercise was followed by a significant increase ($P < 0.05$) 1-hour post exercise (Figure 1B). A similar, albeit less pronounced, response was observed for the naïve $CD4^+$ T-lymphocyte subset, although the decreased proportion with exercise was trending towards significant ($P < 0.05$). However, the increase from post-exercise to the 1-hour post exercise period was significant ($P < 0.05$) (Figure 1B).

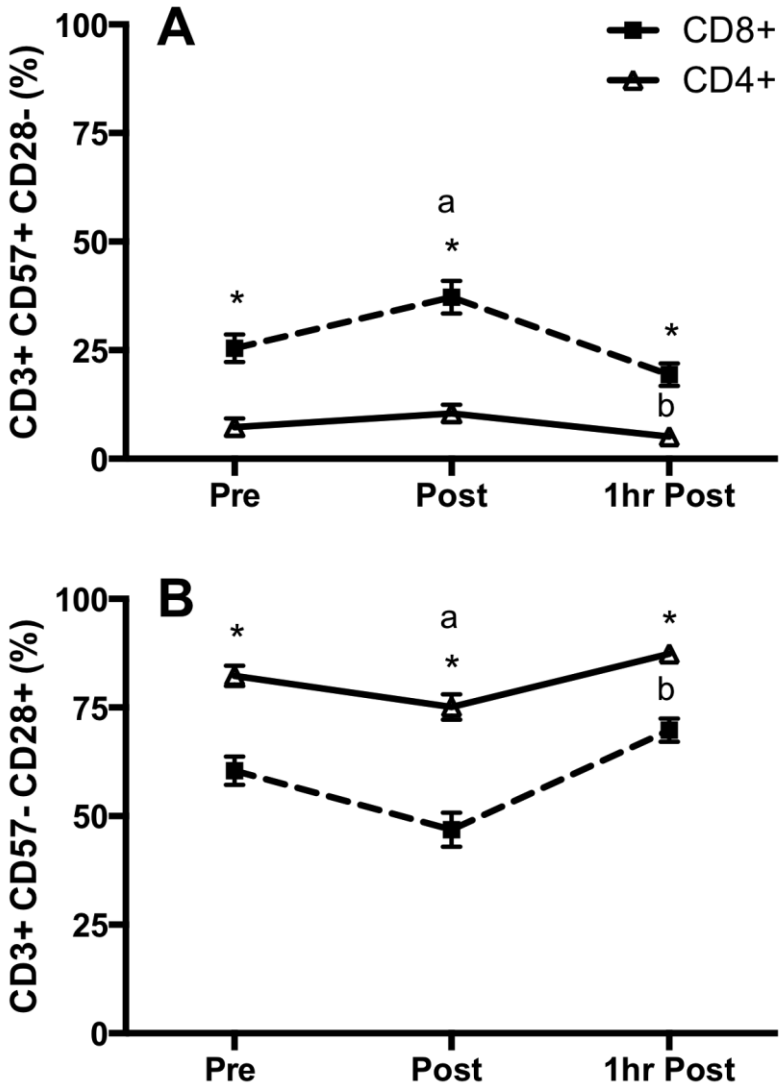

Fig 1 A,B. Values are mean ± SEM per cent of $CD8^+$ and $CD4^+$ T-lymphocyte subsets expressing a senescent phenotype ($CD3^+CD57^+CD28^-$) or a naïve phenotype ($CD3^+CD57^-CD28^+$) at pre, immediately post and 1-hour post exercise. Results are displayed for participants grouped together (n=32). a-indicates significant difference from pre and 1-hour post (P<0.05). b-indicates a significant difference from post (P<0.05).* indicates significant difference between subsets (P<0.05).

Effect of training status on senescent and naïve CD4$^+$ and CD8$^+$ T-lymphocyte redistribution in response to acute exercise

There was a main effect of training status on the senescent CD4$^+$ ($P < 0.05$) and CD8$^+$ ($P < 0.05$) T-lymphocyte subsets, whereby overall, the proportion of senescent T-lymphocytes was higher in UTr vs. Tr (Figure 2). There was a main effect of training status on the naïve CD8$^+$ ($P < 0.05$) T-lymphocyte subset but not the naïve CD4$^+$ T-lymphocyte subset ($P > 0.05$). Overall, the proportion of naïve CD8$^+$ T-lymphocytes was lower in the UTr vs. Tr. No exercise × training status interaction ($P > 0.05$) was observed.

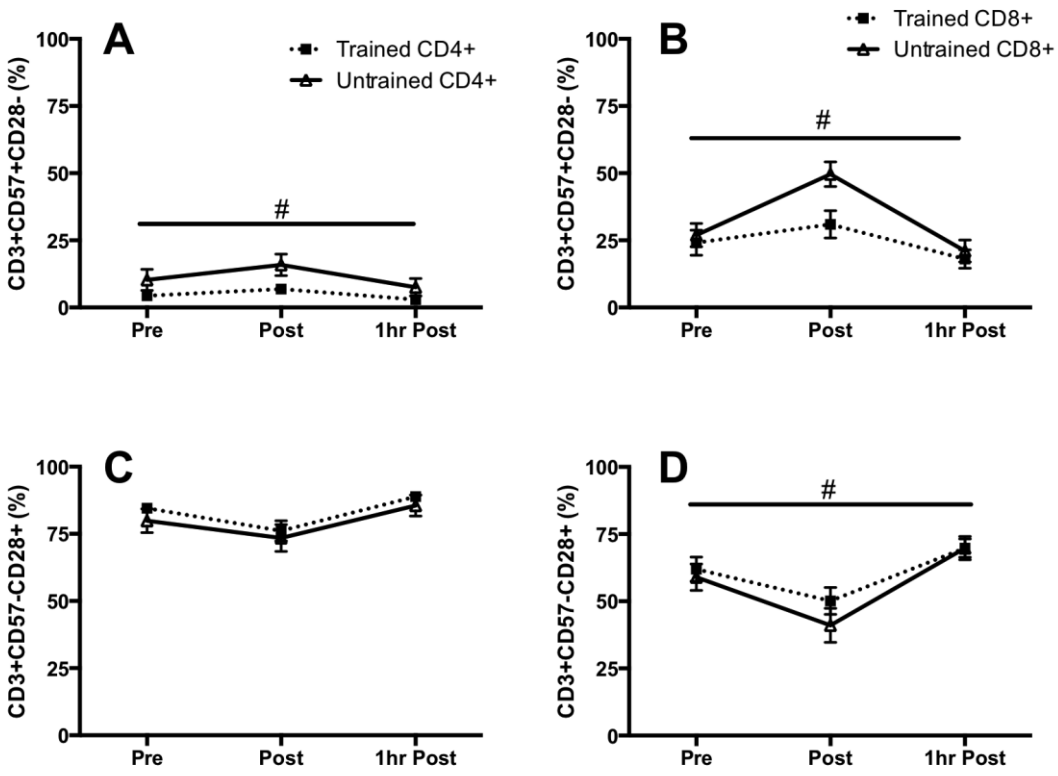

Fig 2 A,B,C,D. Effect of training status on the redistribution of CD4$^+$ and CD8$^+$ T-lymphocyte subsets in response to exercise.
Values are mean ± SEM per cent of CD4$^+$ (A,C) and CD8$^+$ (B,D) T-lymphocyte subsets expressing a senescent phenotype (A,B) (CD3$^+$CD57$^+$CD28$^-$) or a naïve phenotype (C,D) (CD3$^+$CD57$^-$CD28$^+$) at pre, immediately post- and 1-hour post exercise. Results are displayed for trained males and females combined (n=16) and untrained males and females combined (n=16). # indicates significant main effect of training status (P<0.05).

Effect of sex on senescent and naïve $CD4^+$ and $CD8^+$ T-lymphocyte redistribution in response to acute exercise

There was a main effect of sex on the senescent $CD4^+$ ($P < 0.05$) and $CD8^+$ ($P < 0.05$), T-lymphocyte subsets, whereby the overall proportion of senescent T-lymphocytes was higher in males vs. females (Figure 3).

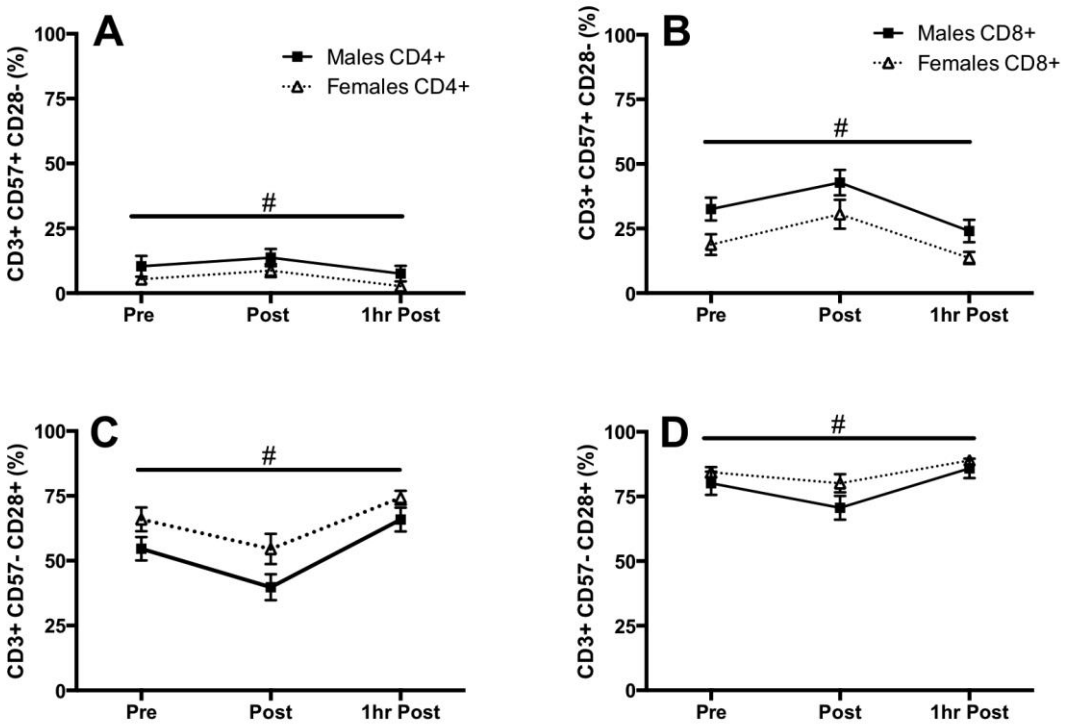

Fig 3 A, B, C, D. Values are mean ± SEM per cent of $CD4^+$ (A,C) and $CD8^+$ (B,D) T-lymphocyte subsets expressing a senescent phenotype (A,B) ($CD3^+CD57^+CD28^-$) or a naïve phenotype (C,D) ($CD3^+CD57^-CD28^+$) at pre, immediately post and 1-hour post exercise. Results are displayed for males grouped together (n=16) and females grouped together (n=16). # indicates significant main effect of gender ($P<0.05$).

A significant sex × training status interaction was observed on the senescent $CD4^+$ ($P < 0.05$) whereby the proportion of senescent $CD4^+$ T-lymphocytes was significantly higher in the UTr males compared to all other groups (Table 3). No sex × training status interaction was observed on the senescent $CD8^+$ T-lymphocyte subset ($P > 0.05$).

There was a main effect of sex on the naïve $CD4^+$ ($P < 0.05$) and $CD8^+$ ($P < 0.05$) T-lymphocyte subsets, whereby the overall proportion of naïve T-lymphocytes was lower in the males vs. females (Figure 3). A significant sex × training status interaction was observed on the naïve $CD4^+$ ($P < 0.05$) T-lymphocyte subset, whereby the proportion of naïve $CD4^+$ T-lymphocytes was lower in the UTr males compared to all other groups. In females, no difference in senescent and naïve T-lymphocyte subsets was observed between the T and UTr groups (Table 3).

Table 3: Percentage of the total $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-lymphocyte population expressing the $CD57^+/CD28^-$ senescent and $CD57^-/CD28^+$ naïve phenotype in response to the maximal exercise protocol

	Phenotype	T-lymphocyte Subset	Pre Exercise	Post Exercise	1-hour Post Exercise
UTr Males	Senescent	$CD4^+$	17.7 ± 23.5#	26.0 ± 18.2#	12.6 ± 17.6#
		$CD8^+$	33.9 ± 15.5	53.7 ± 14.2**	25.5 ± 19.2**
	Naïve	$CD4^+$	73.4 ± 24.6#	63.2 ± 22.2#	81.1 ± 20.5#
		$CD8^+$	51.2 ± 16.1	32.2 ± 16.6	64.3 ± 20.2†
UTr Females	Senescent	$CD4^+$	5.1 ± 3.5	4.9 ± 1.7	2.3 ± 2.2*
		$CD8^+$	20.9 ± 17.3	34.1 ± 27.2	13.0 ± 10.6
	Naïve	$CD4^+$	85.6 ± 5.4	86.3 ± 3.0	90.8 ± 4.6
		$CD8^+$	65.7 ± 20.9	52.2 ± 32.9	76.2 ± 12.1
Tr Males	Senescent	$CD4^+$	5.2 ± 2.5	8.2 ± 4.8	4.1 ± 3.2*
		$CD8^+$	31.4 ±19.7	35.9 ± 19.5	22.7 ± 16*
	Naïve	$CD4^+$	85.9 ± 3.7	75.2 ± 14.1	90.2 ± 4.3†
		$CD8^+$	57.7 ± 19.0	44.6 ± 20.4	67.2 ± 16.5†
Tr Females	Senescent	$CD4^+$	5.8 ± 6.0	10.3 ± 11.7	3.3 ± 2.9
		$CD8^+$	16.8 ±15.9	26.0 ± 21.2*	12.7 ± 9.1
	Naïve	$CD4^+$	83.1 ± 10.2	77.0 ± 16.6	87.4 ± 6.0
		$CD8^+$	66.3 ± 17.3	55.7 ± 19.7	72.7 ± 9.9

Values are percentage means ± SD of the total $CD3^+$ T-lymphocyte population that express the $CD57^+CD28^-$ phenotype. Statistically significant difference from pre-exercise values indicated by *P<0.05, **P<0.01. Statistically significant difference from post-exercise values indicated by $^\dagger P$<0.05. # indicates a significant sex x training status interaction (P<0.01). The values do not add up to 100% because we have only included the senescent (CD28-CD57+) and naïve (CD28+CD57-) phenotypes. If the intermediate phenotypes (CD28-CD57-) and (CD28+CD57+) were included the values would add up to 100%.

CMV serostatus status

Twenty-five per cent of the untrained group was defined as positive for CMV. Fifty-six per cent of the trained group was defined as positive for CMV. CMV serostatus was evenly distributed between the sexes, forty-four per cent of the female and thirty-eight per cent of the male group was defined as positive for CMV. Covariate analysis revealed CMV to be a

significant covariate for both senescent ($P < 0.05$) and naïve ($P < 0.05$) $CD8^+$ T-lymphocyte subset redistribution, but not for senescent ($P > 0.05$) or naïve ($P > 0.05$) $CD4^+$ T-lymphocyte redistribution.

2.5 Discussion

This study investigated the influence of training status and sex on the redistribution of senescent and naïve $CD4^+$ and $CD8^+$T-lymphocytes in response to an acute bout of exhaustive treadmill exercise. We observed that the redistribution of senescent $CD4^+$ and $CD8^+$T-lymphocytes was lower in the trained compared with untrained group. Conversely, the redistribution of naïve $CD8^+$T-lymphocytes was higher in the trained compared to the untrained group. In addition, irrespective of training status, the redistribution of senescent $CD4^+$ and $CD8^+$ T-lymphocytes was lower in females compared with males. Naïve $CD4^+$ and $CD8^+$ T-lymphocyte redistribution mirrored the senescent response. Untrained males, in particular, appeared to have a higher proportion of senescent, and a concomitant lower proportion of naïve $CD4^+$T-lymphocytes at rest and in response to exercise. In the senescent and naïve $CD8^+$ T-lymphocyte subsets, CMV serostatus was a significant covariate indicating that a latent CMV infection influences the effect of training status and sex on the redistribution of senescent and naïve $CD8^+$ T-lymphocytes. However, this observation must be interpreted with caution due to the small number of CMV positive subjects in the sub-groups, in particular the 25% in the untrained group is equivalent to four of sixteen subjects.

We have demonstrated that in males training status influences the proportion of senescent T-lymphocytes at rest and redistribution of senescent T-lymphocytes in response to acute exercise. A previous study demonstrated that, at rest, participants with a high maximal aerobic capacity exhibited more naïve $CD8^+$ T-lymphocytes and less senescent $CD4^+$ and $CD8^+$ T-lymphocytes, compared to a group with a lower maximal aerobic capacity (Spielmann et al, 2011). However, maximal aerobic capacity is not an accurate measure of training status and resting blood measurements do not indicate how senescent or naïve T-lymphocytes respond to exercise. Accordingly, the present study observed lower senescent $CD4^+$ and $CD8^+$ T-lymphocytes, and higher naïve $CD8^+$ T-lymphocytes at rest and in

response to exercise in trained compared to untrained participants. Latent CMV infection status does not appear to impact the senescent or naïve $CD4^+$ T-lymphocyte response to exercise. This is not unexpected since it has been previously reported that $CD4^+$ T-lymphocyte responses to exercise are not influenced by CMV serostatus (Turner et al, 2010). It is also well known that the $CD8^+$ T-lymphocyte subset is more responsive to exercise than the $CD4^+$ subset (Anane et al, 2009; Campbell et al, 2009; Simpson et al, 2007; Simpson et al, 2008). Thus, it would appear that exercise training for competitive soccer reduces the redistribution of senescent $CD4^+$ and $CD8^+$ T-lymphocytes and increases the redistribution of naïve $CD8^+$ T-lymphocytes in response to exercise, independent of CMV serostatus.

Our observation of a blunted senescent T-lymphocyte response, together with an increased naïve T-lymphocyte response to exercise in trained compared to untrained, lend support to the theory that regular exercise training mobilizes senescent T-lymphocytes and thus frees up 'immune space' for naïve T-lymphocytes (Simpson, 2011). In response to exercise, senescent T-lymphocytes are mobilized and subsequently egress to the peripheral tissues where they experience a proapoptotic environment (Kruger et al, 2008). Simpson et al (2011) propose a negative feedback loop that controls the peripheral T-lymphocyte numbers. Hence, after senescent T-lymphocytes undergo apoptosis, peripheral T-lymphocyte numbers are low. The subsequent feedback loop increases the output of naïve T-lymphocytes from the thymus and thus restores the peripheral T-lymphocyte pool. If the feedback loop were to be apparent, it would occur more frequently in trained participants, who undergo regular bouts of exercise. While this is not a widely accepted theory and further investigation is warranted, our data do provide support for this theory.

Another novel observation in this study was that males exhibited a greater proportion of senescent $CD4^+$ and $CD8^+$ T-lymphocytes at rest and in response to exercise than females.

Conversely males exhibited a lower proportion of naïve $CD4^+$ and $CD8^+$ T-lymphocytes at rest and in response to exercise. Exercise duration for the trained females was the same as the trained males and untrained males. Furthermore, absolute exercise intensity was the same for trained females and untrained females and groups were similar in age. Hence, differences in the proportion of senescent and naïve $CD4^+$ and $CD8^+$ cells at rest and in response to exercise observed herein do not appear to be attributed to age differences.

Alternatively, body composition may be a salient factor to explain the sex-differences in senescent and naïve T lymphocytes. Indeed, a higher body fat percentage has been associated with an increased proportion of senescent T-lymphocytes (Tchkonia et al, 2010). However, in the present study the opposite association was observed whereby males exhibited greater proportions of senescent T-lymphocytes (both $CD4^+$ and $CD8^+$) and a lower body fat percentage. Hence, these data suggest that body composition is likely only an important factor in the redistribution of senescent T-lymphocytes within, rather than between sexes. Therefore, sex-differences in the redistribution of senescent and naïve $CD4^+$ and $CD8^+$ T-lymphocytes must be attributed to another, yet to be determined, factor, possibly contributing to the sex- specific differences observed in autoimmune and inflammatory diseases like rheumatoid arthritis (Tengstrand et al, 2004), asthma, cystic fibrosis and chronic obstructive pulmonary disease (Tam et al, 2011).

Speculatively, the sex-difference in T-lymphocyte redistribution could be explained by hormones, namely oestrogen concentrations. T-lymphocytes are known to express oestrogen receptors and the $CD8^+$ T-lymphocyte subset binds oestrogen with high affinity (Cutolo et al, 1995). Indeed, cell culture studies reveal that in the presence of 17 B-estradiol (E2), the ratio of activated $CD4^+/CD8^+$ human T-lymphocytes is decreased (Athreya et al, 1993). Moreover, the promoter region of interferon gamma (IFN-y) appears to be positively

modulated by oestrogen (Betz and Fox, 1991). In turn, IFN-y is produced by senescent T-lymphocytes and has been reported to up-regulate the enzyme telomerase in lymphoid cell lines (Xu et al, 2000). Hormones change cyclically with the menstrual cycle (Oertelt-Prigione, 2012). Reductions in CD4+ T-lymphocyte number have been observed in the luteal compared to early follicular phase of the cycle (Lee et al, 2010). It is unknown what influence cycling hormones have on senescent T-lymphocytes. Interestingly, we observed no difference in the redistribution of senescent T-lymphocytes in response to exercise between trained and untrained females, whereas a difference was observed between trained and untrained males. This observation suggests a sex-specific effect of training status on the redistribution of senescent T-lymphocytes and supports the notion that oestrogen may provide a preventative mechanism against T-lymphocyte telomere shortening in females that possibly contributed to the lower proportion of senescent $CD4^+$ and $CD8^+$ T-lymphocytes at rest and in response to exercise. However, without assessment of menstrual cycle phase or hormonal responses, this supposition cannot be confirmed.

Stress hormones influence the differential redistribution of T-lymphocytes (Landmann, 1992). Epinephrine and norepinephrine mobilise T-lymphocytes via activation of B adrenergic receptors located on the cell surface of T-lymphocytes (Dhabhar et al, 2012). Due to the descriptive nature of this study design, no measurements of stress hormone (i.e., epinephrine and cortisol) concentrations in blood were collected. Previous work has demonstrated a smaller epinephrine response to submaximal exercise in trained compared to untrained individuals (Hong et al, 2005; Kjaer et al, 1985; Vinten and Galbo, 1983). A lower epinephrine response in trained would be expected to be associated with a lower redistribution of senescent cells into the blood. In the present study, it is worth highlighting that one group (untrained females) ran for a significantly shorter duration than the other groups. However, despite a shorter running duration, all groups exercised to volitional exhaustion and there was no difference between groups for maximum heart rate and final

lactate concentration. At maximal exercise intensity, we would expect trained participants to have a higher epinephrine response (Kjaer et al, 1985). Thus, the present data imply, albeit indirectly, that training status may override the impact of stress hormones on the redistribution of senescent cells in response to exercise.

To conclude, the redistribution of senescent and naïve T-lymphocytes is influenced by training status and sex. The sex-differences in lymphocyte redistribution cannot be fully explained by our dataset. Differences between the sexes observed in the incidence of various autoimmune and inflammatory diseases supports the lymphocyte redistribution sex-differences we observed. However, we speculate that training status does not influence the redistribution of senescent T-lymphocytes in females due to inherent sex-differences in oestrogen concentration.

2.6 References

Akbar, A.N. , Henson, S.M., 2011. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? Nat. Rev. Immunol. 11, 289-295.

Anane, L.H., Edwards, K.M., Burns, V.E., Drayson, M.T., Riddell, N.E., van Zanten, J.J., Wallace, G.R., Mills, P.J., Bosch, J.A., 2009. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. Brain Behav. Immun. 23, 823-829.

Appay, V., Dunbar, P.R., Callan, M., Klenerman, P., Gillespie, G.M., Papagno, L., Ogg, G.S., King, A., Lechner, F., Spina, C.A., Little, S., Havlir, D.V., Richman, D.D., Gruener, N., Pape, G., Waters, A., Easterbrook, P., Salio, M., Cerundolo, V., McMichael, A.J., Rowland-Jones, S.L., 2002c. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat. Med. 8, 379-385.

Appay, V., van Lier, R.A., Sallusto, F., Roederer, M., 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A 73, 975-983.

Athreya, B.H., Pletcher, J., Zulian, F., Weiner, D.B., Williams, W.V., 1993. Subset-specific effects of sex hormones and pituitary gonadotropins on human lymphocyte proliferation in vitro. Clin. Immunol. Immunopathol. 66, 201-211.

Betz, M. , Fox, B.S., 1-1-1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J. Immunol. 146, 108-113.

Borg, G., 1982a. Ratings of perceived exertion and heart rates during short-term cycle exercise and their use in a new cycling strength test. Int. J. Sports Med. 3, 153-158.

Borg, G.A., 1982b. Psychophysical bases of perceived exertion. Med. Sci. Sports Exerc. 14, 377-381.

Bosch, J.A., Berntson, G.G., Cacioppo, J.T., Dhabhar, F.S., Marucha, P.T., 2003. Acute stress evokes selective mobilization of T cells that differ in chemokine receptor expression: a potential pathway linking immunologic reactivity to cardiovascular disease. Brain Behav. Immun. 17, 251-259.

Bouman, A., Schipper, M., Heineman, M.J., Faas, M.M., 2004. Gender difference in the non-specific and specific immune response in humans. Am. J. Reprod. Immunol. 52, 19-26.

Brenchley, J.M., Karandikar, N.J., Betts, M.R., Ambrozak, D.R., Hill, B.J., Crotty, L.E., Casazza, J.P., Kuruppu, J., Migueles, S.A., Connors, M., Roederer, M., Douek, D.C., Koup, R.A., 1-4-2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 101, 2711-2720.

Campbell, J.P., Guy, K., Cosgrove, C., Florida-James, G.D., Simpson, R.J., 2008. Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human peripheral blood following an acute bout of high-intensity exercise. Brain Behav. Immun. 22, 375-380.

Campbell, J.P., Riddell, N.E., Burns, V.E., Turner, M., van Zanten, J.J., Drayson, M.T., Bosch, J.A., 2009. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. Brain Behav. Immun. 23, 767-775.

Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., Masi, A.T., 1995. Estrogens, the immune response and autoimmunity. Clin. Exp. Rheumatol. 13, 217-226.

Effros, R.B., 2004. Replicative senescence of CD8 T cells: potential effects on cancer immune surveillance and immunotherapy. Cancer Immunol. Immunother. 53, 925-933.

Giltay, E.J., Fonk, J.C., von Blomberg, B.M., Drexhage, H.A., Schalkwijk, C., Gooren, L.J., 2000. In vivo effects of sex steroids on lymphocyte responsiveness and immunoglobulin levels in humans. J. Clin. Endocrinol. Metab 85, 1648-1657.

Hong, S., Johnson, T.A., Farag, N.H., Guy, H.J., Matthews, S.C., Ziegler, M.G., Mills, P.J., 2005. Attenuation of T-lymphocyte demargination and adhesion molecule expression in response to moderate exercise in physically fit individuals. J. Appl. Physiol 98, 1057-1063.

Ibegbu, C.C., Xu, Y.X., Harris, W., Maggio, D., Miller, J.D., Kourtis, A.P., 15-5-2005. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. J. Immunol. 174, 6088-6094.

Jones, A.M. , Doust, J.H., 1997. The Conconi test in not valid for estimation of the lactate turnpoint in runners. J. Sports Sci. 15, 385-394.

Kjaer, M., Christensen, N.J., Sonne, B., Richter, E.A., Galbo, H., 1985a. Effect of exercise on epinephrine turnover in trained and untrained male subjects. J. Appl. Physiol 59, 1061-1067.

Koch, S., Larbi, A., Ozcelik, D., Solana, R., Gouttefangeas, C., Attig, S., Wikby, A., Strindhall, J., Franceschi, C., Pawelec, G., 2007. Cytomegalovirus infection: a driving force in human T cell immunosenescence. Ann. N. Y. Acad. Sci. 1114, 23-35.

Kruger, K., Lechtermann, A., Fobker, M., Volker, K., Mooren, F.C., 2008. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. Brain Behav. Immun. 22, 324-338.

Landmann, R., 1992. Beta-adrenergic receptors in human leukocyte subpopulations. Eur. J. Clin. Invest 22 Suppl 1, 30-36.

Lee, S., Kim, J., Jang, B., Hur, S., Jung, U., Kil, K., Na, B., Lee, M., Choi, Y., Fukui, A., Gilman-Sachs, A., Kwak-Kim, J.Y., 1-7-2010. Fluctuation of peripheral blood T, B, and NK cells during a menstrual cycle of normal healthy women. J. Immunol. 185, 756-762.

Mallia, P., Message, S.D., Contoli, M., Gray, K., Telcain, A., Laza-Stanca, V., Papi, A., Stanciu, L.A., Elkin, S., Kon, O.M., Johnson, M., Johnstona, S.L., 2014. Lymphocyte subsets in experimental rhinovirus infection in chronic obstructive pulmonary disease. Respir Med. 108(1): 78-85.

Oertelt-Prigione, S., 2012. Immunology and the menstrual cycle. Autoimmun. Rev. 11, A486-A492.

Pawelec, G., Derhovanessian, E., Larbi, A., Strindhall, J., Wikby, A., 2009. Cytomegalovirus and human immunosenescence. Rev. Med. Virol. 19, 47-56.

Romero, P., Zippelius, A., Kurth, I., Pittet, M.J., Touvrey, C., Iancu, E.M., Corthesy, P., Devevre, E., Speiser, D.E., Rufer, N., 1-4-2007. Four functionally distinct populations of human effector-memory CD8+ T lymphocytes. J. Immunol. 178, 4112-4119.

Simpson, R.J., 2011. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc. Sport Sci. Rev. 39, 23-33.

Simpson, R.J., Cosgrove, C., Ingram, L.A., Florida-James, G.D., Whyte, G.P., Pircher, H., Guy, K., 2008. Senescent T-lymphocytes are mobilised into the peripheral blood compartment in young and older humans after exhaustive exercise. Brain Behav. Immun. 22, 544-551.

Simpson, R.J., Florida-James, G.D., Cosgrove, C., Whyte, G.P., Macrae, S., Pircher, H., Guy, K., 2007. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. J. Appl. Physiol 103, 396-401.

Simpson, R.J., Florida-James, G.D., Whyte, G.P., Guy, K., 2006. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur. J. Appl. Physiol 97, 109-121.

Spielmann, G., McFarlin, B.K., O'Connor, D.P., Smith, P.J., Pircher, H., Simpson, R.J., 2011. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav. Immun. 25, 1521-1529.

Tam, A., Morrish, D., Wadsworth, S., Dorscheid, D., Man, S.F., Sin, D.D., 2011. The role of female hormones on lung function in chronic lung diseases. BMC. Womens Health 11, 24-

Tchkonia, T., Morbeck, D.E., Von, Z.T., Van, D.J., Lustgarten, J., Scrable, H., Khosla, S., Jensen, M.D., Kirkland, J.L., 2010. Fat tissue, aging, and cellular senescence. Aging Cell 9, 667-684.

Tengstrand, B., Ahlmen, M., Hafstrom, I., 2004. The influence of sex on rheumatoid arthritis: a prospective study of onset and outcome after 2 years. J. Rheumatol. 31, 214-222.

Turner, J.E., Aldred, S., Witard, O.C., Drayson, M.T., Moss, P.M., Bosch, J.A., 2010. Latent cytomegalovirus infection amplifies CD8 T-lymphocyte mobilisation and egress in response to exercise. Brain Behav. Immun. 24, 1362-1370.

Turner, J.E., Campbell, J.P., Edwards, K.M., Howarth, L.J., Pawelec, G., Aldred, S., Moss, P., Drayson, M.T., Burns, V.E., Bosch, J.A., 12-7-2013. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr.)

Vinten, J. , Galbo, H., 1983. Effect of physical training on transport and metabolism of glucose in adipocytes. Am. J. Physiol 244, E129-E134.

Voehringer, D., Koschella, M., Pircher, H., 15-11-2002. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). Blood 100, 3698-3702.

Witard, O.C., Turner, J.E., Jackman, S.R., Tipton, K.D., Jeukendrup, A.E., Kies, A.K., Bosch, J.A., 2012. High-intensity training reduces CD8+ T-cell redistribution in response to exercise. Med. Sci. Sports Exerc. 44, 1689-1697.

Xu, D., Erickson, S., Szeps, M., Gruber, A., Sangfelt, O., Einhorn, S., Pisa, P., Grander, D., 15-12-2000. Interferon alpha down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. Blood 96, 4313-4318.

Yan, J., Greer, J.M., Hull, R., O'Sullivan, J.D., Henderson, R.D., Read, S.J., McCombe, P.A., 2010. The effect of ageing on human lymphocyte subsets: comparison of males and females. Immun. Ageing 7, 4-

CHAPTER 3

CHAPTER 3: The response of T-lymphocyte populations to a period of increased volume of training in trained females vs habitual activity in female controls.

3.1 Abstract

PURPOSE: To investigate the T-lymphocyte response to a period of increased volume training in trained females compared to a period of habitual activity in female controls METHODS: Thirteen trained female (19.8 ± 1.9 yrs) soccer players (Tr) were monitored during a 2 week long intensified training period and thirteen female untrained (20.5 ± 2.2 yrs) controls (UTr) were monitored during 2 weeks of habitual activity. Training/habitual activity and diet was monitored during the 2 week period. Blood lymphocytes were isolated before and after the 2 week period for assessment of cell surface expression of CD28 and CD57 on $CD4^+$ and $CD8^+$ T-lymphocytes and $\gamma\delta^+$ T-lymphocyte subsets. Plasma was used to determine Cytomegalovirus (CMV) serostatus. RESULTS: Training was increased by 39% in the Tr group during the intensified training period and the Tr did significantly more physical activity than the UTr. The total energy, carbohydrate and protein intake was greater in the Tr compared to the UTr. The number of $CD3^+$ T-lymphocytes was greater in the Tr compared to the UTr ($P<0.05$). No significant main effects ($P>0.05$) were observed for the proportion of senescent ($CD28^-$ $CD57^+$) or naïve ($CD28^+$ $CD57^-$) $CD8^+$ or $CD4^+$ T-lymphocytes between the Tr and the UTr. The proportion of $CD4^+$T-lymphocytes was greater in the UTr compared to the Tr ($P<0.05$). The proportion of $\gamma\delta^+$ T-lymphocytes was greater in the Tr compared to the UTr ($P<0.05$). The $CD4^+$:$CD8^+$ ratio was greater in the UTr compared to the Tr ($P<0.05$). 8% of the UTr were defined as positive for CMV. 23% of the Tr were defined as positive for CMV. Covariate analysis revealed CMV to be a significant covariate in the analysis of $CD8^+$ ($P<0.05$), $CD28^+$ $CD8^+$ ($P<0.05$) and naïve $CD8^+$ ($P<0.05$) T-lymphocyte cell numbers. However, CMV was not a significant covariate in the analysis of T-lymphocyte proportions. CONCLUSION: The increased volume training period had no effect on T-lymphocyte populations in Tr females, and T-lymphocyte populations also did not change with 2 weeks of habitual exercise in UTr. Differences in the proportions of $\gamma\delta^+$, $CD4^+$ and the ratio of $CD4^+$:$CD8^+$ T-lymphocytes observed between the Tr and UTr warrants further investigation.

Keywords: $CD4^+$ T-lymphocytes; $CD8^+$ T-lymphocytes; Trained; Untrained; Diet; CMV infection; $\gamma\delta^+$ T-lymphocytes.

3.2 Introduction

Senescent T-lymphocytes are mitotically older T-lymphocytes that have undergone multiple rounds of division, and thus fail to respond to antigenic stimulation (Brenchley et al, 2003). However, senescent T-lymphocytes remain effective killers of virally infected cells (Effros, 2004). It has recently been observed that seven days of intensified overload training results in a blunted mobilisation of effector memory $CD8^+$ T-lymphocytes in trained males (Witard et al, 2012). However, intensified training had no effect on resting T-lymphocytes in a trained population during the cross-over design (Witard et al, 2012). This study by Witard et al had no untrained group for comparison, but it seems clear that an increase in volume and intensity of training modulated the immune response to exercise in trained males when fed a carbohydrate restricted diet (Witard et al, 2012).

Further to this, we have recently shown that the redistribution of senescent T-lymphocytes in response to exercise is influenced by sex and training status (Brown et al, 2013). At rest and in response to exercise the proportion of senescent T-lymphocytes was greater in untrained males compared to trained males. However, there were no differences at rest or in response to exercise between trained and untrained females. As well as a specific sex effect another potential explanation for this discrepancy in observations between sexes is the greater difference in weekly training volume between trained and untrained males (12 hrs/wk vs.3 hrs/wk, respectively) compared to the difference between trained and untrained females (9hrs/wk vs. 2hrs/wk, respectively) in our previous work. The higher training volume in the trained males could account for the lower senescent T-lymphocytes at rest compared with untrained males. Regular mobilisation and egress of these cells to a proapoptotic environment (Kruger et al, 2008) in response to exercise could drive the resting proportions down in a trained population. Therefore, if training volume is an explanation we would expect to see lower senescent T-lymphocyte proportions in a trained group after a period of

intensified training. An influence of training status has been recently corroborated by Teixeira and colleagues who observed a greater proportion of effector memory T-lymphocytes in an untrained population compared to trained swimmers (Teixeira et al, 2014). Therefore, to confirm whether there is a training volume effect on senescent T-lymphocyte proportions it seems prudent to compare trained females who have undergone a period of intensified training (increased volume and intensity) with a control group of untrained females.

To fully identify any training status difference, or explain any lack thereof, it is also of interest to examine other exercise responsive cells with similar properties to senescent and effector memory T-lymphocytes, for example gamma delta ($\gamma\delta^+$) T-lymphocytes. $\gamma\delta^+$ T-lymphocytes have high cytotoxic capabilities, tissue homing potential, and a capacity for rapid innate-like target recognition. They are also preferentially mobilised following acute exercise, psychological stress and β-agonist infusion (Anane et al, 2009), indicating that they provide protection in situations where tissue damage and antigen exposure are more likely to occur (Anane et al, 2009; Anane et al, 2010). Investigating senescent T-lymphocytes alongside $\gamma\delta^+$ T-lymphocytes in trained and untrained females will hopefully provide a greater understanding of previously observed training status differences in immune response.

The aim of this study was to examine the effects of 14-days of increased volume training on the composition of the blood T-lymphocyte pool in highly trained soccer players. We hypothesized that the training period would result in marked reductions in the proportions of senescent blood T-lymphocytes in soccer players with no change in controls.

3.3 Methods

Subjects

A group of 13 well trained female soccer players [mean ± SD age: 19.8 ± 1.9 yr., height: 162.7 ± 5.7 cm, mass: 60.9 ± 8.6 kg, body fat: 18.2 ± 3.4 %] were monitored before and after a two week long intensified training camp and compared to 13 age matched untrained females [age: 20.5 ± 2.2 yr., height: 169.0 ± 6.5 cm, mass: 64.8 ± 6.7 kg, body fat: 25.1 ± 4.1 %] monitored during habitual living for two weeks. All subjects were non-smokers who were not taking any medication and were free from infectious illness for 6 wk. prior to the study. Each participant completed a pre-participation health screen questionnaire and provided their written informed consent. Ethical approval was granted by the University of Stirling Research Ethics Committee. To be included in the study it was important that the untrained group were not exercising any more than the recommended healthy living guidelines of 2-3 hours per week.

Experimental protocol

All participants visited the laboratory on two separate occasions. The first visit was before the training camp for the trained females, or before the two weeks of monitored activity for the untrained females. This visit consisted of the completion of a pre-screening questionnaire followed by measurements of height, body mass and body fat percentage (using bioelectrical impedance analysis) and a 10ml fasted blood sample. The second visit consisted of a second 10ml blood sample obtained within two days after the training camp for the trained or immediately after the two weeks of recorded activity for the untrained. On both visits the participants arrived at the laboratory in the morning (between 7:30-9:30am) after a >10h fast and after 24 h of rest and were instructed to rest supine on a treatment couch for approximately 5 min before a baseline 10mL blood sample was collected from a forearm vein. The blood sample was collected into a K_2EDTA blood collection tube (Becton

Dickson, Oxford, UK) and placed horizontally on a mixer at room temperature for later separation of cells.

Training camp monitoring

Heart rate (HR) data was used to monitor intensity and volume of training during every session throughout the training camp. This was completed using the Activio team sport HR system (Activio AB, Sweden). This system enabled simultaneous monitoring of HR data for multiple players during each session of the training camp. Twenty sessions in total were monitored including field based training (12 sessions), pool based recovery (2 sessions), competitive matches (2 matches) and strength sessions (4 sessions). Not all players participated in the full seventy minutes of match-play in each match situation and thus HR data for any thirty-five minute half spent on the pitch was used for the analysis of results. Maximum HR was assessed prior to the training camp. Subjects wore a numbered HR belt around their chest (directly contacting the skin) for the duration of each session. Data was recorded with a 5-s sample rate throughout the session, and included any time spent warming up and cooling down. Reports were then created on an individual basis at a later stage once the HR values associated with each individuals training zones had been determined.

Determination of Intensity zones and training load

Within the two week period after the training camp (to allow for recovery), each trained participant completed an incremental exercise test to volitional exhaustion on a motorised treadmill (Powerjog G100). Starting speed was 8km/h. The incline was set at 1% to best simulate outdoor running conditions (Jones and Doust, 1997). Treadmill speed was increased by 1km/h at 3 min intervals. At the end of each 3 min increment blood lactate was

measured. Heart rate (HR) during the test was recorded using Polar RS200sd HR monitors (Finland). Rating of perceived exertion (RPE) also was recorded at the end of each increment using the category-ratio Borg (CR10) scale (Borg, 1982a; Borg, 1982b). Participants were verbally encouraged to run to volitional exhaustion. At exhaustion, participants straddled the running belt and treadmill speed was decreased to 3.5km/hr for a short recovery walk (2 mins). Participants were allowed to consume water ad libitum during the test.

By plotting blood lactate against work intensity, a specific lactate curve was determined and lactate threshold (LT1) and lactate turn-point (LT2) markers estimated for each subject using a 3 part linear regression. Through knowledge of lactate markers and use of the Activio computer software package, the HR values associated with LT1 and LT2 were entered for each specific subject enabling HR data to be separated into time spent in Z1, Z2 and Z3 for each session. The percentage of time spent in each intensity zone for every subject was then calculated for all training sessions during the camp. Time spent in each intensity zone was then converted into a training impulse (TRIMP) value using the calculation.

Total TRIMP= (Frequency * Duration)* Intensity

Intensity was given a number to signify intensity zone, 1 for low intensity (Z1), 2 for moderate intensity (Z2) and 3 for vigorous intensity (Z3).

Determination of physical activity intensity and activity load during habitual exercise period

Physical activity in the untrained females was monitored during two weeks of habitual activity using the long form, self-administered version of the international physical activity questionnaire (IPAQ) detailed elsewhere (Craig et al, 2003). The IPAQ long form evaluates habitual physical activity performed in everyday life in the four domains, namely leisure, work, transport and domestic/gardening. Participants were asked to report frequency and

duration of vigorous, moderate and low intensity activities lasting for more than 10 minutes. To allow for comparison with the trained activity levels, the untrained leisure time activity was broken down into intensity zones and allocated a corresponding number. 1 for low, 2 for moderate intensity and 3 for vigorous intensity. TRIMP values were then calculated using the same calculation.

Dietary analysis

During the training camp the players recorded food intake for four days, two training days, one match day and one recovery day, using portable electronic scales (CS series, Pine Brook, NJ, USA). During the two weeks of monitored activity the untrained females recorded food intake for three days, two week days and one weekend day using the same type of portable electronic scales. Energy, macronutrient, and micronutrient intake was then analysed using Microdiet software (Salford, UK).

Peripheral blood mononuclear cells (PBMC) isolation

The methods used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood have been described elsewhere (Simpson et al, 2006). The isolated PBMCs were then stored in 70% (700µl) foetal bovine serum (Sigma-Aldrich, Ltd, UK), 20% (200µl) RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK) and 10% (100µl) DMSO (Sigma-Aldrich, Ltd, UK) that was added to the samples on ice. Samples were snap frozen in liquid nitrogen and stored until later analysis. Plasma was also obtained from whole blood centrifugation for later analysis of latent CMV infection serostatus.

Labelling of cell-surface antigens

Overlapping phenotypes exist for senescent T-lymphocyte identification as shown in Chapter 2. Hence, prior studies have used several different markers for characterisation of the same distinct T-lymphocyte subsets. In this study we used the $CD57^{+}$ $CD28^{-}$ phenotype to identify senescent T-lymphocytes and the $CD57^{-}CD28^{+}$ phenotype to identify naïve T-lymphocytes.

PMBC's were labelled with an APC conjugated anti-CD3 (Clone SK7) or anti-CD4 (Clone SK3) monoclonal antibody (mAb), a PerCP Cy-5.5 conjugated anti-CD8 (Clone RPA-T8) mAb, a PE conjugated anti-CD28 (Clone CD28.2) or anti-γδ (Clone B1.1) mAb and a FITC conjugated anti-CD3 (Clone MEM-57) or anti-CD57 (Clone NK-1) mAb in preparation for a four-colour direct immunofluorescence procedure. Cells were incubated with 50µl of each pre-diluted mAb for 1-hour at room temperature, protected from the light. The anti-CD28 mAb was purchased from BD Pharmingen (San Jose, CA, USA). The anti-CD3, anti-CD4, anti-γδ and anti-CD8 mAbs were purchased from Ebioscience (San Diego, CA, USA) and the anti-CD57 mAb from Abcam (Cambridge, UK). Cells also were labelled with each mAb in a one-color immunofluorescence procedure to account for spectral overlap and adjust compensation settings during flow cytometry analysis. Appropriately conjugated isotype controls also were used in each assay to account for background binding of immunoglobulin (Ig). After incubation the cell and mAb mixture was vortexed to resuspend the cells in solution before analysis using flow cytometry.

Flow cytometry

Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA), equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter mode using Accuri C6 (CFlow ® v1) software. Side scatter was used to identify and gate the $CD3^+$ cells. The $CD4^+$, $CD8^+$ and $\gamma\delta^+$ populations were then identified in the $CD3^+$ cell population. For each sample, 40,000 $CD3^+/CD4^+$, $CD3^+/CD8^+$ or $CD3^+/\gamma\delta^+$ events were collected for analysis. The expression of CD57 and CD28 was assessed on the $CD4^+$ and $CD8^+$ T-lymphocytes by four colour flow cytometry. The percentage of all $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T-lymphocyte subsets expressing the markers of interest was tabulated for statistical analysis.

CMV serostatus

CMV serostatus was defined for all participants. Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) and a SpectromaxM2 plate reader (Molecular Devices, CA, USA).

Data presentation and statistical analysis

All data are presented as means ± standard deviation (SD), unless otherwise stated. Physical characteristics and exercise capacity were compared between groups by independent sample t-tests. Lymphocyte data were analysed using a 3 factor ANCOVA with cell type as the dependent variable and sample time and training status as independent factors. CMV serostatus was the covariate. Post-hoc analysis used the Tukey test and 95%

confidence. Main effects and interactions were accepted as statistically significant at the $p<0.05$ level. Data were analysed using Mini-tab v-16 software.

3.4 Results

Physical characteristics

The trained and untrained groups were age matched and there was no difference in body mass ($P>0.05$), but the untrained were taller 169.0 ± 6.5cm than the trained 162.7 ± 5.7cm ($P<0.05$), and had significantly higher body fat percentage than the trained (25.1 ± 4.1% compared to 18.2 ± 3.4%; $P<0.001$).

Physical activity monitoring

Prior to the intensified training camp participants in the trained group routinely trained for 9 hr/wk. During the study training volume was increased to 12 hr/wk. Over the duration of the study the time spent in low, moderate and vigorous physical activity, measured in minutes and converted to total TRIMP score, differed between groups (Table 1). Total training time as well as time spent in low and vigorous activity was higher in the trained compared to the untrained ($P<0.05$). However, time in moderate intensity activity was not significantly different between the trained and untrained ($P>0.05$). When converted to total TRIMP scores the trained total TRIMP score was significantly greater ($P<0.05$) than the untrained.

Table 1: Physical activity volume and intensity recorded during 2 weeks of habitual activity (untrained group) or 2 weeks of increased volume training (trained group). Values are mean ± SD

	Untrained	Trained	P-value
Total (hrs/wk)	3.6 ± 5.8	12.5 ± 3.1	<0.05
Low (min)	131 ± 274	618 ± 74	<0.01
Moderate (min)	87 ± 74	96 ± 80	>0.05
Vigorous (min)	0 ± 0	36 ± 31	<0.05
Total TRIMPS	306 ± 303	918 ± 325	<0.01

Dietary analysis

Energy intake

The overall mean energy intake in the trained group for the recorded days was 3077 ± 574 Kcal/day. The untrained group overall mean energy intake was 1741 ± 483 Kcal/day and was significantly lower than the trained (P<0.05, Table 2).

Table 2: Dietary intake Table 2: Values are means ± SD of macronutrient intake per day or percent of total energy intake in trained during the increased volume training period and untrained during the period of activity monitoring.

	Trained	% Energy intake	Untrained	% Energy intake	P-value
Energy intake (Kcal/day)	3077 ± 716		1741 ± 603		<0.01
Carbohydrate (g/day)	547 ± 169	67	261 ± 107	57	<0.01
Carbohydrate (g/kg/day)	9.3 ± 3.3		4.1 ± 1.8		<0.01
Protein (g/day)	104 ± 25	13	67 ± 24	16	<0.01
Protein (g/kg/day)	1.8 ± 0.5		1.0 ± 0.4		<0.05
Fat (g/day)	67 ± 27	20	52 ± 26	27	>0.05

Macro-nutrients

The overall mean carbohydrate (CHO) intake for the recorded days in the trained group was 9.3 ± 2.9 $g \cdot kg^{-1} \cdot day^{-1}$. The untrained group overall mean CHO intake was significantly lower at 4.1 ± 1.5 $g \cdot kg^{-1} \cdot day^{-1}$ ($P<0.05$).

The trained overall mean protein intake was 1.8 ± 0.5 $g \cdot kg^{-1} \cdot day^{-1}$. The untrained overall mean protein intake was significantly less at 1.0 ± 0.4 $g \cdot kg^{-1} \cdot day^{-1}$ ($P<0.05$). Overall there was no significant difference in total fat intake between the trained (67 ± 20 g/day) and the untrained (52 ± 18 g/day) ($P>0.05$). When the constituent fats are examined, saturated (SFA) fat intake was not significantly different between trained 35% (24 ± 8g/day) and untrained 37% (19 ± 7g/day) ($P>0.05$). However, the trained consumed significantly more monounsaturated (MUFA) fats (35% (24 ± 8g) vs 30% (16 ± 6g)) and polyunsaturated (PUFA) fats (30% (20 ± 8) vs 16% (8 ± 6g)) than the untrained ($P<0.05$). SFA, MUFA and PUFA data may be underestimated due to incomplete data in the McCance and Widdowson's reference database for all foods. As a result the total fat reported may not match the breakdown of SFA, MUFA and PUFA reported.

Micro-nutrients

The trained and untrained showed no significant difference in intake of the micro-nutrients examined: iron (22 ± 8mg and 17 ± 18), calcium (985 ± 221mg and 874 ± 364) and vitamin D (4 ± 3µg and 4 ± 7µg) intake ($P>0.05$). However, the trained consumed more vitamin C than the untrained (1269 ± 693 mg vs. 371 ± 544 mg) during the dietary analysis period, respectively.

$CD3^+$ T-lymphocytes

All $CD3^+$ T-lymphocyte population cell counts are presented in Table 3. A significant main effect of training status was observed in the $CD3^+$ population. The number of $CD3^+$ T-lymphocytes was greater in the trained compared to the untrained ($P<0.05$). Time was not a significant main effect ($P>0.05$), therefore trained had more $CD3^+$ T-lymphocytes than untrained irrespective of the intensified training camp intervention. No interaction between training status and time was observed ($P>0.05$).

Table 3: T-lymphocyte counts pre and post camp. Values are means ± SD count in x10^6/L of the T-lymphocyte populations in the trained at pre and post period of intensified training compared to the untrained at pre and post period of activity monitoring.

		Trained		Untrained	
Phenotype	**T-lymphocyte Subset**	**Pre**	**Post**	**Pre**	**Post**
$CD3^+$	T-lymphocytes	1610.5 ± 484.7	1569.5 ± 340.5	1314.0 ± 272.4*	1473.1 ± 230.2*
$CD3^+CD8^+$	Cytotoxic	538.8 ± 193.6	567.5 ± 188.3	411.6 ± 119.1*	472.2 ± 151.4*
$CD8^+CD28^+$ $CD57^-$	Naïve	346.4 ± 145.9	380.7 ± 114.4	265.8 ± 95.3*	305.4 ± 86.1*
$CD8^+CD28^+$ $CD57^+$	Intermediate	26.3 ± 17.0	22.3 ± 6.3	18.5 ± 9.9*	17.6 ± 7.6*
$CD8^+CD28^-$ $CD57^-$	Intermediate	95.9 ± 70.8	90.1 ± 81.7	67.9 ± 20.5	78.6 ± 30.4
$CD8^+CD28^-$ $CD57^+$	Senescent	70.2 ± 46.4	74.5 ± 73.4	59.4 ± 46.5	70.6 ± 69.1
$CD3^+CD4^+$	Helper	924.7 ± 332.3	855.2 ± 189.5	820.0 ± 201.1	905.9 ± 193.8
$CD4^+CD28^+$ $CD57^-$	Naïve	841.0 ± 325.7	773.9 ± 176.8	748.1 ± 202.4	821.7 ± 201.2
$CD4^+CD28^+$ $CD57^+$	Intermediate	26.2 ± 14.1	25.3 ± 7.1	23.8 ± 8.6	26.6 ± 11.5
$CD4^+CD28^-$ $CD57^-$	Intermediate	38.0 ± 23.4	32.1 ± 28.5	32.0 ± 9.7	34.6 ± 15.7
$CD4^+CD28^-$ $CD57^+$	Senescent	15.5 ± 11.8	15.2 ± 14.6	16.2 ± 9.4	23.1 ± 18.7
$CD3^+\gamma\delta^+$	Gamma-delta	153.4 ± 64.2$^{\$}$	155.0 ± 36.3$^{\$}$	99.3 ± 30.4*	111.3 ± 42.2*

* indicates a significant main effect of training status, total (pre and post) is significantly different from the trained total (pre and post). $^{\$}$ indicates a significant difference from the untrained at pre (P<0.05).

Senescent and naïve T-lymphocytes

No significant main effect of time (P>0.05), training status (P>0.05) or any time-by-training status interactions (P>0.05) were observed for the proportion of senescent ($CD28^{-}$ $CD57^{+}$) or naïve ($CD28^{+}$ $CD57^{-}$) $CD8^{+}$ or $CD4^{+}$ T-lymphocytes (Figure 1 and 2).

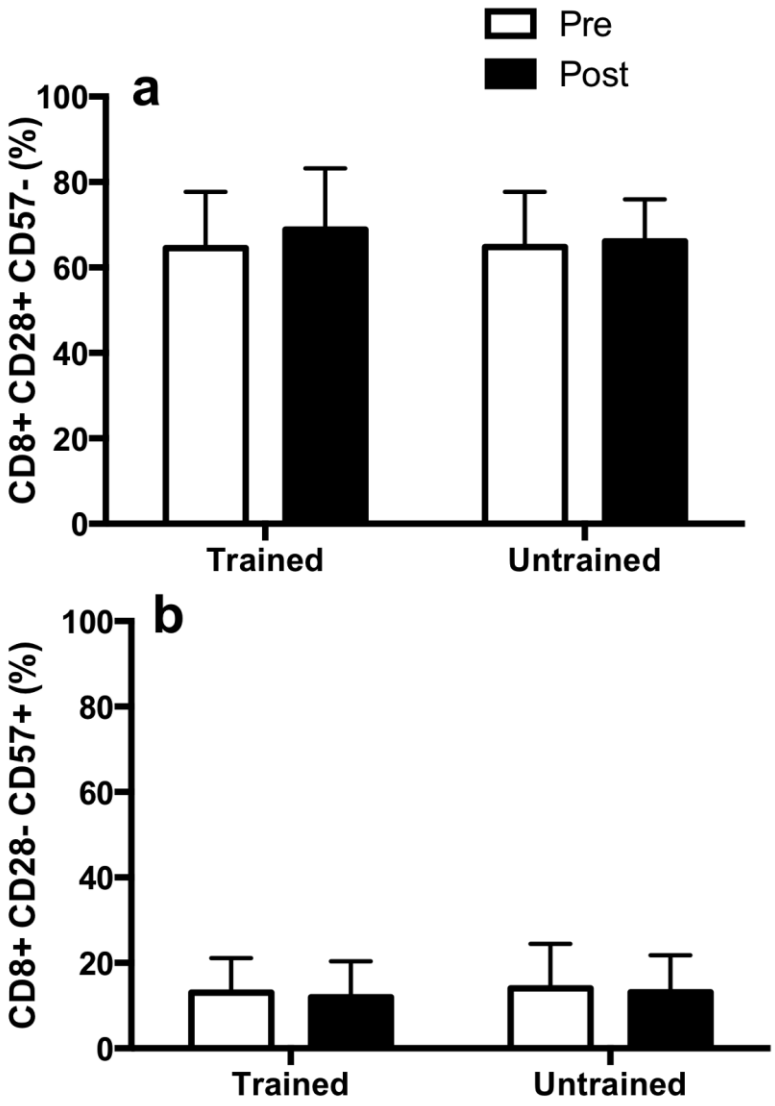

Fig 1. Values are means ± SD per cent of $CD8^{+}$ T-lymphocyte subset expressing a naïve phenotype (a) ($CD8^{+}CD28^{+}CD57^{-}$) and a senescent (b) phenotype ($CD8^{+}CD28^{-}CD57^{+}$) in the trained group pre and post the period of intensified training and in the untrained group at pre and post period of activity monitoring.

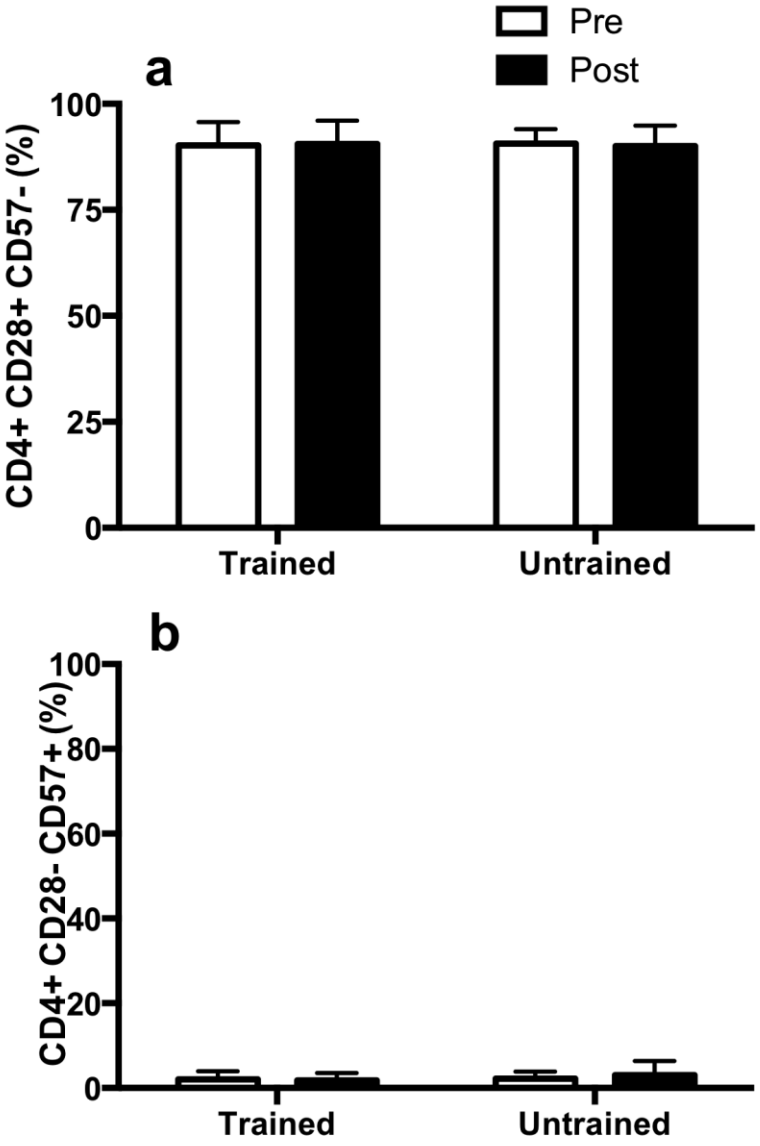

Fig 2. Values are means ± SD per cent of $CD4^+$ T-lymphocyte subset expressing a naïve phenotype (a) ($CD8^+CD28^+CD57^-$) and a senescent (b) phenotype ($CD8^+CD28^-CD57^+$) in the trained group pre and post the period of intensified training and in the untrained group at pre and post period of activity monitoring.

$CD4^+$ T-lymphocytes

A significant main effect of training status was observed on the proportion of $CD4^+$T-lymphocytes to total lymphocytes. The proportion of $CD4^+$T-lymphocytes was greater in the untrained compared to the trained ($P<0.05$). The mean trained was 56.5% and the mean untrained was 61.2% with a mean difference of 4.7% (-8.7<-4.7<-0.7). However, time was not a main effect ($P>0.05$) and no time-by-training status interaction ($P>0.05$) was observed on the proportion of $CD4^+$T-lymphocytes (Figure 3).

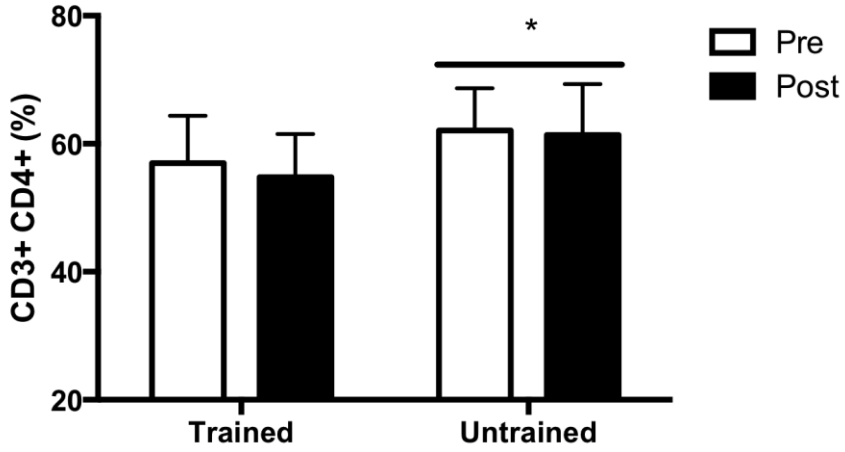

Fig 3. Values are means ± SD per cent of T-lymphocytes that are $CD4^+$ in the trained group pre and post the period of intensified training and in the untrained group at pre and post period of activity monitoring. * indicates a main effect of training status ($P<0.05$). Total untrained (pre and post) is greater than the total (pre and post) trained.

$\gamma\delta^+$ T-lymphocytes

A significant main effect of training status was observed on the proportion of $\gamma\delta^+$ T-lymphocytes. The proportion of $\gamma\delta^+$ T-lymphocytes was greater in the trained compared to the untrained ($P<0.05$). The mean trained was 10.2% and the mean untrained was 7.9% with a mean difference of 2.3% (0.1<2.3<4.5). However, time was not a main effect ($P>0.05$) and there was also no time-by-training status interaction ($P>0.05$) observed on the $\gamma\delta^+$ T-lymphocytes (Figure 4).

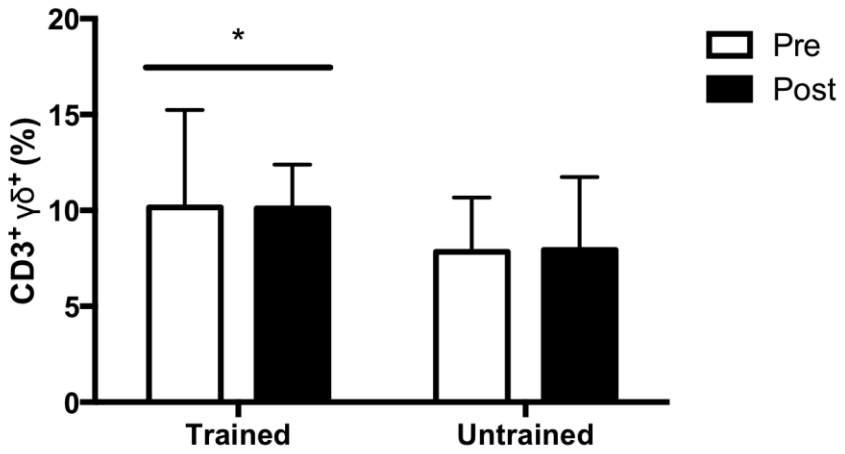

Fig 4. Values are means ± SD per cent of T-lymphocytes that are $\gamma\delta^+$ in the trained group pre and post the period of intensified training and in the untrained group at pre and post period of activity monitoring. * indicates a main effect of training status ($P<0.05$). Total trained (pre and post) is greater than the total (pre and post) untrained.

$CD4^+$:$CD8^+$ ratio

A significant main effect of training status was observed on the ratio of % $CD4^+$T-lymphocytes to % $CD8^+$ T-lymphocytes ($CD4^+$:$CD8^+$). The $CD4^+$:$CD8^+$ ratio was greater in the untrained compared to the trained ($P<0.05$). Time was not a main effect ($P>0.05$). No time-by-training status interaction ($P>0.05$) was observed on the $CD4^+$:$CD8^+$ (Figure 5).

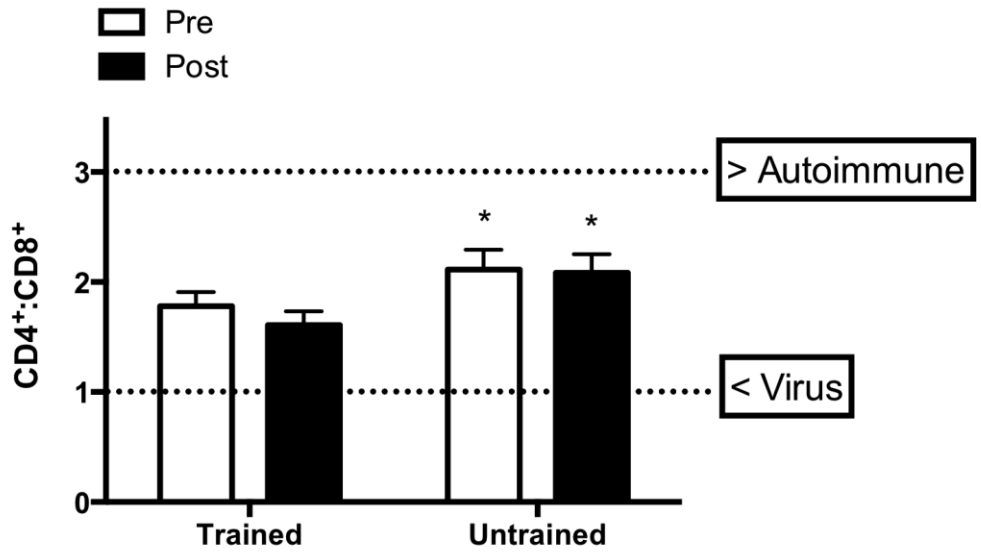

Fig 5. Values are the mean ± SD ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ in the trained at pre and post period of intensified training compared to the untrained at pre and post period of activity monitoring. * indicates a significant main effect of training status ($P<0.05$) the total untrained (pre and post) is greater than the total trained (pre and post). A ratio of > 3 occurs in autoimmune diseases (Pender et al, 2011; Salman and Al-Rubeaan, 2009). A ratio of <1 occurs in viral infection (e.g HIV) (Zijenah et al, 2005).

CMV serostatus

Eight per cent of the untrained group was defined as positive for CMV. Twenty-three per cent of the trained group was defined as positive for CMV. Covariate analysis revealed CMV to be a significant covariate in the analysis of $CD8^+$ ($P<0.05$), $CD28^+$ $CD8^+$ ($P<0.05$) and naïve $CD8^+$ ($P<0.05$) T-lymphocyte cell numbers. Participants with CMV positive serostatus had increased numbers of $CD8^+$, $CD28^+$ $CD8^+$ and naïve $CD8^+$ T-lymphocytes. However, CMV was not a significant covariate in the analysis of T-lymphocyte proportions.

3.5 Discussion

This study investigated resting T-lymphocyte populations in a trained female group before and after a period of increased training volume compared to an untrained female group before and after a period of habitual physical activity. We observed that physical activity levels were 3-fold greater in the trained group compared to the untrained group for the duration of the study. The training volume in the trained group was increased by 39% from the normal weekly training volume during the study. The increase in training volume succeeded in matching that of trained males in our previously reported study (Brown et al, 2013) in which we observed a greater proportion of senescent T-lymphocytes at rest in untrained males compared to trained males. The present study highlights that a two week period of increased training volume has no effect on resting proportions of senescent T-lymphocytes in trained females. However, we did observe a lower proportion of $CD4^+$ T-lymphocytes at rest in the trained group compared to the untrained group. Additionally, a higher proportion of $\gamma\delta^+$ T-lymphocytes was observed at rest in the trained group compared to the untrained group.

Total lymphocyte numbers were greater in the trained compared to the untrained for $CD3^+$, $CD8^+$, $CD8^+CD28^+$, naïve $CD8^+$ ($CD8^+CD28^+CD57^-$), intermediate $CD8^+$ ($CD8^+CD28^+CD57^+$) and $\gamma\delta^+$ T-lymphocytes. This appears to be driven primarily by the fact that trained participants tended to have more T-lymphocytes at rest than those who were untrained (1.6 ± 0.5 $x10^9$/L vs 1.3 ± 0.3 $x10^9$/L respectively, $P<0.05$). Therefore analysis was carried out on the lymphocyte proportions as a percentage of total $CD3^+$ T-lymphocytes to allow for a fair comparison within and between groups to be made. In addition, participants who were CMV positive had greater numbers of resting $CD8^+$, $CD8^+CD28^+$ and $CD8^+CD28^+CD57^-$ T-lymphocytes compared to CMV negative participants. But with CMV not being a significant

covariate for the T-lymphocyte proportions this potential confounding variable drops out of consideration.

No difference was observed for the resting proportions of naïve, intermediate or senescent phenotypes between the trained and untrained participants. This contradicts previous literature in males in which greater resting proportions of senescent T-lymphocytes were observed in untrained compared to trained (Brown et al, 2013; Spielmann et al, 2011), and confirms our earlier observation in females (Brown et al, 2013). It is also interesting that there was no difference observed for the resting proportions of naïve, intermediate or senescent phenotypes from pre-camp to post-camp in the trained group despite the increased training volume. Recently, we observed that seven days of high intensity training resulted in an impaired exercise induced $CD8^+$ T-lymphocyte mobilisation in trained males (Witard et al, 2012). In a trained group of swimmers it has been shown that terminal effector memory RA (TEMRA) $CD8^+$ T-lymphocytes decreased for the first 7 weeks of the season (Teixeira et al, 2014). Therefore, the increase in training volume during the present study could have impaired the $CD8^+$ T-lymphocyte mobilisation response, similar to Witard's study, but the duration or intensity of the training was likely not long enough or high enough to observe any changes in resting $CD8^+$ T-lymphocyte proportions.

Many factors influence the T-lymphocyte compartment, training volume, baseline fitness and macronutrient intake. The current study was not designed to manipulate or control diet, it was just recorded. However, since it is one of the factors that could influence the T-lymphocyte compartment the observations could provide a further explanation for the lack of differences between pre-camp and post-camp in the trained group. The carbohydrate consumption of the trained females during the two week intensified training period meets the recommended 8-10 $g \cdot kg^{-1}$ body $mass \cdot day^{-1}$ for athletes during periods of high volume training (Burke et al, 2004; Burke et al, 2007). Protein intake of 1.8g/kg is at the upper end of

recommended intake for athletes and is well above the 1.0g/kg ingested by untrained or 0.66g/kg recommended for the general population (Pedersen et al, 2013). Previous analysis of the diet of the trained footballers in the present study indicates that total energy intake, carbohydrate and protein intake were increased on the camp compared with normal intake. Total energy intake increased from 1932 kcal/day to 3077 kcal/day, carbohydrate increased from 268 g/day to 547 g/day and protein increased from 71 g/day to 103 g/day. Therefore, the increase in carbohydrate consumption in this study was likely sufficient to prevent impaired T-lymphocyte function post-exercise (Henson et al, 1998; Nieman et al, 1998). This hypothesis is strengthened by the findings that increasing dietary protein intake restores the impaired mobilisation of $CD8^+$ T-lymphocytes during high intensity exercise training (Witard et al, 2013). When a high-protein diet is ingested, gluconeogenesis can increase by 40% (Linn et al, 2000) thus providing the potential for gluconeogenic pathways to increase muscle and liver glycogen availability during periods of high intensity exercise training. Thus, the impaired $CD8^+$ T-lymphocyte mobilisation observed during high intensity exercise training by Witard et al (2012) was likely due to suboptimal carbohydrate consumption, and could probably be restored by directly increasing the carbohydrate intake or indirectly increasing carbohydrate availability through increasing protein intake (Witard et al, 2013).

Resting $CD4^+$ T-lymphocyte proportions were greater in the untrained compared to the trained in the current study. Existing literature on resting immune cell numbers between trained and untrained is very variable (Gleeson et al, 1995; Hong et al, 2005; Hooper and Mackinnon, 1995; LaPerriere et al, 1994; Matthews et al, 1995; Pedersen and Toft, 2000). There were no differences in circulating $CD4^+$ T, $CD8^+$ T, or NK cells at rest between elite female rowers (Nieman et al, 2000) or trained female gymnasts (Eliakim et al, 1997) compared with age-matched untrained females. The discrepancies between proportions of resting $CD4^+$ T-lymphocytes observed in this study could be a result of the menstrual cycle phase. Reductions in $CD4^+$ T-lymphocyte number have been observed in the luteal

compared to the early follicular phase of the cycle (Lee et al, 2010). Although we did not directly record menstrual cycle phase in this study it is unlikely that all untrained were in the follicular phase and all trained were in the luteal phase at the time of sampling. Therefore, the training status difference we observed in the $CD4^+$ T-lymphocyte proportions cannot be fully explained.

The greater proportions of $CD4^+$ T-lymphocytes observed in the untrained compared to the trained drove the $CD4^+$:$CD8^+$ ratio up, thus contributing to the significantly higher $CD4^+$:$CD8^+$ ratio observed in the untrained group. People with autoimmune diseases tend to have an increased $CD4^+$:$CD8^+$ ratio, while those with viral infections have a decreased ratio. A further explanation for the higher $CD4^+$:$CD8^+$ ratio observed in the untrained compared to the trained group in the current study could be due to the higher body fat percentage observed in the untrained group compared to the trained group. Obesity is associated with increased inflammation due to increased fat tissue (Tchkonia et al, 2010), while the untrained group in the current study are not obese, the higher body fat percentage could be contributing to the higher $CD4^+$:$CD8^+$ ratio as a result of increased inflammation. However, without measurement of inflammatory markers this is merely an observation and needs further investigation before any conclusions can be made. In the current study, the difference between the ratio in trained and untrained could also be speculatively explained by Cytomegalovirus (CMV) infection. Infection with CMV is associated with a lower $CD4^+$:$CD8^+$ ratio. A higher percentage (23% (3 of 13)) of the trained population compared to (8% (1 of 13) the untrained population were positive for CMV. CMV infection was also a significant covariate in the $CD8^+$ T-lymphocyte population. Thus, suggesting that increased numbers of $CD8^+$ T-lymphocytes in the CMV positive participants (Chidrawar et al, 2009; Khan et al, 2002; Weinberger et al, 2007) could be driving the $CD4^+$:$CD8^+$ ratio down in the trained group due to the greater number of CMV positive participants. However, CMV was not a significant covariate in the $CD4^+$:$CD8^+$ ratio and we did not observed any significant

differences between the trained and untrained $CD8^+$ T-lymphocytes so any conclusions made based on CMV must be interpreted with care due to the small number of CMV positive participants. Therefore, the significantly higher $CD4^+$:$CD8^+$ ratio in the untrained compared to the trained observed in this study was a result of the greater proportion of $CD4^+$ T-lymphocytes observed respectively.

The proportion of resting $\gamma\delta^+$ T-lymphocytes was greater in the trained compared to the untrained group. Similarly, a previous study observed an increase in the percentage of $\gamma\delta^+$ T-lymphocytes from week 7 until the end of the season in swimmers (Teixeira et al, 2014). It is known that the resting population of $\gamma\delta^+$ T-lymphocytes is also influenced by age (Pistillo et al, 2013), and CMV infection (Pistillo et al, 2013). Age was matched between groups in the present study and CMV was not a significant covariate in the $\gamma\delta^+$ T-lymphocyte differences observed in our study. However, the lack of a CMV effect cannot be considered conclusive, since CMV positive participants were a very small number and not matched between groups. Speculatively, the reason for the greater proportion of $\gamma\delta^+$ T-lymphocytes in the trained compared to the untrained could be a result of the frequent mobilisation of this lymphocyte subset in response to the regular exercise training stimulus. Mobilisation of this lymphocyte subset would provide protection in situations where tissue damage and antigen exposure are more likely to occur (Anane et al, 2009; Anane et al, 2010) such as during the open window of opportunity immediately after exercise when athletes are reputedly more susceptible to URTIs.

To conclude, increasing the training volume in a group of trained females does not reduce resting senescent T-lymphocyte proportions as hypothesised from data obtained in trained vs. untrained males. The training intervention had no effect on resting T-lymphocyte subsets, the reason for this is unclear. We can however, speculate from our observations of dietary

intake that appropriate increases in consumption of key macronutrients during the increased training volume warrants further investigation. The resting proportion of $CD4^+$, $\gamma\delta^+$ T-lymphocytes and $CD4^+$:$CD8^+$ ratio differed between trained and untrained participants, highlighting the importance of training status/volume, and potentially CMV infection status when exploring immune responses to exercise training interventions.

3.6 References

Anane, L.H., Edwards, K.M., Burns, V.E., Drayson, M.T., Riddell, N.E., van Zanten, J.J., Wallace, G.R., Mills, P.J., Bosch, J.A., 2009. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. Brain Behav. Immun. 23, 823-829.

Anane, L.H., Edwards, K.M., Burns, V.E., Zanten, J.J., Drayson, M.T., Bosch, J.A., 2010. Phenotypic characterization of gammadelta T cells mobilized in response to acute psychological stress. Brain Behav. Immun. 24, 608-614.

Borg, G., 1982. Ratings of perceived exertion and heart rates during short-term cycle exercise and their use in a new cycling strength test. Int. J. Sports Med. 3, 153-158.

Borg, G.A., 1982. Psychophysical bases of perceived exertion. Med. Sci. Sports Exerc. 14, 377-381.

Brenchley, J.M., Karandikar, N.J., Betts, M.R., Ambrozak, D.R., Hill, B.J., Crotty, L.E., Casazza, J.P., Kuruppu, J., Migueles, S.A., Connors, M., Roederer, M., Douek, D.C., Koup, R.A., 1-4-2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 101, 2711-2720.

Brown, F.F., Bigley, A.B., Sherry, C., Neal, C.M., Witard, O.C., Simpson, R.J., Galloway, S.D., 4-11-2013. Training status and sex influence on senescent T-lymphocyte redistribution in response to acute maximal exercise. Brain Behav. Immun.

Burke, L.M., Kiens, B., Ivy, J.L., 2004. Carbohydrates and fat for training and recovery. J. Sports Sci. 22, 15-30.

Burke, L.M., Millet, G., Tarnopolsky, M.A., 2007. Nutrition for distance events. J. Sports Sci. 25 Suppl 1, S29-S38.

Chidrawar, S., Khan, N., Wei, W., McLarnon, A., Smith, N., Nayak, L., Moss, P., 2009. Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals. Clin. Exp. Immunol. 155, 423-432.

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., Oja, P., 2003. International physical activity questionnaire: 12-country reliability and validity. Med. Sci. Sports Exerc. 35, 1381-1395.

Effros, R.B., 2004. T cell replicative senescence: pleiotropic effects on human aging. Ann. N. Y. Acad. Sci. 1019, 123-126.

Eliakim, A., Wolach, B., Kodesh, E., Gavrieli, R., Radnay, J., Ben-Tovim, T., Yarom, Y., Falk, B., 1997. Cellular and humoral immune response to exercise among gymnasts and untrained girls. Int. J. Sports Med. 18, 208-212.

Gleeson, M., McDonald, W.A., Cripps, A.W., Pyne, D.B., Clancy, R.L., Fricker, P.A., 1995. The effect on immunity of long-term intensive training in elite swimmers. Clin. Exp. Immunol. 102, 210-216.

Henson, D.A., Nieman, D.C., Parker, J.C., Rainwater, M.K., Butterworth, D.E., Warren, B.J., Utter, A., Davis, J.M., Fagoaga, O.R., Nehlsen-Cannarella, S.L., 1998. Carbohydrate supplementation and the lymphocyte proliferative response to long endurance running. Int. J. Sports Med. 19, 574-580.

Hong, S., Johnson, T.A., Farag, N.H., Guy, H.J., Matthews, S.C., Ziegler, M.G., Mills, P.J., 2005. Attenuation of T-lymphocyte demargination and adhesion molecule expression in response to moderate exercise in physically fit individuals. J. Appl. Physiol 98, 1057-1063.

Hooper, S.L. , Mackinnon, L.T., 1995. Monitoring overtraining in athletes. Recommendations. Sports Med. 20, 321-327.

Jones, A.M. , Doust, J.H., 1997. The Conconi test in not valid for estimation of the lactate turnpoint in runners. J. Sports Sci. 15, 385-394.

Khan, N., Shariff, N., Cobbold, M., Bruton, R., Ainsworth, J.A., Sinclair, A.J., Nayak, L., Moss, P.A., 15-8-2002. Cytomegalovirus seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J. Immunol. 169, 1984-1992.

Kruger, K., Lechtermann, A., Fobker, M., Volker, K., Mooren, F.C., 2008. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. Brain Behav. Immun. 22, 324-338.

LaPerriere, A., Antoni, M.H., Ironson, G., Perry, A., McCabe, P., Klimas, N., Helder, L., Schneiderman, N., Fletcher, M.A., 1994. Effects of aerobic exercise training on lymphocyte subpopulations. Int. J. Sports Med. 15 Suppl 3, S127-S130.

Lee, S., Kim, J., Jang, B., Hur, S., Jung, U., Kil, K., Na, B., Lee, M., Choi, Y., Fukui, A., Gilman-Sachs, A., Kwak-Kim, J.Y., 1-7-2010. Fluctuation of peripheral blood T, B, and NK cells during a menstrual cycle of normal healthy women. J. Immunol. 185, 756-762.

Linn, T., Santosa, B., Gronemeyer, D., Aygen, S., Scholz, N., Busch, M., Bretzel, R.G., 2000. Effect of long-term dietary protein intake on glucose metabolism in humans. Diabetologia 43, 1257-1265.

Matthews, K.A., Caggiula, A.R., McAllister, C.G., Berga, S.L., Owens, J.F., Flory, J.D., Miller, A.L., 1995. Sympathetic reactivity to acute stress and immune response in women. Psychosom. Med. 57, 564-571.

Nieman, D.C., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Henson, D.A., Shannon, M., Hjertman, J.M., Schmitt, R.L., Bolton, M.R., Austin, M.D., Schilling, B.K., Thorpe, R., 2000. Immune function in female elite rowers and non-athletes. Br. J. Sports Med. 34, 181-187.

Nieman, D.C., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Henson, D.A., Utter, A., Davis, J.M., Williams, F., Butterworth, D.E., 1998. Effects of mode and carbohydrate on the granulocyte and monocyte response to intensive, prolonged exercise. J. Appl. Physiol (1985.) 84, 1252-1259.

Pedersen, A.N., Kondrup, J., Borsheim, E., 2013. Health effects of protein intake in healthy adults: a systematic literature review. Food Nutr. Res. 57,

Pedersen, B.K. , Toft, A.D., 2000. Effects of exercise on lymphocytes and cytokines. Br. J. Sports Med. 34, 246-251.

Pender, M.P., Csurhes, P.A., Pfluger, C.M., Burrows, S.R., 2011. Decreased CD8+ T cell response to Epstein-Barr virus infected B cells in multiple sclerosis is not due to decreased HLA class I expression on B cells or monocytes. BMC. Neurol. 11, 95-

Pistillo, M., Bigley, A.B., Spielmann, G., LaVoy, E.C., Morrison, M.R., Kunz, H., Simpson, R.J., 2013. The effects of age and viral serology on gammadelta T-cell numbers and exercise responsiveness in humans. Cell Immunol. 284, 91-97.

Salman, R.A. , Al-Rubeaan, K.A., 2009. Incidence and risk factors of hypertension among Saudi type 2 diabetes adult patients: an 11-year prospective randomized study. J. Diabetes Complications 23, 95-101.

Simpson, R.J., Florida-James, G.D., Whyte, G.P., Guy, K., 2006. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur. J. Appl. Physiol 97, 109-121.

Spielmann, G., McFarlin, B.K., O'Connor, D.P., Smith, P.J., Pircher, H., Simpson, R.J., 2011. Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. Brain Behav. Immun. 25, 1521-1529.

Tchkonia, T., Morbeck, D.E., Von, Z.T., Van, D.J., Lustgarten, J., Scrable, H., Khosla, S., Jensen, M.D., Kirkland, J.L., 2010. Fat tissue, aging, and cellular senescence. Aging Cell 9, 667-684.

Teixeira, A.M., Rama, L., Carvalho, H.M., Borges, G., Carvalheiro, T., Gleeson, M., Alves, F., Trindade, H., Paiva, A., 10-1-2014. Changes in naive and memory T-cells in elite swimmers during a winter training season. Brain Behav. Immun.

Weinberger, B., Lazuardi, L., Weiskirchner, I., Keller, M., Neuner, C., Fischer, K.H., Neuman, B., Wurzner, R., Grubeck-Loebenstein, B., 2007. Healthy aging and latent infection with CMV lead to distinct changes in CD8+ and CD4+ T-cell subsets in the elderly. Hum. Immunol. 68, 86-90.

Witard, O.C., Turner, J.E., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., Bosch, J.A., Tipton, K.D., 10-10-2013. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav. Immun.

Witard, O.C., Turner, J.E., Jackman, S.R., Tipton, K.D., Jeukendrup, A.E., Kies, A.K., Bosch, J.A., 2012. High-intensity training reduces CD8+ T-cell redistribution in response to exercise. Med. Sci. Sports Exerc. 44, 1689-1697.

Zijenah, L.S., Katzenstein, D.A., Nathoo, K.J., Rusakaniko, S., Tobaiwa, O., Gwanzura, C., Bikoue, A., Nhembe, M., Matibe, P., Janossy, G., 1-2-2005. T lymphocytes among HIV-infected and -uninfected infants: CD4/CD8 ratio as a potential tool in diagnosis of infection in infants under the age of 2 years. J. Transl. Med. 3, 6-

CHAPTER 4

CHAPTER 4: The effect of omega-3 fatty acid supplementation on T-lymphocyte populations at rest and in response to acute exercise.

4.1 Abstract

PURPOSE: To investigate the influence of different dietary fatty acids on T-lymphocyte differentiated populations at rest and in response to exercise. METHODS: Twenty trained male soccer players visited the lab on three occasions to participate in a battery of soccer specific exercise tests. The first visit was baseline (BL). The second visit 4 weeks later (control period) was pre supplementation (Pre Sup) at this visit participants were pair matched and randomized to a polyunsaturated fatty acid (PUFA) fish oil (FO) (n=10) or a saturated fatty acid (SFA) coconut oil (CO) (n=10) supplement at 0.1g/kg body mass/day for 4 weeks (supplementation period) based upon their initial baseline blood analysis for n-3 fatty acids. The third visit 4 weeks later was post supplementation (Post Sup). Whole blood was collected at each visit for blood lipid analysis. Blood lymphocytes were isolated before (Pre), immediately post (Post) and 1-hour post (1h) the exercise tests for assessment of cell surface expression of CD28 and CD57 on $CD4^+$ and $CD8^+$ T-lymphocytes and $\gamma\delta^+$ T-lymphocyte subsets. Plasma was used to determine Cytomegalovirus (CMV) serostatus. RESULTS: There was no difference in blood lipid analysis from BL to Pre Sup in the FO or CO groups (P>0.05). The *n*-3 PUFAs, EPA (20:5n-3), DHA (22:6n-3) and DPA (22:5n-3) increased from Pre Sup to Post Sup in the FO group (P<0.05) but not in the CO group (P>0.05). During the control and supplementation period a main effect of exercise (P<0.05) was observed in all the $CD3^+$ and $\gamma\delta^+$ T-lymphocytes subsets, increasing from Pre to Post and decreasing from Post to 1-hr. During the control period an interaction of group-by-day (P<0.05) was observed in the senescent $CD8^+$ T-lymphocytes from BL to Pre Sup the proportion and number decreased in the FO group and increased in the CO group. Inclusion of CMV as a covariate introduced a main effect of group (P<0.05) on the $CD4^+$ naïve proportions and cell counts and the group-by-day interaction observed on the $CD8^+$ senescent T-lymphocyte proportions and cell counts disappeared. During the supplementation period no main effect of exercise (P<0.05) was observed on the $CD8^+$ naïve T-lymphocyte proportion but there was a day-by-exercise interaction (P<0.05). The proportion of $CD8^+$ naïve T-lymphocytes was lower at rest and in response to exercise in FO and CO groups after supplementation. A main effect of group (P<0.05) was observed on senescent $CD4^+$ T-lymphocyte cell counts when CMV was included as a covariate. There was trend towards a group-by-exercise interaction on $\gamma\delta^+$ T-lymphocyte counts in the supplementation period. At the post exercise time point the $\gamma\delta^+$ T-lymphocyte count increased in the FO group but decreased in the CO group following the supplementation period. CMV was not a significant covariate (P>0.05) for the $\gamma\delta^+$ T-lymphocyte population, during the control or supplementation periods. CONCLUSION: Although a difference between the groups was evident for $\gamma\delta^+$ T-lymphocyte count and proportion there was insufficient evidence to conclude whether the difference was supplement related. Dose, duration and type of fatty acids may be important in the response but these require further study.

Keywords: PUFA , SFA, $CD4^+$ T-lymphocytes; $CD8^+$ T-lymphocytes; Trained; Untrained;

Diet; CMV infection; $\gamma\delta^+$ T-lymphocytes.

4.2 Introduction

Previously we have shown at rest and in response to acute exercise that senescent T-lymphocytes are influenced by sex and training status (Brown et al, 2013), but a period of increased volume training has no effect on senescent T-lymphocyte number or proportion at rest (Brown et al, in review). Omega-3 (*n*-3) polyunsaturated fatty acids (PUFAs), that consist primarily of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), are abundant in oily fish sources. These *n*-3 PUFAs are known to exhibit strong anti-inflammatory properties (Thies et al, 2003) leading to dietary fish oils being implicated in neurological and chronic inflammatory diseases, such as Alzheimer's (Samieri et al, 2008), diabetes mellitus (Nettleton and Katz, 2005) and cardiovascular disease (Lemaitre et al, 2003). However, the effect of these *n*-3 PUFAs on T-lymphocyte populations at rest and in response to exercise is currently unknown.

Like other cells the membrane of many immune cells is composed of both omega-3 (*n*-3) and omega-6 (*n*-6) PUFAs. These PUFAs are essential precursors for inflammatory mediators; increased circulating *n*-6 can be pro-inflammatory, whereas *n*-3 is thought to be pro-resolution (Calder, 2006; Galli and Calder, 2009). Increasing dietary consumption of *n*-3 PUFAs eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) is known to alter the lipid composition of the immune cell membrane (Yaqoob et al, 2000). In addition within T-lymphocytes EPA and DHA interrupt inflammatory signalling by disrupting the formation of lipid rafts, important platforms on which proteins are combined for signalling (Stulnig et al, 2001; Stulnig and Zeyda, 2004; Zeyda et al, 2002; Zeyda et al, 2003; Zeyda and Stulnig, 2006). Despite the beneficial health-related characteristics of *n*-3 PUFA's, there is limited evidence examining the effects of *n*-3 PUFA supplementation on T-lymphocyte differentiated subsets despite the evidence that *n*-3 PUFA supplementation reduces T-lymphocyte proliferation (Rossetti et al, 1997; Stulnig et al, 1998; Zeyda et al, 2002; Zurier et al, 1999).

Supplementation with a low level of *n*-3 0.77 or 1.7g EPA+DHA/day over 6 months has no effect on proliferation of T-lymphocytes by mitogens *in vitro* (Kew et al, 2003). With a higher dose of *n*-3 supplementation, and *in vivo* measurement of T-lymphocyte differentiated populations at rest and in response to exercise, it might be possible to observe a more global view. Whether dietary supplementation with *n*-3 PUFA or short chain saturated fatty acids (SFA) influences the mobilisation of T-lymphocyte populations in response to exercise over a 4 week supplementation period during exercise training has not been investigated. PUFAs are incorporated into lipid rafts in the T-lymphocyte membrane leading to altered signalling within the T-lymphocyte; This altered signalling impairs the production of IL-2 from the lymphocyte itself and impairs the cell surface expression of IL-2R in turn reducing T-lymphocyte proliferation in vitro (Zeyda et al, 2003). This reduction in T-lymphocyte proliferation could alter the T-lymphocyte compartment. Proliferation is the clonal expansion of T-lymphocytes via cell division which leads to the differentiation of T-lymphocytes to effector, memory and senescent T-lymphocytes. Therefore, a PUFA related decrease in T-lymphocyte proliferation could result in a decrease in senescent T-lymphocytes in response to PUFA supplementation when investigated in vivo in humans. Acute exercise is also known to alter the T-lymphocyte compartment, but the effects of PUFA supplementation and acute exercise are unknown.

Thus, to fully understand the influence of different dietary fatty acids on T-lymphocyte differentiated populations at rest and in response to exercise we pair-matched participants for baseline blood *n*-3 status and then supplemented the diet of trained males with either short chain saturated fatty acids (coconut oil) or *n*-3 polyunsaturated fatty acids (fish oil).

4.3 Methods

Participants

A group of 20 (20.3 ± 4.6 yrs) well-trained male soccer players participated in this study. All participants were non-smokers, not taking any medication and were free from infectious illness for 6 wk prior to the study. Each participant completed a pre-participation health screen questionnaire and provided their written informed consent. Ethical approval was granted by the University of Stirling Research Ethics Committee.

Experimental design and protocol.

The study was conducted as a single blind trial over 8 consecutive weeks. All participants entered the study on their normal diet with each participant acting as their own control during a 4 week control period. After this control period participants were assigned to receive 0.1g/kg body mass/day of either fish oil (n=10) containing polyunsaturated fatty acids EPA (70%), DHA (20%), DPA (2%) and Vitamin E (0.2%), or coconut oil (n=10) containing short chain saturated fatty acids (Caprylic acid 8:0 (7.06%), Capric acid 10:0 (91.75%) Lauric acid 12:0 (0.91%) and Palmitic acid 16:0 (0.28%)) for 4 weeks. Fish oil and coconut oil capsules were matched for size shape and were provided by Glasgow Health Solutions Ltd. Participants were pair matched and randomized to fish oil or coconut oil based upon their initial baseline blood analysis for n-3 fatty acids.

The timeline of the study is shown in Figure 1. All participants reported to the laboratory, in the morning after an overnight fast at the beginning of the study (baseline), at the end of the control period (pre-supplementation), and at the end of the fish oil or coconut oil supplementation period (post-supplementation). On each visit a venous cannula was inserted into a forearm vein and fasted venous blood samples were drawn before exercise, immediately post exercise and 1hr post-exercise. Blood samples were used for peripheral blood cell count, T-lymphocyte activation, and for analysis of plasma and T-lymphocyte

cytokine production. The morning fasted blood samples were also used for analysis of full blood lipid profile. Training and competition load was quantified during the 8 weeks using the pre-published method of Foster et al (2001) based on volume and intensity of exercise undertaken.

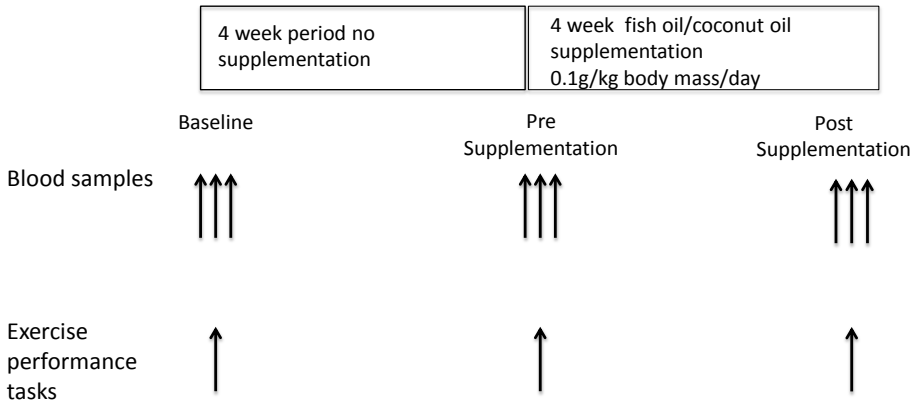

Fig 1. Timeline of the experimental protocol.

Exercise performance tasks

At baseline, pre-supplementation and post-supplementation time points participants completed football specific exercise tasks. These tasks were conducted on the same surface at the same time of day and in exactly the same order. The tasks were designed to assess strength, power, speed and aerobic endurance. The football specific exercise task data are reported elsewhere (Gravina et al., in preparation). No differences were observed in the scores obtained on any task from the male players in the present study between the baseline, pre-supplementation and post-supplementation sample time points.

Analysis of whole blood sample lipid profile

Samples of whole blood were placed onto two circular collection spots on Whatman 903 collection cards. The cards were left open and allowed to dry for 3 h after which the dried whole blood sample was detached from the collection device using forceps and placed into a screw-cap vial containing 1 ml of methylating solution (1.25M methanol/HCl). The vials were placed in a hot block at 70^{o}C for 1 h. The vials were allowed to cool to room temperature and then 2 ml of distilled water and 2 ml of saturated KCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 2 ml of iso-hexane + butylated hydroxytoluene (BHT) followed by a second extraction using 2 ml of isohexane alone.

FAME were separated and quantified by gas chromatography (ThermoFisher Trace, Hemel Hempstead, England) using a 60 m x 0.32 mm x 0.25 µm film thickness capillary column (ZB Wax, Phenomenex). Hydrogen was used as carrier gas at a flow rate of 4.0 ml/min and the temperature programme was from 50 to 150^{o}C at 40^{o}C/min then to 195^{o}C at 2^{o}C/min and finally to 215^{o}C at 0.5^{o}C/min. Individual FAME were identified compared to well characterised in house standards as well as commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England).

Peripheral blood cell count and peripheral blood mononuclear cell (PBMC) isolation

Total lymphocyte counts were determined using a Sysmex XP300 automated haematology analyser. Absolute cell numbers of the lymphocyte subset populations were determined by multiplying the percentage of all lymphocytes expressing $CD3^{+}/CD4^{+}$ or $CD3^{+}/CD8^{+}$ (as determined by flow cytometry) by the total lymphocyte count. The methods used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood have been described elsewhere (Simpson et al, 2006). The isolated PBMCs were stored in 70% (700µl) foetal bovine serum (Sigma-Aldrich, Ltd, UK), 20% (200µl) RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK) and 10% (100µl) DMSO (Sigma-Aldrich, Ltd, UK) that was added to the

samples on ice. Samples were snap frozen in liquid nitrogen and stored at -80 °C until later analysis.

Labelling of cell-surface antigens

Overlapping phenotypes exist for senescent T-lymphocyte identification as shown in Chapter 2. Hence, prior studies have used several different markers for characterisation of the same distinct T-lymphocyte subsets. In this study we used the $CD57^{+}$ $CD28^{-}$ phenotype to identify senescent T-lymphocytes and the $CD57^{-}CD28^{+}$ phenotype to identify naïve T-lymphocytes.

PMBC's were labelled with an APC conjugated anti-CD3 (Clone UCHT1) (BD cat 555335, lot 15521) monoclonal antibody (mAb), a PE-CY5 conjugated anti-CD8 (Clone HIT8a BD cat 555636, lot 63949) mAb or a PE-CY5 conjugated anti-CD4 (Clone RPA-T4 BD cat 555348, lot 10591), a PE conjugated anti-CD28 (Clone L293) (BD cat 348047, lot 3028673) mAb or a PE conjugated anti-gamma-delta (Clone B1.1 Ebio cat 129959, lot E021659) mAb, a FITC conjugated anti-CD4 (Clone RPA-T4 BD cat 555346, lot 75982) mAb or a FITC conjugated anti-CD57 (Clone HNK-1 BD cat 333169, lot 3056577) mAb, in preparation for a four-colour direct immunofluorescence procedure. Cells were incubated with 50µl of each pre-diluted mAb for 1-hour at room temperature, protected from the light. The anti-CD28, -CD57, -CD4, and -CD8 mAb's were purchased from BD Pharmingen (San Jose, CA, USA). The anti-gamma-delta mAb was purchased from Ebioscience (San Diego, CA, USA). Cells also were labelled with each mAb in a one-color immunofluorescence procedure to account for spectral overlap and adjust compensation settings during flow cytometry analysis. Appropriately conjugated isotype controls also were used in each assay to account for background binding of immunoglobulin (Ig). After incubation the cell and mAb mixture was vortexed to resuspend the cells in solution before analysis using flow cytometry.

Flow cytometry

Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA), equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter mode using Accuri C6 (CFlow ® v1) software. Side scatter was used to identify and gate the $CD3^+$ cells. The $CD4^+$, $CD8^+$ and $\gamma\delta^+$ populations were then identified in the $CD3^+$ cell population. For each sample, 40,000 $CD3^+/CD4^+$, $CD3^+/CD8^+$ and $CD3^+/\gamma\delta^+$ events were collected for analysis. The expression of CD57 and CD28 was assessed on the $CD4^+$ and $CD8^+$ T-lymphocytes by four colour flow cytometry. The percentage of all $CD3^+/\gamma\delta^+$ T-lymphocytes and the $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-lymphocyte subsets expressing the markers of interest was tabulated for statistical analysis.

CMV serostatus

CMV serostatus was defined for all participants. Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) and a SpectromaxM2 plate reader (Molecular Devices, CA, USA).

Data presentation and statistical analysis

All data are presented as means ± standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Mini-tab v-16 statistical software. Physical characteristics and dietary intake were compared between groups by independent sample t-tests. Repeated measures analysis of variance (ANOVA) was used to compare between condition changes over time in the blood fatty acid profile with n-3%, n-6% and n-3: total

(highly unsaturated fatty acids) HUFA% as dependent variables and test day as independent variable. Lymphocyte data were analysed using a 3 factor ANCOVA with cell type as the dependent variable and time, training status and test day as independent factors. CMV serostatus was the covariate. Post-hoc analysis was performed using the Tukey HSD test and 95% confidence. Main effects and interactions were accepted as statistically significant at the $p<0.05$ level.

4.4 Results

Participant Characteristics and dietary analysis

There was no significant difference between the fish oil and coconut oil groups for any physical characteristics, exercise training, habitual dietary intake or baseline blood lipid parameters (Table 1).

Fatty Acid Profile

Blood fatty acid profile was analysed at baseline, pre and post supplementation (Table 2). No significant differences were observed during the control period for any fatty acids in the fish oil or coconut oil group. A significant main effect of treatment ($P < 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n-3% whereby participants in the fish oil group had an increase n-3% (105% ± 52) compared to the coconut oil group (3% ± 23). A significant main effect of treatment ($P< 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n-6% whereby participants in the fish oil group observed a decrease in n-6% (-6% ± 9) compared to the coconut oil group (2% ± 5). A significant main effect of treatment ($P < 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n3: total HUFA (%) whereby participants in the fish oil group observed an increase the ratio of n3: total HUFA % (83% ± 43) compared to the coconut oil group (2% ± 6).

Table 1. Participant characteristics, dietary intake, and blood n-3 and n-6 fatty acids (% of total blood lipids) at baseline. Values are means ± SD. n=10 in each group

	Fish oil	Coconut oil	*P* Value
Age (years)	21.1 ± 5.9	19.4 ± 3.3	0.41
Height (cm)	180.6 ± 8.0	178.2 ± 6.9	0.48
Body Mass (kg)	76.9 ± 9.6	75.6 ± 12.1	0.79
Training (Hrs/Week)	6.3 ± 1.0	5.7 ± 0.9	0.15
Dietary Intake (Kcal/day)	1773 ± 516	2049 ± 1053	0.49
CHO Intake (g/day)	226 ± 89	259 ± 143	0.56
PRO Intake (g/day)	70 ± 15	88 ± 39	0.23
FAT Intake (g/day)	68 ± 22	77 ± 36	0.56
N-3 (g) Diet	1 ± 1.2	0.5 ± 0.2	0.14
N-6 (g) Diet	3 ± 2	3 ± 1	0.52
N-3 (%) Blood	5.12 ± 0.84	4.81 ± 0.69	0.41
N-6 (%) Blood	33.35 ± 1.99	32.81 ± 2.19	0.90
N3:total HUFA (%)	25.03 ± 3.16	23.35 ± 2.50	0.58

Table 2: Blood fatty acid profile (% of total lipids) at pre supplementation (pre) and post supplementation (post). Values are mean ± SD. * represents a significant change from pre supplementation (p <0.05). NS represents not significant from pre supplementation.

Variable	Group	Pre	Post	Difference	% Difference	P Value
n-3 (%)	**Fish oil**	5.18 ± 0.95	10.61 ± 2.96*	5.43 ± 2.60	104.8 ± 50.2	<0.05
	Coconut oil	5.07 ± 0.82	5.09 ± 0.65	0.02 ± 1.12	0.39 ± 22.09	NS
n-6 (%)	**Fish oil**	32.32 ± 2.25	30.16 ± 2.84*	-2.16 ± 2.67	-6.68 ± 8.26	<0.05
	Coconut oil	33.16 ± 1.62	33.93 ± 1.24	0.77 ± 1.60	2.32 ± 4.83	NS
n3: total	**Fish oil**	25.01 ± 3.14	45.62 ± 11.07*	20.61 ± 0.18	82.4 ± 0.72	<0.05
HUFA (%)	**Coconut oil**	23.64 ± 2.61	23.73 ± 2.35	0.09 ± 3.76	0.38 ± 15.9	NS

A significant main effect of treatment (P < 0.05) and treatment-by-time interaction (P < 0.05) was observed in the fish oil group for other blood lipids (Tables 3-4). The active omega-3 fish oil ingredients EPA (20:5n-3), DHA (22:6n-3) and DPA (22:5n-3) increased from pre supplementation to post supplementation, whereas the omega-6 fatty acids linolenic acid (18:3n-6), eicosadienoic acid (20:2n-6), dihomo-gamma-linolenic acid (20:n-3n-6), arachidonic acid (20:4n-6), adrenic acid (22:4n-6) and docosapentaenoic acid (22:5n-6) all decreased from pre to post supplementation in the fish oil group. No significant differences were observed from pre to post supplementation in the coconut oil group for any omega-3 (n-3) or omega-6 (n-6) fatty acids.

Table 3: Blood lipid membrane composition. The breakdown and total saturated and monosaturated fatty acids in the fish oil and coconut oil groups before and after the control and supplementation periods. Values are % of total lipid expressed as mean ± SD. * indicates a significant difference between the start and the end of the control or supplementation period.

	Fish Oil Control Period			Fish Oil Supplementation Period			Coconut Oil Control Period			Coconut Oil Supplementation Period		
	Start	End	P	Start	End	P	Start	End	P	Start	End	P
14:0	0.7 ± 0.2	0.8 ± 0.5	0.36	0.8 ± 0.5	0.6 ± 0.2	0.16	0.5 ± 0.1	0.6 ± 0.1	0.07	0.6 ± 0.1	0.6 ± 0.2	0.91
15:0	0.2 ± 0.03	0.2 ± 0.04	0.58	0.2 ± 0.04	0.2 ± 0.03	0.13	0.1 ± 0.03	0.2 ± 0.02	0.03*	0.2 ± 0.02	0.2 ± 0.03	0.11
16:0	21.2 ± 1.2	21.9 ± 1.4	0.26	21.9 ± 1.4	21.1 ± 0.1	0.17	21.5 ± 1.0	21.2 ± 0.5	0.50	21.2 ± 0.5	21.1 ± 0.9	0.73
18:0	12.4 ± 1.1	12.2 ± 0.9	0.77	12.2 ± 0.9	11.9 ± 0.6	0.32	12.5 ± 1.4	12.1 ± 0.6	0.41	12.1 ± 0.6	12.2 ± 0.7	0.67
20:0	0.2 ± 0.03	0.3 ± 0.03	0.21	0.3 ± 0.03	0.2 ± 0.02	0.40	0.2 ± 0.02	0.3 ± 0.02	0.14	0.3 ± 0.02	0.2 ± 0.02	0.34
22:0	0.7 ± 0.1	0.7 ± 0.1	0.42	0.7 ± 0.1	0.7 ± 0.1	0.57	0.7 ± 0.05	0.7 ± 0.1	0.46	0.7 ± 0.1	0.7 ± 0.1	0.90
24:0	1.2 ± 0.2	1.3 ± 0.2	0.34	1.3 ± 0.2	1.1 ± 0.1	0.02*	1.2 ± 0.2	1.2 ± 0.2	0.99	1.2 ± 0.2	1.2 ± 0.1	0.85
Total Sat Fat	36.5 ± 2.1	37.4 ± 2.0	0.38	37.4 ± 2.0	35.8 ± 1.3	0.03*	36.8 ± 2.4	36.2 ± 1.1	0.57	36.2 ± 1.1	36.2 ± 1.1	0.95
16:1n-9	0.2 ± 0.03	0.2 ± 0.08	0.72	0.2 ± 0.08	0.3 ± 0.1	0.61	0.2 ± 0.1	0.3 ± 0.02	0.66	0.3 ± 0.02	0.3 ± 0.06	0.64
16:1n-7	0.9 ± 0.4	1.1 ± 0.7	0.43	1.1 ± 0.7	0.8 ± 0.2	0.20	1.1 ± 0.7	1.0 ± 0.2	0.59	1.0 ± 0.2	0.9 ± 0.3	0.29
18:1n-9	16.7 ± 1.4	16.5 ± 1.7	0.81	16.5 ± 1.7	15.7 ± 1.9	0.20	16.6 ± 1.2	17.0 ± 1.1	0.27	17.0 ± 1.1	16.3 ± 1.5	0.27
18:1n-7	1.5 ± 0.1	1.4 ± 0.1	0.29	1.4 ± 0.1	1.4 ± 0.2	0.50	1.5 ± 0.2	1.5 ± 0.1	0.95	1.5 ± 0.1	1.4 ± 0.1	0.42
20:1n-9	0.3 ± 0.1	0.3 ± 0.1	0.31	0.3 ± 0.1	0.3 ± 0.1	0.50	0.3 ± 0.05	0.3 ± 0.05	0.19	0.3 ± 0.05	0.3 ± 0.06	0.11
24:1n-9	1.5 ± 0.3	1.6 ± 0.5	0.51	1.6 ± 0.5	1.2 ± 0.4	0.06	1.6 ± 0.2	1.6 ± 0.3	0.83	1.6 ± 0.3	1.6 ± 0.3	0.74
Total monounsat Fat	21.2 ± 1.5	21.3 ± 2.2	0.92	21.3 ± 2.2	19.7 ± 2.2	0.04*	21.3 ± 1.8	21.7 ± 1.1	0.49	21.7 ± 1.1	20.8 ± 1.7	0.16

1

Table 4. Blood lipid membrane composition. The breakdown and total omega 6 and omega 3 fatty acids in the fish oil and coconut oil groups before and after the control and supplementation periods. Values are % of total lipid expressed as mean ± SD. * indicates a significant difference between the start and the end of the control or supplementation period.

	Fish Oil Control Period			Fish Oil Supplementation Period			Coconut Oil Control Period			Coconut Oil Supplementation Period		
	Start	End	P	Start	End	P	Start	End	P	Start	End	P
18:2n-6	19.0 ± 2.3	17.9 ± 2.0	0.21	17.9 ± 2.0	18.1 ± 1.3	0.67	18.2 ± 2.5	18.0 ± 2.5	0.78	18.0 ± 2.5	18.6 ± 1.4	0.43
18:3n-6	0.2 ± 0.1	0.2 ± 0.1	0.82	0.2 ± 0.1	0.1 ± 0.1	0.03*	0.2 ± 0.1	0.2 ± 0.1	0.54	0.2 ± 0.1	0.2 ± 0.1	0.67
20:2n-6	0.3 ± 0.1	0.3 ± 0.1	0.52	0.3 ± 0.1	0.3 ± 0.04	0.00*	0.3 ± 0.1	0.3 ± 0.04	0.54	0.3 ± 0.04	0.3 ± 0.1	0.17
20:3n-6	1.6 ± 0.4	1.7 ± 0.3	0.34	1.7 ± 0.3	1.2 ± 0.3	0.00*	1.6 ± 0.3	1.7 ± 0.3	0.42	1.7 ± 0.3	1.7 ± 0.4	0.72
20:4n-6	10.3 ± 1.5	10.3 ± 1.0	0.99	10.3 ± 1.0	9.2 ± 1.6	0.02*	10.8 ± 1.1	11.0 ± 0.9	0.62	11.0 ± 0.9	11 ± 1.1	0.93
22:4n-6	1.6 ± 0.4	1.6 ± 0.3	0.99	1.6 ± 0.3	1.2 ± 0.3	0.00*	1.7 ± 0.3	1.8 ± 0.3	0.92	1.8 ± 0.3	1.8 ± 0.2	0.80
22:5n-6	0.3 ± 0.1	0.3 ± 0.1	1.00	0.3 ± 0.1	0.2 ± 0.1	0.00*	0.3 ± 0.1	0.4 ± 0.1	0.54	0.4 ± 0.1	0.3 ± 0.03	0.41
Total n-6	33.4 ± 1.9	32.3 ± 2.3	0.18	32.3 ± 2.3	30.2 ± 2.8	0.03*	33.2 ± 2.3	33.2 ± 1.6	0.98	33.2 ± 1.6	33.9 ± 1.2	0.17
18:3n-3	0.5 ± 0.1	0.4 ± 0.1	0.35	0.4 ± 0.1	0.5 ± 0.1	0.09	0.4 ± 0.1	0.5 ± 0.2	0.26	0.5 ± 0.2	0.4 ± 0.1	0.61
20:4n-3	0.1 ± 0.1	0.1 ± 0.03	0.95	0.1 ± 0.03	0.1 ±0.03	0.70	0.1 ± 0.05	0.1 ± 0.04	0.48	0.1 ± 0.04	0.1 ± 0.04	0.60
EPA 20:5n-3	0.6 ± 0.01	0.6 ± 0.2	0.39	0.6 ± 0.2	4.4 ± 1.2	0.00*	0.6 ± 0.2	0.6 ± 0.2	0.17	0.6 ± 0.2	0.6 ± 0.1	0.81
22:5n-3	1.4 ± 0.3	1.5 ± 0.3	0.69	1.5 ± 0.3	2.0 ± 0.5	0.00*	1.4 ± 0.2	1.5 ± 0.2	0.47	1.5 ± 0.2	1.5 ± 0.2	0.60
DHA 22:6n-3	2.5 ± 0.7	2.6 ± 0.8	0.63	2.6 ± 0.8	3.6 ± 0.8	0.00*	2.4 ± 0.5	2.5 ± 0.5	0.43	2.5 ± 0.5	2.5 ± 0.6	0.87
Total n-3	5.1 ± 0.8	5.2 ± 0.9	0.66	5.2 ± 0.9	10.6 ± 3.0	0.00*	4.8 ± 0.7	5.1 ± 0.8	0.24	5.1 ± 0.8	5.1 ± 0.7	0.95
20:4n-6/20:5n-3	18.0 ± 4.5	18.2 ± 4.2	0.91	18.2 ± 4.2	4.4 ± 7.0	0.00*	20.1 ± 5.7	18.3 ± 4.0	0.32	18.3 ± 4.0	18.3 ± 3.1	0.99
%n-3HUFA/Total HUFA	24.7 ± 3.2	25.2 ± 3.2	0.32	25.2 ± 3.2	45.6 ± 11.1	0.00*	23.2 ± 2.4	23.6 ± 2.6	0.35	23.6 ± 2.6	23.7 ± 2.4	0.94

1

T-lymphocyte data

Control period

During the control period a main effect of exercise was observed in the total $CD3^+$ T-lymphocytes whereby the number of $CD3^+$ T-lymphocytes increased in response to acute exercise followed by a decrease in the 1-hour post exercise period. As a result a main effect of exercise was observed for all of the T-lymphocyte subsets. All subsets followed the same pattern of an immediate post exercise increase followed by a 1-hour post exercise decrease (Table 5).

Table 5: T-lymphocyte counts at Baseline, Pre-supplementation and Post-supplementation. Values are means ± SD count in $x10^6$/L of the T-lymphocyte populations. A box round the data indicates a significant main effect of exercise in the total $CD3^+$ T-lymphocytes that results in a main effect of exercise in all T-lymphocyte subsets (*P*<0.05). ** indicates a significant difference from pre and 1hr post exercise (P<0.05). $ indicates a significant difference between groups at the post exercise time point (P<0.05). * indicates a significant difference from post exercise (P<0.05).

			Baseline		Pre-Supplementation		Post-Supplementation	
Phenotype	**T-lymphocyte Subset**	**Exercise**	**FO**	**CO**	**FO**	**CO**	**FO**	**CO**
$CD3^+$	T-lymphocytes	Pre	1135 ± 359	1170 ± 547	1394 ± 670	1174 ± 253	1302 ± 597	1134 ± 453
		Post	2059 ± 517	1666 ± 572	2169 ± 900	1809 ± 652	2317 ± 866	1919 ± 811
		1hour	787 ± 384	804 ± 354	990 ± 428	857 ± 428	979 ± 422	899 ± 387
$CD3^+$ $CD8^+$	Cytotoxic	Pre	299 ± 121	294 ± 167	364 ± 224	285 ± 144	353 ± 203	292 ± 157
		Post	535 ± 173	381 ± 132	537 ± 270	469 ± 276	541 ± 363	492 ± 328
		1hour	204 ± 120	200 ± 106	271 ± 148	228 ± 170	263 ±116	243 ± 136
$CD3^+CD4^+$	Helper	Pre	632.9 ± 225.3	590.4 ± 288.3	677.3 ± 350.6	592.6 ± 229.5	708.2 ± 381.7	534.5 ± 236.6
		Post	899.8 ± 376.3	752.8 ± 323.2	894.3 ± 418.8	777.9 ± 327.5	1012.2 ± 480.4	747.9 ± 412.7
		1hour	403.8 ± 179.2	399.2 ± 246.5	466.8 ± 217.1	381.4 ± 196.6	474.9 ± 256.8	389.0 ± 232.7
$CD3^+\gamma\delta^+$	Gamma-delta	Pre	34.8 ± 26.0	20.7 ± 19.3	43.6 ± 28.0	24.4 ± 19.9	47.7 ± 42.0	14.8 ± 10.8
		Post	121.3 ± 137.9**	41.5 ± 36.7$	121.3 ± 107.9**	60.0 ± 42.6	160.3 ± 180.2**	51.0 ± 40.6$
		1hour	23.9 ± 18.6	12.5 ± 9.6	36.3 ± 35.3	13.4 ± 8.4	27.6 ± 19.9	14.9 ± 11.8

There was no significant effect of group or any interactions for the naïve $CD4^+$, $CD8^+$ or senescent $CD4^+$ T-lymphocyte proportions (Figure 2 and 3) or cell counts (Table 6) during the control period. However during the control period the senescent $CD8^+$ T-lymphocytes responded differently. There was an additional interaction of group-by-day on the senescent $CD8^+$ T-lymphocytes. From baseline to pre-supplementation the proportion and number of senescent $CD8^+$ T-lymphocytes decreased in the fish oil group and increased in the coconut oil group (Table 6). During the control period covariate analysis revealed infection with CMV to be a significant covariate ($P<0.05$) for both the $CD4^+$ and $CD8^+$ naïve and senescent T-lymphocyte proportions and cell counts. Inclusion of CMV as a covariate introduced a main effect of group ($P<0.05$) on the $CD4^+$ naïve proportions and cell counts. The group-by-day interaction observed on the $CD8^+$ senescent T-lymphocyte proportions and cell counts disappeared when CMV was included as a covariate.

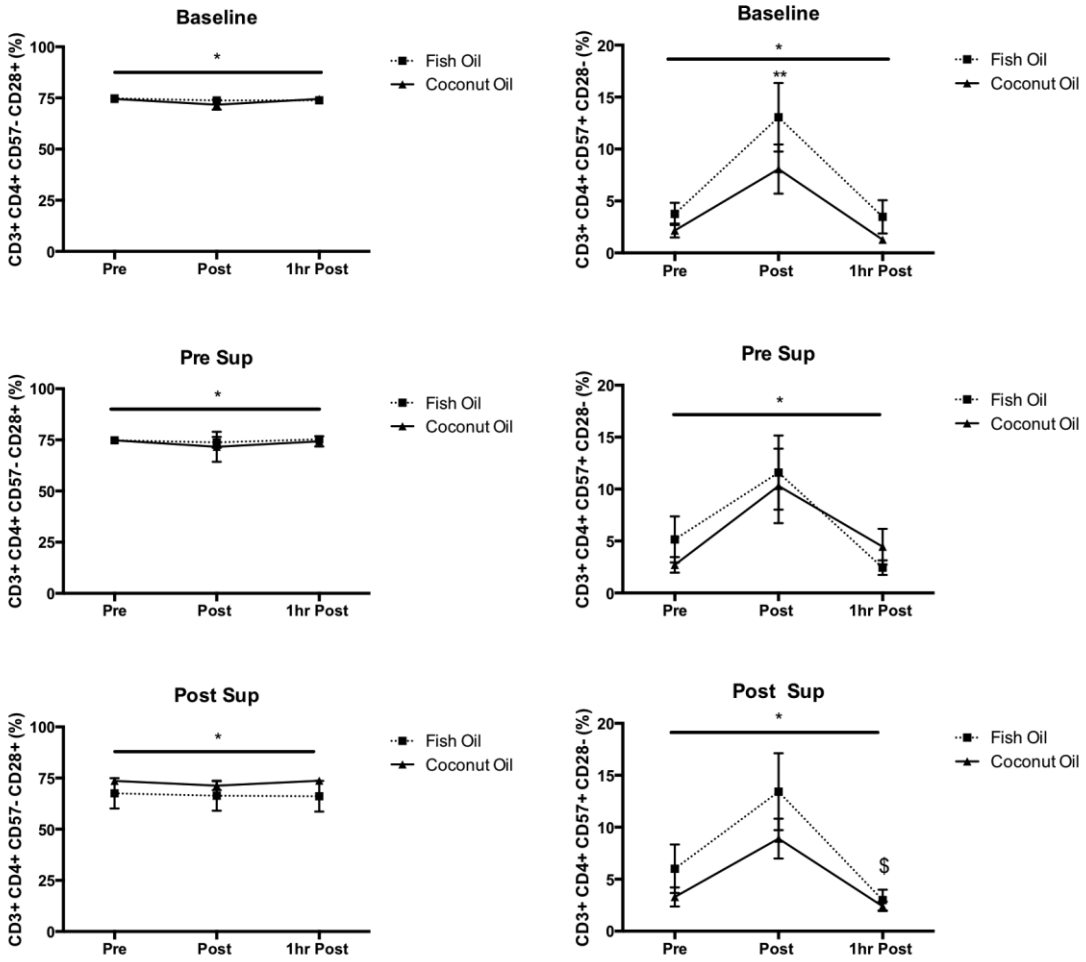

Fig 2. Values are mean (± SEM) percent of $CD4^+$ T-lymphocyte subset expressing a naïve phenotype ($CD3^+CD4^+CD57^-CD28^+$) or a senescent phenotype ($CD3^+CD4^+CD57^+CD28^-$) at pre, immediately post and 1-h post exercise. Results are displayed for the fish oil group (n=10) and the coconut oil group (n=10) at baseline, pre supplementation (Pre Sup) and post supplementation (Post Sup). * indicates a significant main effect of exercise. ** indicates a significant difference from pre and 1-hour post exercise in the fish oil group. $ indicates a significant difference from post exercise in the fish oil group.

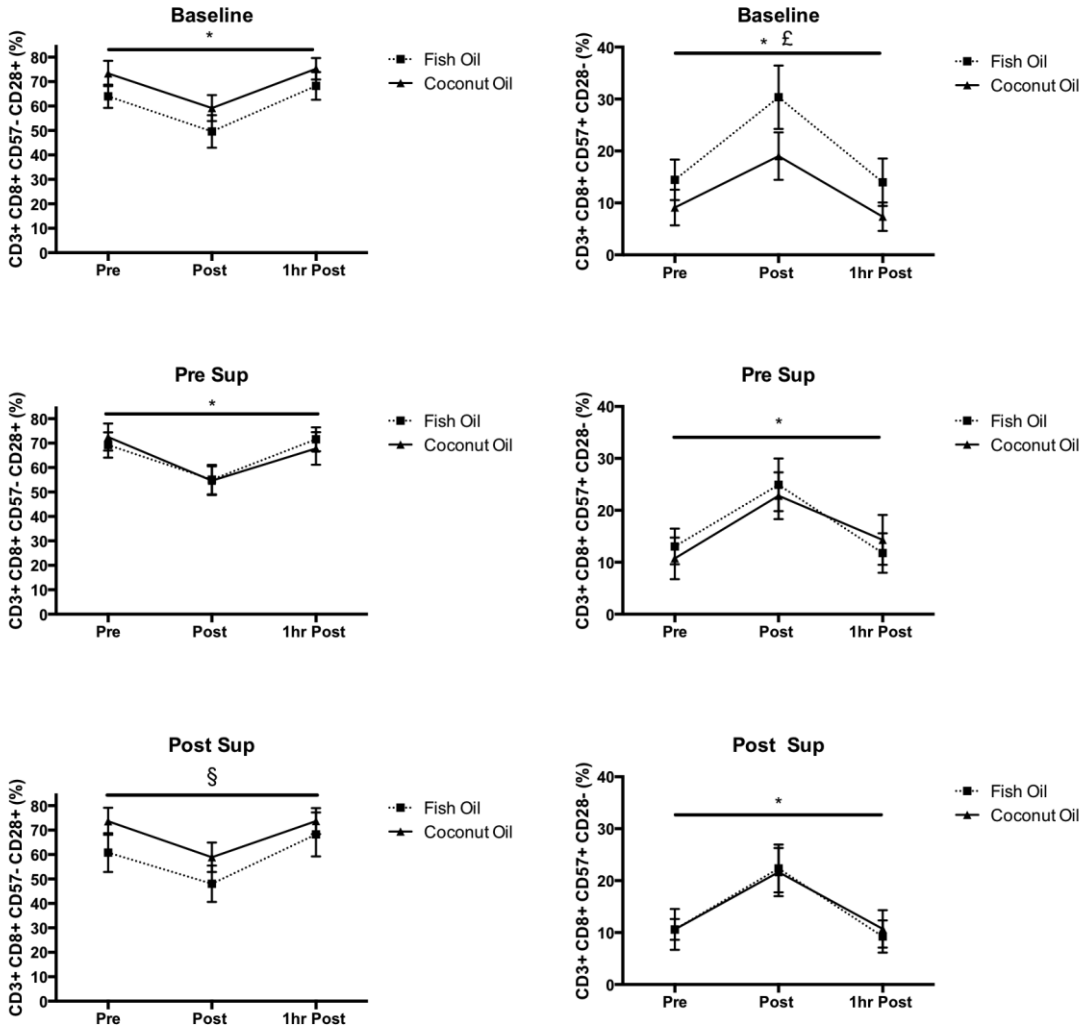

Fig 3. Values are mean (± SEM) percent of $CD8^+$ T-lymphocyte subset expressing a naïve phenotype ($CD3^+CD8^+CD57^-CD28^+$) or a senescent phenotype ($CD3^+CD8^+CD57^+CD28^-$) at pre, immediately post and 1-h post exercise. Results are displayed for the fish oil group (n=10) and the coconut oil group (n=10) at baseline, pre supplementation (Pre Sup) and post supplementation (Post Sup). * indicates a significant main effect of exercise ($P<0.05$). £ indicates a significant main effect of group ($P<0.05$). § indicates a significant day-by-exercise interaction ($P<0.05$).

Table 6: $CD4^+$ and $CD8^+$ T-lymphocyte subsets expressed as naïve, intermediate and senescent phenotypes. Values are means (±SD) count $x10^6$/L of the T-lymphocyte populations ** indicates a significant difference from pre and 1hr post exercise (P<0.05). $ indicates a significant difference between groups at the post exercise time point (P<0.05). * indicates a significant difference from post exercise (P<0.05).

			Baseline		Pre-Supplementation		Post-Supplementation	
Phenotype	**T-lymphocyte Subset**	**Exercise**	**FO**	**CO**	**FO**	**CO**	**FO**	**CO**
$CD8^-CD28^+$ $CD57^-$	Naïve	Pre	200 ± 108	225 ± 152	273 ± 209	214 ± 127	239 ± 188	213 ± 133
		Post	262 ± 128	228 ± 112	313 ± 197	254 ± 178	268 ± 194	284 ± 237
		1hour	131 ± 63	156 ± 104	201 ± 126	157 ± 125	187 ± 124	179 ± 120
$CD8^+CD28^+$ $CD57^+$	Intermediate	Pre	9.9 ± 9.3	7.0 ± 8.2	11.3 ± 11.5	7.0 ± 7.6	10.5 ± 9.3	6.6 ± 6.4
		Post	24.1 ± 20.8	14.3 ± 15.5	22.4 ± 20.3	21.8 ± 25.0	19.4 ± 23.3	23.8 ± 26.9
		1hour	7.1 ± 6.7	5.1 ± 4.7	9.0 ± 9.4	4.8 ± 3.1	7.3 ± 8.8	6.1 ± 3.6
$CD8^+CD28^-$ $CD57^-$	Intermediate	Pre	48.9 ± 22.0	39.8 ± 25.4	61.4 ± 50.2	40.5 ± 34.2	69.4 ± 60.3	41.1 ± 36.5
		Post	77.8 ± 43.6	66.2 ± 28.6	78.1 ± 43.1	79.3 ± 48.1	115.0 ± 79.4	73.2 ± 64.1
		1hour	27.9 ± 22.6	27.6 ± 18.8	32.1 ± 15.7	37.8 ± 44.4	48.3 ± 77.0	31.2 ± 23.9
$CD8^+CD28^-$ $CD57^+$	Senescent	Pre	40.2 ± 32.5	22.3 ± 29.1	36.3 ± 23.6	23.6 ± 22.5	33.9 ± 26.5	31.5 ± 42.3
		Post	161.3 ± 104.9**	71.7 ± 63.4$	123.9 ± 73.0	114.4 ± 152.7	138.3 ± 166.4	111.5 ± 116.4
		1hour	38.9 ± 70.1	12.0 ± 12.8	29.7 ± 25.7	29.3 ± 41.1	19.7 ± 19.9*	26.1 ± 31.4
$CD4^+CD28^+$ $CD57^-$	Naïve	Pre	615.7 ± 221.6	576.4 ± 290.0	660.4 ± 347.8	577.1 ± 229.0	649.1 ± 433.8	513.4 ± 235.8
		Post	861.9 ± 348.1*	712.2 ± 324.3	863.5 ± 411.2*	729.4 ± 335.8	917.8 ± 556.9	706.6 ± 417.5
		1hour	348.8 ± 167.8*	389.2 ± 248.6	456.8 ± 212.3*	368.9 ± 194.2	417.2 ± 293.2	374.2 ± 231.3
$CD4^+CD28^+$ $CD57^+$	Intermediate	Pre	5.8 ± 2.8	5.8 ± 3.1	8.1 ± 4.4	6.0 ± 5.1	7.2 ± 4.1	5.6 ± 3.1
		Post	14.6 ± 20.4	10.2 ± 8.0	13.8 ± 11.2	12.7 ± 9.6	15.4 ± 13.0	10.1 ± 4.8
		1hour	3.7 ± 1.8	3.8 ± 2.3	6.1 ± 4.2	4.7 ± 3.4	6.3 ± 3.9	5.0 ± 4.4
$CD4^+CD28^-$ $CD57^-$	Intermediate	Pre	10.1 ± 10.3	6.9 ± 4.5	7.2 ± 10.0	6.4 ± 5.3	6.2 ± 6.0	11.1 ± 11.2
		Post	12.9 ± 12.5	21.7 ± 19.7	11.2 ± 9.3	15.2 ± 17.6	11.7 ± 7.9	14.9 ± 11.5
		1hour	14.6 ± 24.6	5.5 ± 5.8	3.2 ± 3.8	3.6 ± 2.6	2.2 ± 1.7	7.4 ± 9.9
$CD4^+CD28^-$ $CD57^+$	Senescent	Pre	1.2 ± 1.6**	1.3 ± 3.1	1.7 ± 2.8**	3.1 ± 7.0	1.6 ± 2.4**	4.4 ± 9.8
		Post	10.4 ± 21.9**	8.7 ± 22.2	5.7 ± 9.7**	20.4 ± 40.8	8.5 ± 16.1**	16.6 ± 34.4
		1hour	0.7 ± 1.1	0.8 ± 2.0	0.7 ± 1.4	4.3 ± 10.3	0.9 ± 1.2	2.3 ± 4.8*

Supplementation period

During the supplementation period there was a main effect of exercise on the total $CD3^+$ T-lymphocytes whereby the number of $CD3^+$ T-lymphocytes increased in response to acute exercise followed by a decrease in the 1-hour post exercise period. As a result a main effect of exercise was observed for all of the T-lymphocyte subsets and all subsets followed the same pattern of an immediate post exercise increase followed by a 1-hour post exercise decrease (Table 5). No main effect of exercise was observed on the $CD8^+$ naïve T-lymphocyte proportion. There was however a day-by-exercise interaction. Whereby the proportion of $CD8^+$ naïve T-lymphocytes was lower at rest and in response to exercise in both groups after supplementation (Figure 3). During the supplementation period CMV was a significant covariate ($P<0.05$) for the $CD4^+$ naïve cell count and senescent proportions and cell counts. CMV was a significant covariate ($P<0.05$) for the $CD8^+$ naïve cell count and senescent T-lymphocyte proportions. A main effect of group ($P<0.05$) was observed on senescent $CD4^+$ T-lymphocyte cell counts when CMV was included as a covariate.

Gamma delta ($\gamma\delta^+$)T-lymphocytes

A main effect of exercise ($P<0.05$) was observed during both the control and supplementation period for both the proportions (Figure 4) and numbers of $\gamma\delta^+$ T-lymphocytes (Table 5). The proportion and number of $\gamma\delta^+$ T-lymphocytes increased in response to exercise, followed by a decrease, one hour post in both groups. No interactions were observed on the $\gamma\delta^+$ T-lymphocyte proportions in the control or supplementation period (Figure 4). However, there was trend towards a group-by-exercise interaction on $\gamma\delta^+$ T-lymphocyte counts in the supplementation period. At the post exercise time point the $\gamma\delta^+$ T-lymphocyte count increased in the fish oil group but decreased in the coconut oil group following the supplementation period (Table 5). CMV was not a significant covariate ($P>0.05$) for the $\gamma\delta^+$ T-lymphocyte population, during the control or supplementation periods.

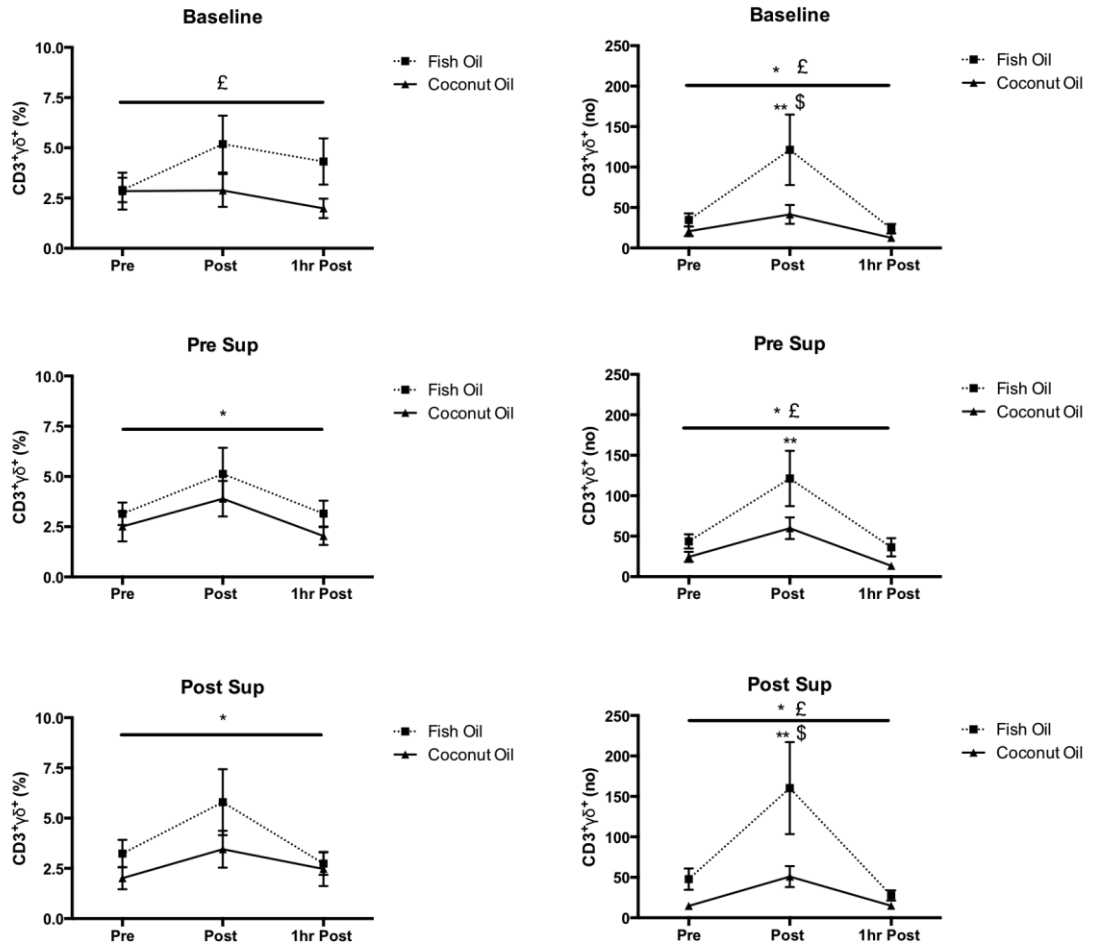

Fig 4. Values are mean (± SEM) cell number (x10^6/L) and percent and of the $CD3^+$ T-lymphocyte subset expressing a $\gamma\delta^+$ phenotype at pre, immediately post and 1-h post exercise. Results are displayed for the fish oil group (n=10) and the coconut oil group (n=10) at baseline, pre supplementation (Pre Sup) and post supplementation (Post Sup). $^{£}$ indicates a significant main effect of group (P<0.05). * indicates a significant main effect of exercise (P<0.05). ** indicates a significant difference from pre and 1hr post exercise in the fish oil group (P<0.05). $^{\$}$ indicates a significant difference between groups at the post exercise time point (P<0.05).

CMV serostatus

Forty percent of the fish oil group was defined as positive for CMV, four out of the ten participants. Twenty percent of the coconut oil group was defined as positive for CMV, two out of the ten participants.

4.5 Discussion

The immunomodulatory effects of *n*-3 PUFAs are well established (Calder, 2011). Despite the evidence of reduced T-lymphocyte proliferation as a result of PUFAs (Zeyda et al, 2002) the effect of PUFA on mobilisation of T-lymphocyte differentiated populations has not been characterised. In the present study, although we observed some differences between groups, most likely as a result of differences in CMV infection history, it is clear that four weeks ingestion of short chain saturated fats (coconut oil) or *n*-3 PUFA's (fish oil) has little effect on T-lymphocyte differentiated subsets at rest and in response to acute exercise in an active male sample.

During the control period no significant differences were observed for any of the blood fatty acids in either fish oil or coconut oil groups, confirming that no underlying major dietary differences existed between the groups. There was also no difference observed for any of the *n*-3 or *n*-6 fatty acids in blood during the supplementation period in the coconut oil group. However, during the supplementation period we observed an increase in *n*-3 PUFAs with a concomitant decrease in *n*-6 PUFAs in the fish oil group. As confirmed by our results supplementation with *n*-3 PUFAs like EPA and DHA results in an alteration of the blood cell lipid membrane composition whereby EPA and DHA are incorporated into the cell membrane at the expense of AA (Calder et al, 1994; Kew et al, 2003; Kew et al, 2004). Two studies have previously investigated supplementation with EPA (2.1g/day) and DHA (1.1g/day) for one week (Faber et al, 2011) and 12 weeks (Yaqoob et al, 2000). Faber et al. observed significant incorporation of EPA and DHA into the blood cell lipid membrane after 1 day of supplementation. Together both studies suggest maximum incorporation of PUFAs into human PBMCs occurs after about 7 days of supplementation. Incorporation is quicker and with a lower dose than previously thought (Faber et al, 2011). If maximum incorporation into the PBMC membrane occurred after 7 days, with 2.1g/day EPA and 1.1g/day of DHA,

the four weeks supplementation period used in the current study may have been too long and the dose (0.1g/kg/day) too high to identify any differences in the T-lymphocyte populations.

We observed that the acute mobilisation of senescent $CD4^+$ and $CD8^+$ T-lymphocytes was not influenced by quite marked changes in the lipid profile of blood. Although it would appear that the subsequent egress of senescent $CD4^+$ T-lymphocytes was more pronounced in the fish oil group at the post-supplementation visit, it is more likely a result of natural variation in response between baseline, pre and post supplementation time points. During both the control and supplementation periods in the fish oil and coconut oil groups we observed that acute exercise elicits an increase in the proportions and numbers of total $CD3^+$ T-lymphocytes, senescent $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T-lymphocyte subsets as previously reported (Anane et al, 2009; Brown et al, 2013; Campbell et al, 2009; Simpson et al, 2007). We also observed a concomitant decrease in the proportion of T-lymphocytes and measured subsets in the 1-hr post exercise. The numbers of naïve and senescent $CD4^+$ and $CD8^+$ and $\gamma\delta^+$ T-lymphocytes increased post exercise reflected the increase in total $CD3^+$ T-lymphocytes mobilised in response to exercise. This response could be explained by a feedback loop, as previously discussed in Chapter 2, in which senescent T-lymphocytes migrate to peripheral tissues for immunosurveillance after acute exercise, creating vacant immune space that is then filled by naïve T-lymphocytes (Simpson, 2011). It is known that the post exercise T-lymphocyte response is improved by carbohydrate supplementation (Henson et al, 1998; Nieman, 1998). It has recently been observed that the aforementioned exercise induced mobilisation was impaired during 7 days of high-intensity training (Witard et al, 2012) and subsequently restored with increased protein consumption (Witard et al, 2013). We have now observed that supplementing the diet with short chain SFA or n-3 PUFA has no effect on the mobilisation and egress of T-lymphocyte subsets in response to acute strenuous exercise.

During the control period the proportion and number of senescent $CD8^+$ T-lymphocytes decreased in the fish oil group and increased in the coconut oil group. This observation highlights a particular strength of our study design. Participants training, age and body composition were matched between groups. Therefore, including the control period allowed us to observe any natural variation between participants as a result of genetic polymorphisms, latent CMV infection, or natural fluctuations in T-lymphocyte subsets within and between groups that occurred in the T-lymphocyte responses over the 4 weeks. Thus, our design precluded any false interpretation of responses observed during the 4 week supplementation period. The difference observed between groups during the control period is likely a result of CMV infection history, since there were a greater number of CMV+ individuals in the fish oil group. CMV was a significant covariate in the $CD4^+$ and $CD8^+$ naïve and senescent T-lymphocyte numbers and proportions. Adding CMV to the statistical analysis model as a covariate introduced a main group effect on the naïve $CD4^+$ T-lymphocyte counts and proportions, suggesting the greater $CD4^+$ T-lymphocytes observed between treatment groups was a result of latent CMV infection. Inclusion of CMV as a covariate also removed the group-by-day interaction on the senescent $CD8^+$ T-lymphocyte counts and proportions suggesting this interaction was an artefact of latent CMV infection.

We observed a lower proportion of $CD8^+$ naïve T-lymphocytes at rest and in response to exercise in both groups after supplementation but no effect on the senescent T-lymphocyte population. T-lymphocyte activation is triggered by the activation of the T-cell receptor (TCR)-CD3 complex. This elicits a signalling cascade that is partially dependent on CD28 a co-stimulatory molecule on the surface of naïve T-lymphocytes (Ward, 1996). Eventually, in the presence of a signal from the TCR-CD3 complex and co-stimulation from CD28, transcription factors like NF-AT and NF- κB are switched on inducing the transcription of the T-lymphocyte proliferation factor IL-2 (Cantrell, 1996). Previous studies have observed a reduction in the CD3/CD28 induced activation of NF-AT with PUFA supplementation

reducing T-lymphocyte proliferation (Zeyda et al, 2003). Perhaps the reduction in CD3/CD28 induced activation previously observed is due to a reduction in available naïve T-lymphocytes to provide the co-stimulatory signal, which would be supported by the lower proportions of $CD8^+$ naïve T-lymphocytes observed in the current study. However, the data on cell counts does not fully support this speculative argument. CMV was a significant covariate in the $CD4^+$ and $CD8^+$ naïve and senescent T-lymphocyte numbers and proportions. Including CMV as a covariate adds a significant main effect of group to the naïve $CD4^+$ T-lymphocyte count suggesting the greater number of naïve $CD4^+$ T-lymphocytes observed in the fish oil group was a result of latent CMV infection.

At the post exercise time point the $\gamma\delta^+$ T-lymphocyte count increased in the fish oil group but decreased in the coconut oil group following the supplementation period. It is not fully understood if the observed increase in $\gamma\delta^+$ T-lymphocytes post exercise is a beneficial or detrimental observation. In the context of this study the higher post exercise count of $\gamma\delta^+$ T-lymphocytes in the fish oil group could be beneficial for immunosurveillance. The increased $\gamma\delta^+$ T-lymphocytes attract and stimulate more immune cells (Tikhonov et al, 2006), promote wound healing (Jameson et al, 2002) and present antigen for recognition by other T-lymphocytes (Brandes et al, 2005). Conversely the increased $\gamma\delta^+$ T-lymphocyte count could enhance atherosclerotic plaque formation by attracting immune cells into the inflamed sub endothelia in healthy active participants (Bosch et al, 2003; Dyugovskaya et al, 2003). The increased $\gamma\delta^+$ T-lymphocyte count in response to exercise in the group supplemented with fish oil is not a result of CMV infection therefore warrants further investigation to fully understand the relationship between fatty acid supplementation, cytokines and $\gamma\delta^+$ T-lymphocytes.

The present study examined 4 weeks of supplementation with fish oil at a dose of 0.1g/kg body mass/day compared to coconut oil at a dose of 0.1g/kg body mass/day on T-

lymphocyte subsets and the subset responses to acute strenuous exercise. Although a difference between the groups was evident for $\gamma\delta^+$ T-lymphocyte counts and proportion there was insufficient evidence to conclude whether the difference was supplement related. Dose, duration and type of fatty acids may be important in the response but these require further study. Additional work examining specific cytokine responses to supplementation with saturated fat or PUFA's is warranted to explore whether the changes we have observed in lymphocyte numbers/proportions are potentially supplement related.

4.6 References

Anane, L.H., Edwards, K.M., Burns, V.E., Drayson, M.T., Riddell, N.E., van Zanten, J.J., Wallace, G.R., Mills, P.J., Bosch, J.A., 2009. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. Brain Behav. Immun. 23, 823-829.

Bosch, J.A., Berntson, G.G., Cacioppo, J.T., Dhabhar, F.S., Marucha, P.T., 2003. Acute stress evokes selective mobilization of T cells that differ in chemokine receptor expression: a potential pathway linking immunologic reactivity to cardiovascular disease. Brain Behav. Immun. 17, 251-259.

Brandes, M., Willimann, K., Moser, B., 8-7-2005. Professional antigen-presentation function by human gammadelta T Cells. Science 309, 264-268.

Brown, F.F., Bigley, A.B., Sherry, C., Neal, C.M., Witard, O.C., Simpson, R.J., Galloway, S.D., 4-11-2013. Training status and sex influence on senescent T-lymphocyte redistribution in response to acute maximal exercise. Brain Behav. Immun.

Calder, P.C., 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot. Essent. Fatty Acids 75, 197-202.

Calder, P.C., 2011. Fatty acids and inflammation: the cutting edge between food and pharma. Eur. J. Pharmacol. 668 Suppl 1, S50-S58.

Calder, P.C., Yaqoob, P., Harvey, D.J., Watts, A., Newsholme, E.A., 1-6-1994. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. Biochem. J. 300 (Pt 2), 509-518.

Campbell, J.P., Riddell, N.E., Burns, V.E., Turner, M., van Zanten, J.J., Drayson, M.T., Bosch, J.A., 2009. Acute exercise mobilises CD8+ T lymphocytes exhibiting an effector-memory phenotype. Brain Behav. Immun. 23, 767-775.

Cantrell, D.A., 1996. T cell antigen receptor signal transduction pathways. Cancer Surv. 27, 165-175.

Dyugovskaya, L., Lavie, P., Lavie, L., 15-7-2003. Phenotypic and functional characterization of blood gammadelta T cells in sleep apnea. Am. J. Respir. Crit Care Med. 168, 242-249.

Faber, J., Berkhout, M., Vos, A.P., Sijben, J.W., Calder, P.C., Garssen, J., van, H.A., 2011. Supplementation with a fish oil-enriched, high-protein medical food leads to rapid incorporation of EPA into white blood cells and modulates immune responses within one week in healthy men and women. J. Nutr. 141, 964-970.

Foster, C., Florhaug, J.A., Franklin, J., Gottschall, L., Hrovatin, L.A., Parker, S., Doleshal, P., Dodge, C., 2001. A new approach to monitoring exercise training. J. Strength. Cond. Res. 15, 109-115.

Galli, C. , Calder, P.C., 2009. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. Ann. Nutr. Metab 55, 123-139.

Henson, D.A., Nieman, D.C., Parker, J.C., Rainwater, M.K., Butterworth, D.E., Warren, B.J., Utter, A., Davis, J.M., Fagoaga, O.R., Nehlsen-Cannarella, S.L., 1998. Carbohydrate supplementation and the lymphocyte proliferative response to long endurance running. Int. J. Sports Med. 19, 574-580.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., Havran, W.L., 26-4-2002. A role for skin gammadelta T cells in wound repair. Science 296, 747-749.

Kew, S., Banerjee, T., Minihane, A.M., Finnegan, Y.E., Williams, C.M., Calder, P.C., 2003. Relation between the fatty acid composition of peripheral blood mononuclear cells and measures of immune cell function in healthy, free-living subjects aged 25-72 y. Am. J. Clin. Nutr. 77, 1278-1286.

Kew, S., Mesa, M.D., Tricon, S., Buckley, R., Minihane, A.M., Yaqoob, P., 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am. J. Clin. Nutr. 79, 674-681.

Lemaitre, R.N., King, I.B., Mozaffarian, D., Kuller, L.H., Tracy, R.P., Siscovick, D.S., 2003. n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study. Am. J. Clin. Nutr. 77, 319-325.

Nettleton, J.A. , Katz, R., 2005. n-3 long-chain polyunsaturated fatty acids in type 2 diabetes: a review. J. Am. Diet. Assoc. 105, 428-440.

Nieman, D.C., 1998. Influence of carbohydrate on the immune response to intensive, prolonged exercise. Exerc. Immunol. Rev. 4, 64-76.

Rossetti, R.G., Seiler, C.M., DeLuca, P., Laposata, M., Zurier, R.B., 1997. Oral administration of unsaturated fatty acids: effects on human peripheral blood T lymphocyte proliferation. J. Leukoc. Biol. 62, 438-443.

Samieri, C., Jutand, M.A., Feart, C., Capuron, L., Letenneur, L., Barberger-Gateau, P., 2008. Dietary patterns derived by hybrid clustering method in older people: association with cognition, mood, and self-rated health. J. Am. Diet. Assoc. 108, 1461-1471.

Simpson, R.J., 2011. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc. Sport Sci. Rev. 39, 23-33.

Simpson, R.J., Florida-James, G.D., Cosgrove, C., Whyte, G.P., Macrae, S., Pircher, H., Guy, K., 2007. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. J. Appl. Physiol 103, 396-401.

Simpson, R.J., Florida-James, G.D., Whyte, G.P., Guy, K., 2006. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur. J. Appl. Physiol 97, 109-121.

Stulnig, T.M., Berger, M., Sigmund, T., Raederstorff, D., Stockinger, H., Waldhausl, W., 2-11-1998. Polyunsaturated fatty acids inhibit T cell signal transduction by modification of detergent-insoluble membrane domains. J. Cell Biol. 143, 637-644.

Stulnig, T.M., Huber, J., Leitinger, N., Imre, E.M., Angelisova, P., Nowotny, P., Waldhausl, W., 5-10-2001. Polyunsaturated eicosapentaenoic acid displaces proteins from membrane rafts by altering raft lipid composition. J. Biol. Chem. 276, 37335-37340.

Stulnig, T.M. , Zeyda, M., 2004. Immunomodulation by polyunsaturated fatty acids: impact on T-cell signaling. Lipids 39, 1171-1175.

Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C., Grimble, R.F., 8-2-2003. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 361, 477-485.

Tikhonov, I., Deetz, C.O., Paca, R., Berg, S., Lukyanenko, V., Lim, J.K., Pauza, C.D., 2006. Human Vgamma2Vdelta2 T cells contain cytoplasmic RANTES. Int. Immunol. 18, 1243-1251.

Ward, S.G., 1-9-1996. CD28: a signalling perspective. Biochem. J. 318 (Pt 2), 361-377.

Witard, O.C., Turner, J.E., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., Bosch, J.A., Tipton, K.D., 10-10-2013. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav. Immun.

Witard, O.C., Turner, J.E., Jackman, S.R., Tipton, K.D., Jeukendrup, A.E., Kies, A.K., Bosch, J.A., 2012. High-intensity training reduces CD8+ T-cell redistribution in response to exercise. Med. Sci. Sports Exerc. 44, 1689-1697.

Yaqoob, P., Pala, H.S., Cortina-Borja, M., Newsholme, E.A., Calder, P.C., 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur. J. Clin. Invest 30, 260-274.

Zeyda, M., Staffler, G., Horejsi, V., Waldhausl, W., Stulnig, T.M., 9-8-2002. LAT displacement from lipid rafts as a molecular mechanism for the inhibition of T cell signaling by polyunsaturated fatty acids. J. Biol. Chem. 277, 28418-28423.

Zeyda, M. , Stulnig, T.M., 2006. Lipid Rafts & Co.: an integrated model of membrane organization in T cell activation. Prog. Lipid Res. 45, 187-202.

Zeyda, M., Szekeres, A.B., Saemann, M.D., Geyeregger, R., Stockinger, H., Zlabinger, G.J., Waldhausl, W., Stulnig, T.M., 15-6-2003. Suppression of T cell signaling by polyunsaturated fatty acids: selectivity in inhibition of mitogen-activated protein kinase and nuclear factor activation. J. Immunol. 170, 6033-6039.

Zurier, R.B., Rossetti, R.G., Seiler, C.M., Laposata, M., 1999. Human peripheral blood T lymphocyte proliferation after activation of the T cell receptor: effects of unsaturated fatty acids. Prostaglandins Leukot. Essent. Fatty Acids 60, 371-375.

CHAPTER 5

CHAPTER 5: The effect of fatty acid supplementation on T-lymphocyte activation and plasma cytokine concentration.

5.1 Abstract

PURPOSE: To investigate the influence of different dietary fatty acids on plasma cytokine concentrations and expression of T-lymphocyte activation marker CD69 at rest and in response to exercise. METHODS: Twenty trained male soccer players visited the lab on three occasions to participate in a battery of soccer specific exercise tests. The first visit was baseline (BL). The second visit 4 weeks later (control period) was pre supplementation (Pre Sup) at this visit participants were pair matched and randomized to a polyunsaturated fatty acid (PUFA) fish oil (FO) (n=10) or a saturated fatty acid (SFA) coconut oil (CO) (n=10) supplement at 0.1g/kg body mass/day for 4 weeks (supplementation period) based upon their initial baseline blood analysis for n-3 fatty acids. The third visit 4 weeks later was post supplementation (Post Sup). Whole blood was collected at each visit for blood lipid analysis. Blood lymphocytes were isolated before (Pre) immediately post (Post) and 1-hour post (1h) the exercise tests for assessment of cell surface expression of CD69 on $CD4^+$ and $CD8^+$ T-lymphocytes subsets. Plasma concentration of Th1 cytokines: IL-2, TNF- α and IFN-γ, and Th2 cytokines IL-4, IL-6 and IL-10 was analysed at Pre, Post and 1h. Plasma was also used to determine Cytomegalovirus (CMV) serostatus. RESULTS: There was no difference in blood lipid analysis from BL to Pre Sup in the FO or CO groups ($P>0.05$). The *n*-3 PUFAs, EPA (20:5n-3), DHA (22:6n-3) and DPA (22:5n-3) increased from Pre Sup to Post Sup in the FO group ($P<0.05$) but not in the CO group ($P>0.05$). There was a significant mobilisation of activated $CD4^+$ $CD69^+$ and $CD8^+$ $CD69^+$ ($P<0.05$) T-lymphocyte numbers in response to exercise in both FO and CO groups, but no group, or group x time interaction ($P>0.05$). CMV infection was a significant covariate on the number and proportion of $CD4^+CD69^+$ T-lymphocytes ($P<0.05$) but not on the number or proportion of $CD8^+CD69^+$ T-lymphocytes ($P>0.05$). During the control period there were no significant main effects or interactions ($P>0.05$) on the cytokine concentrations in the FO or CO groups. During the supplementation period there was a significant effect of day on TNF-α, IL-6, IL-4 and IL-2 ($P<0.05$) with IFN-γ and IL-10 trending towards a difference. The plasma cytokine concentration was greater at Post Sup compared to Pre Sup for both FO and CO groups. Latent CMV infection was a significant covariate for TNF-α, IL-6, IL-4, IL-2, IFN-γ and IL-10 ($P<0.05$). CONCLUSION: In the current study we observed no evidence of a difference between the CO and FO groups for early T-lymphocyte activation marker or plasma cytokine concentrations despite the membrane lipid composition change over the 4 week supplementation period.

Keywords: PUFA , SFA, $CD4^+$ T-lymphocytes; $CD8^+$ T-lymphocytes; Trained; Diet; CMV infection; $CD69^+$.

5.2 Introduction

It has been previously shown that supplementation with both long chain polyunsaturated (fish oil) fatty acids (PUFA) and short chain saturated fatty acids had no effect on resting or post exercise T-lymphocyte populations (Chapter 4). T-lymphocyte maturation, proliferation and function is controlled by cytokines (Banchereau et al, 2012). Different fatty acid supplements have previously been shown to alter cytokine expression (Bladbjerg et al, 2011; Ferrucci et al, 2006). Therefore, understanding the cytokine expression pattern and early T-lymphocyte activation that results from manipulating the blood fatty acid profile may help to explain the lack of difference we have observed in the T-lymphocyte populations.

The immune cell membrane includes both n-3 and n-6 PUFAs. PUFAs are essential precursors for inflammatory mediators and eicosanoids (Calder, 2006; Galli and Calder, 2009). Immune cell membranes contain high proportions of the n-6 PUFA arachidonic acid (AA) that provides the precursor for pro-inflammatory mediators such as prostaglandins eg PGE_2. Increasing dietary consumption of n-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) alters the composition of the immune cell membrane with AA in the cell membrane being replaced by EPA and/or DHA (Yaqoob et al, 2000). Thus, since AA is a precursor for pro-inflammatory mediators like PGE_2 a decrease in AA alters the cytokine milieu (Calder, 2006; Calder, 2007; Galli and Calder, 2009; Simopoulos, 2007). In vitro PGE_2 inhibits lymphocyte proliferation (Goodwin et al, 1977) and the production of cytokines (IL-2 and IFN-γ) by T-helper 1(Th1) T-lymphocytes (Betz and Fox, 1991; Fedyk et al, 1997; Hilkens et al, 1996), subsequently indirectly stimulating the production of cytokines (IL-4, IL-5 and IL-10) by T-helper 2 (Th2) T-lymphocytes (Betz and Fox, 1991; Fedyk et al, 1997; Hilkens et al, 1996).

Evidence addressing the cytokine response to exercise with PUFA and SFA supplementation has provided contrasting outcomes. Immune derived IL-6 production was initially shown to be decreased (Meydani et al, 1991) following n-3 fatty acid supplementation in young and old women. However, other studies have observed no effect (Nieman et al, 2009; Toft et al, 2000), during PUFA supplementation. Post exercise peripheral blood mononuclear cell (PBMC) IL-2 production increased in a PUFA supplemented group of healthy active males (Gray et al, 2012). However, in their study no post exercise effect of PUFA supplementation was observed on PBMC production of IL-4 or IFN-γ (Gray et al, 2012). Elsewhere no effect of PUFA supplementation was observed on PBMC production of IL-2 or IFN-γ but an increase in IL-4 was reported, however, this was not an exercise study (Miles et al, 2006). Recently it was observed that exercise results in an increase of both Th1 and Th2 cytokine expression from late differentiated $CD8^+$ T-lymphocytes on an individual cell basis (LaVoy et al, 2013), in Chapter 4 we observed a mobilisation of the same cell type in response to exercise, but it is unknown if the increased cytokine expression per cell relates to whole body expression measured in the plasma and if fatty acid supplementation impacts the response.

In order to understand the influence of n-3 PUFA and indeed short chain fatty acids as biological mediators on the immune response to exercise in athletes, we examined the same population as in Chapter 4 with the same dose of coconut and fish oils and investigated the plasma concentration of Th1 cytokines: IL-2, TNF- α and IFN- γ, and Th2 cytokines IL-4, IL-6 and IL-10 and the early T-lymphocyte activation marker CD69 as a marker for T-lymphocyte function at rest and in response to exercise. We hypothesized that PUFA supplementation would potentially increase lymphocyte activation and Th-1 cytokines with a decrease in the Th-2 cytokines.

5.3 Methods

Participants

A group of 20 (20.3 ± 4.6 yrs) well-trained male soccer players participated in this study. All participants were non-smokers, not taking any medication and were free from infectious illness for 6 wk. prior to the study. Each participant completed a pre-participation health screen questionnaire and provided their written informed consent. Ethical approval was granted by the University of Stirling Research Ethics Committee.

Experimental design and protocol.

The study was conducted as a single blind trial over 8 consecutive weeks. All participants entered the study on their normal diet with each participant acting as their own control during a 4 week control period. After this control period they were assigned to receive either fish oil (n=10) 0.1g/kg body mass of omega-3 (700mg of EPA, 200mg of DHA, 20mg of DPA and 0.02mg of Vitamin E per 1g capsule), or coconut oil (n=10) 0.1g/kg body mass of short chain saturated fats (breakdown) daily for 4 weeks. Fish oil and coconut oil capsules were matched for size shape and were provided by Glasgow Health Solutions.

The timeline of the study is shown in Chapter 4 Figure 1. At the beginning of the study (baseline) at the end of the control period (pre-supplementation) and at the end of the fish oil or coconut oil supplementation period (post-supplementation) all participants reported to the laboratory, in the morning after an overnight fast. A venous cannula was inserted into a forearm vein and fasted venous blood samples were drawn before exercise, immediately post exercise and 1hr post-exercise. Blood samples were used for peripheral blood cell count, T-lymphocyte activation, plasma and T-lymphocyte cytokine production. The morning fasted blood samples were used for analysis of blood lipid profile. Training and competition

load was quantified during the 8 weeks using the pre-published method of Foster et al (2001) based on volume and intensity of exercise undertaken.

Exercise tests

At baseline, pre-supplementation and post-supplementation participants completed exactly the same battery of football specific exercise tasks, on the same surface at the same time of day and in exactly the same order. These tasks were designed to test strength, power, speed and aerobic endurance and the data are reported elsewhere.

Analysis of whole blood sample lipid profile

Samples of whole blood were placed onto two circular collection spots on Whatman 903 collection cards. The cards were left open and allowed to dry for 3 h after which the dried whole blood sample was detached from the collection device using forceps and placed into a screw-cap vial containing 1 ml of methylating solution (1.25M methanol/HCl). The vials were placed in a hot block at 70^{o}C for 1 h. The vials were allowed to cool to room temperature and then 2 ml of distilled water and 2 ml of saturated KCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 1 x 2 ml of iso-hexane + butylated hydroxytoluene (BHT) followed by a second extraction using 2 ml of isohexane alone.

FAME were separated and quantified by gas-liquid chromatography (ThermoFisher Trace, Hemel Hempstead, England) using a 60 m x 0.32 mm x 0.25 µm film thickness capillary column (ZB Wax, Phenomenex). Hydrogen was used as carrier gas at a flow rate of 4.0 ml/min and the temperature programme was from 50 to 150^{o}C at 40^{o}C/min then to 195^{o}C at 2^{o}C/min and finally to 215^{o}C at 0.5^{o}C/min. Individual FAME were identified compared to well characterised in house standards as well as commercial FAME mixtures (Supelco™ 37 FAME mix, Sigma-Aldrich Ltd., Gillingham, England).

Peripheral Blood Cell count and Peripheral blood mononuclear cells (PBMC) isolation

Total lymphocyte counts were determined using a Sysmex XP300 automated haematology analyser. Absolute cell numbers of the lymphocyte subset populations were determined by multiplying the percentage of all lymphocytes expressing $CD3^+/CD4^+$ or $CD3^+/CD8^+$ (as determined by flow cytometry) by the total lymphocyte count. The methods used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood have been described elsewhere (Simpson et al, 2006). The isolated PBMCs were then stored in 70% (700µl) foetal bovine serum (Sigma-Aldrich, Ltd, UK), 20% (200µl) RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK) and 10% (100µl) DMSO (Sigma-Aldrich, Ltd, UK) that was added to the samples on ice. Samples were snap frozen in liquid nitrogen and stored until later analysis.

Plasma was also obtained from whole blood centrifugation for cytokine analysis.

Labelling of cell-surface antigens

Cells were incubated with 20µl of pre-diluted CD3/CD8/CD69 (Clone SK7, SK1, L78) fastimmune kit (BD Biosciences, UK) according to manufacturers instructions for 1-hour at room temperature, protected from the light. After incubation the cell and mAb mixture was vortexed to resuspend the cells in solution before analysis using flow cytometry.

Flow cytometry

Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on a FACs Calibur flow cytometer (BD Biosciences, UK), equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter

mode using Cell Quest software. Side scatter was used to identify and gate the $CD3^+$ cells. The $CD4^+$ and $CD8^+$ populations were then identified in the $CD3^+$ cell population. For each sample, 10,000 $CD3^+/CD4^+$ and $CD3^+/CD8^+$ events were collected for analysis. The expression of CD69 was assessed on the $CD4^+$ and $CD8^+$ T-lymphocytes by four colour flow cytometry. The percentage of all $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T-lymphocyte subsets expressing the markers of interest was tabulated for statistical analysis.

Cytokine analysis

Cytokines TNFα, IFNγ, IL-2, IL-10, IL-6 and IL-4 were measured in the plasma from the pre and post exercise blood samples using a commercially available cytokine bead assay (BD Biosciences, UK) and read on a FACs array (BD Biosciences,UK).

CMV serostatus

CMV serostatus was defined for all participants. Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) and a SpectromaxM2 plate reader (Molecular Devices, CA, USA).

Data presentation and statistical analysis

All data are presented as means ± standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Mini-tab v-16 statistical software. Physical characteristics and dietary intake were compared between groups by independent sample t-tests. Repeated measures analysis of variance (ANOVA) was used to compare between condition changes over time in the blood fatty acid profile with n-3%, n-6% and n-3: total

highly unsaturated fatty acid (HUFA%) as dependent variables and test day as independent variable. Lymphocyte and cytokine data were analysed using a 3 factor ANCOVA with cell type or cytokine as the dependent variable and exercise, group and test day as independent factors. CMV serostatus was the covariate. Post-hoc analysis was performed using the Tukey HSD test and 95% confidence. Main effects and interactions were accepted as statistically significant at the $p<0.05$ level.

5.4 Results

Participant Characteristics and dietary analysis

There was no significant difference between the fish oil and coconut oil groups for any physical characteristics, exercise training or dietary intake as in Chapter 4.

Fatty Acid Profile

Blood fatty acid profile was analysed pre to post supplementation as in Chapter 4. A significant main effect of treatment ($P < 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n-3% whereby participants in the fish oil group had an increase n-3% (105% ± 52) compared to the coconut oil group (3% ± 23). A significant main effect of treatment ($P< 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n-6% whereby participants in the fish oil group observed a decrease in n-6% (-6% ± 9) compared to the coconut oil group (2% ± 5). A significant main effect of treatment ($P < 0.05$) and a treatment-by-time interaction ($P < 0.05$) was observed for n3: total HUFA (%) whereby participants in the fish oil group observed an increase the ratio of n3: total HUFA % (83% ± 43) compared to the coconut oil group (2% ± 6).

T-lymphocytes

A significant effect of exercise was observed for all T-lymphocytes ($CD3^+$) ($P < 0.05$). The mobilisation of $CD3^+$ T-lymphocytes increased in response to exercise as shown in Chapter 4. There was a significant mobilisation of activated $CD4^+$ $CD69^+$ ($P < 0.05$) (Figure 1A) and $CD8^+$ $CD69^+$ ($P<0.05$) (Figure 1B) T-lymphocyte numbers in response to exercise. Despite significant mobilisation there was no significant effect of group and no significant interaction (group x time) observed on the $CD4^+CD69^+$ or $CD8^+CD69^+$ T-lymphocyte numbers. There was no significant effect of exercise, group or interaction on the $CD4^+CD69^+$ or $CD8^+CD69^+$

T-lymphocyte proportions (Figure 2 and 3). CMV infection was a significant covariate for the number and proportion of $CD4^+CD69^+$ T-lymphocytes ($P<0.05$). CMV was not a significant covariate for the number or proportion of $CD8^+CD69^+$ T-lymphocytes ($P>0.05$).

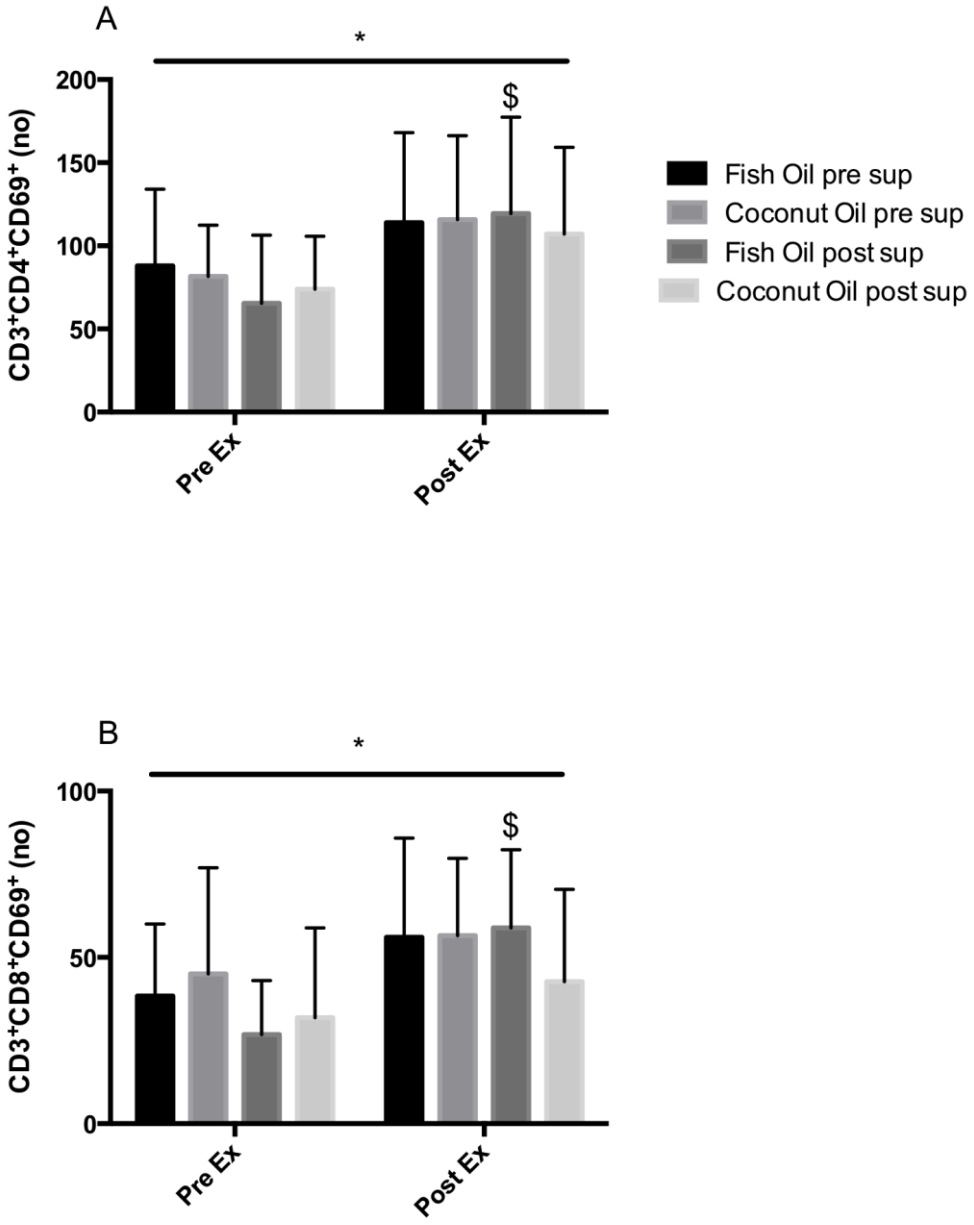

Fig 1. Values are means ± SD of $CD4^+$(A) and $CD8^+$ (B) T-lymphocyte numbers ($x10^6$/L) expressing the early activation marker $CD69^+$ at pre and post exercise. Results are indicated for fish oil and coconut oil groups at pre supplementation (pre sup) and post supplementation (post sup) visits. * Indicates a significant main effect of exercise ($P<0.05$). $ indicates a significant difference from pre exercise ($P<0.05$).

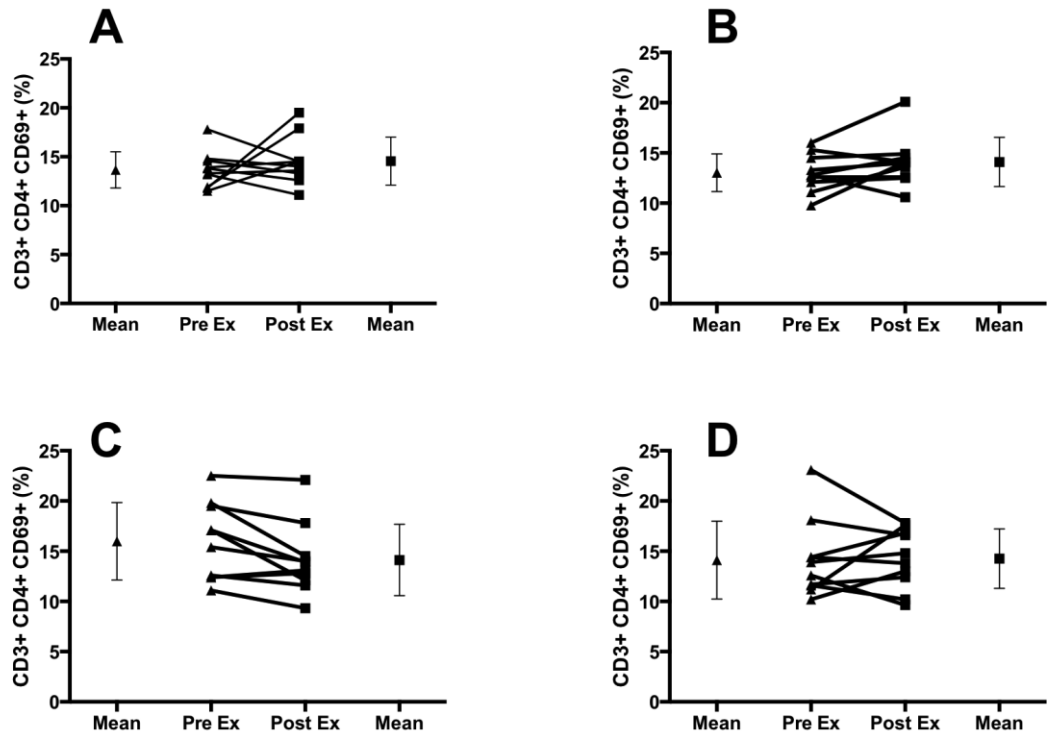

Fig 2. Values are individual responses and mean ± SD percent of $CD4^+$ T-lymphocytes expressing the early activation marker $CD69^+$ pre and post exercise. Results are indicated for fish oil (A, B) and coconut oil (C,D) groups at the pre supplementation (A,C) and post supplementation (B,D) visits.

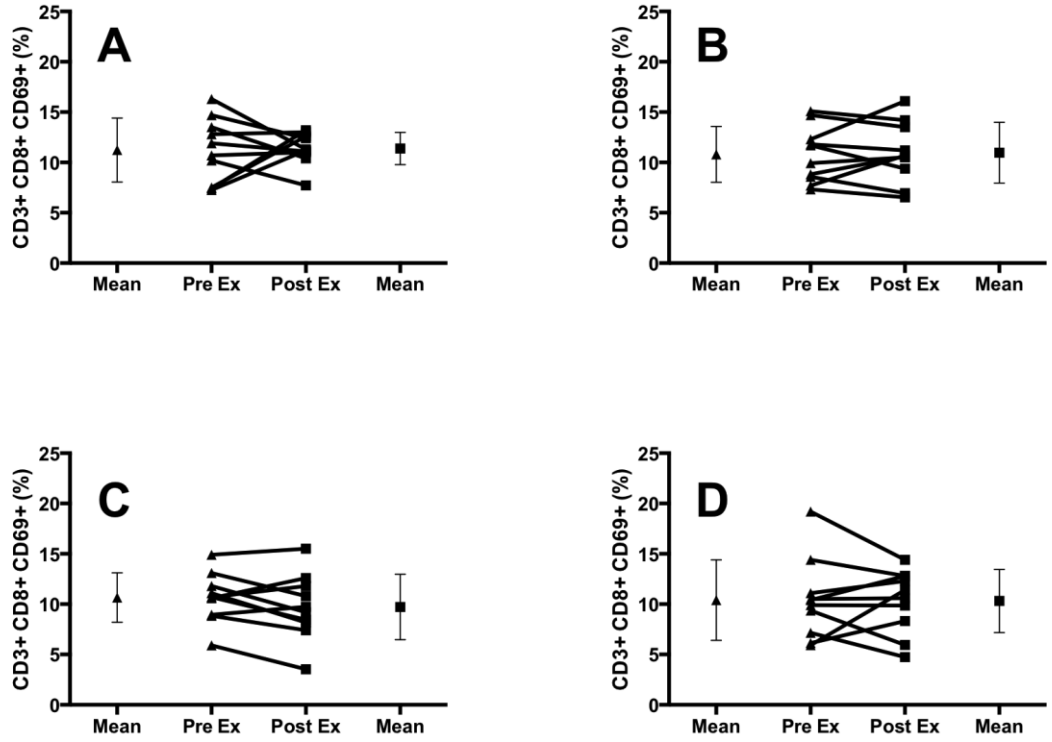

Fig 3. Values are individual responses and mean ± SD percent of $CD8^+$ T-lymphocytes expressing the early activation marker $CD69^+$ pre and post exercise. Results are indicated for fish oil (A, B) and coconut oil (C,D) groups at the pre supplementation (A,C) and the post supplementation (B,D) visits.

Plasma Cytokines

There were no significant main effects or interactions on the cytokine concentrations in the fish oil or coconut oil groups during the control period (baseline to pre-supplementation) (Figure 4, 5). A significant effect of day during the supplementation period (pre-supplementation to post-supplementation) was observed for TNF-α ($P<0.05$), IL-6 ($P<0.05$), IL-4 ($P<0.05$) and IL-2 ($P<0.05$) with IFN-γ ($P<0.05$) and IL-10 ($P<0.05$) tending towards a difference (Figure 4, 5). The plasma concentrations of TNF-α, IL-6, IL-4, IL-2, IFN-γ and IL-10 was greater at post-supplementation compared to pre-supplementation for both fish oil and coconut oil groups (Figure 4, 5). Latent CMV infection was a significant covariate for TNF-α, IL-6, IL-4, IL-2, IFN-γ and IL-10 ($P<0.05$).

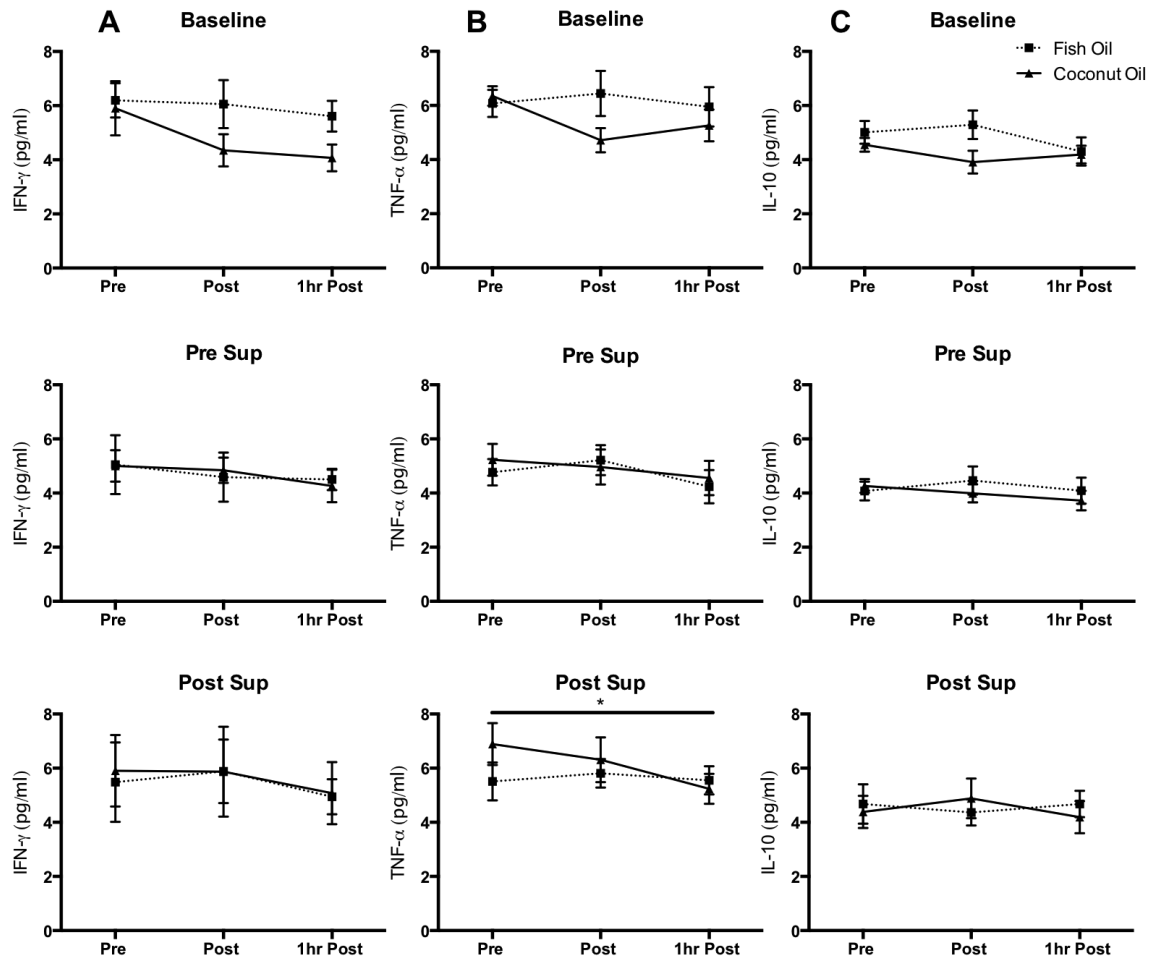

Fig 4. Plasma concentrations of cytokines IFN-γ (A), TNF-α (B), IL-10 (C) values are mean ± SEM in pg/ml at pre, post and 1hr post exercise at the baseline, pre supplementation (Pre Sup) and post supplementation (Post Sup) visits in the fish oil and coconut oil groups. * indicates a significant main effect of day in both fish oil and coconut oil groups ($P<0.05$).

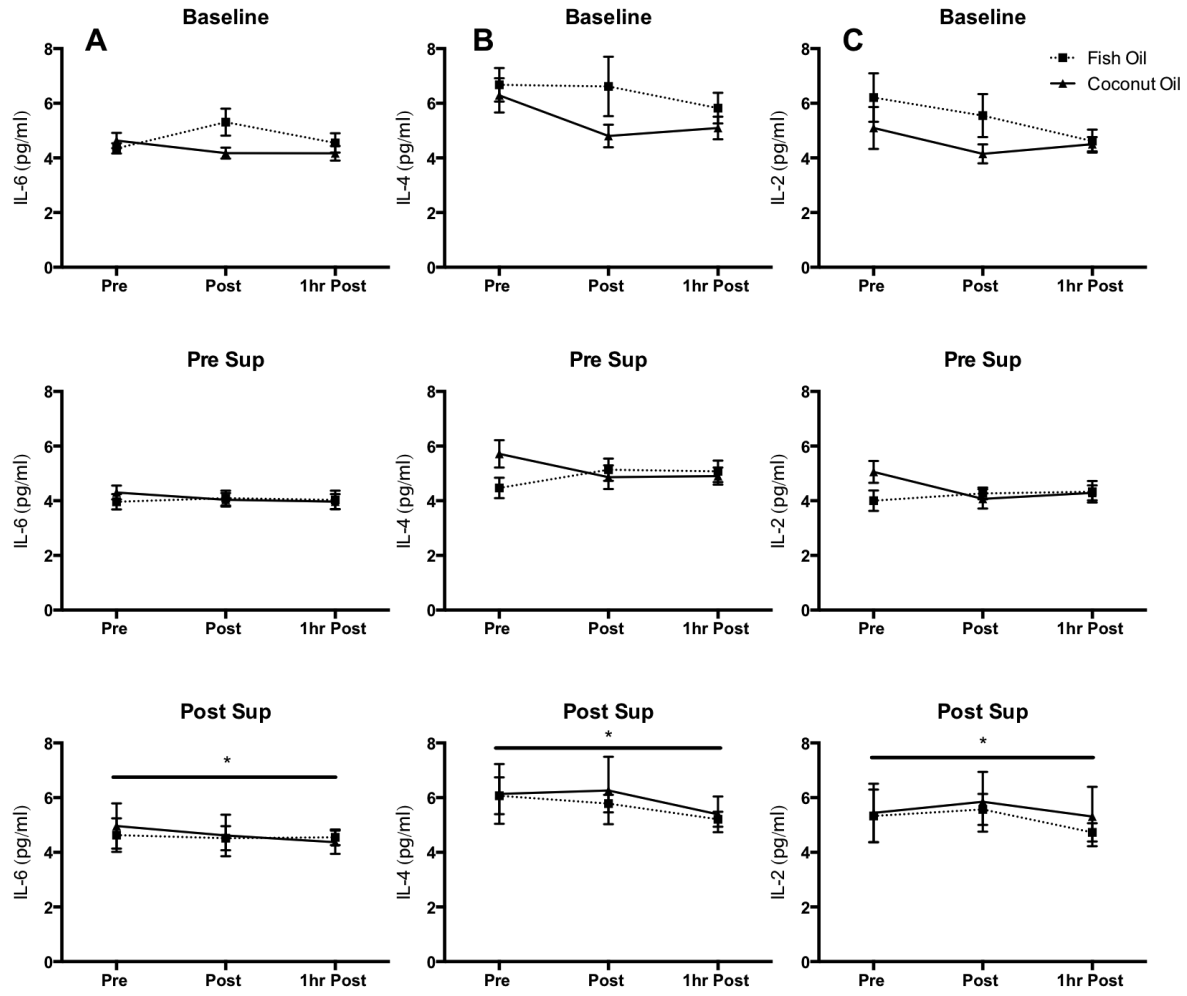

Fig 5. Plasma concentrations of cytokines IL-6 (A), IL-4 (B), IL-2 (C) values are mean ± SEM in pg/ml at pre, post and 1hr post exercise at the baseline, pre supplementation (Pre Sup) and post supplementation (Post Sup) visits in the fish oil and coconut oil groups. * indicates a significant main effect of day in both fish oil and coconut oil groups ($P<0.05$).

5.5 Discussion

Four weeks of supplementation with long chain n-3 PUFA or short chain SFA altered the blood fatty acid profile significantly. Supplementation with PUFA and SFA had no effect on the expression of the early activation marker CD69 on the surface of T-lymphocytes at rest or in the response to exercise. However, plasma concentrations at rest and following exercise of the Th1 cytokines TNF-α, IFN-γ and IL-2 and Th2 cytokines IL-10, IL-6 and IL-4 increased as a result of both PUFA and SFA supplementation compared with the control period.

During the supplementation period we observed an increase in blood n-3 PUFAs with a concomitant decrease in n-6 PUFAs in response to 4 weeks of fish oil supplementation. This observation supports a mechanism that could explain the anti-inflammatory benefits of PUFA's. Membrane lipids consist of both n-3 and n-6 PUFAs and many reviews and primary literature have been published detailing the immunomodulatory effects of fish oil supplementation on cell membrane lipid composition (Calder, 1997; Calder, 1998; Calder, 2006; Calder, 2010; Calder, 2011; Calder, 2012; Calder, 2013a; Calder, 2013b; Calder and Deckelbaum, 2014; Calder and Yaqoob, 2009; Calder and Yaqoob, 2009; Chapkin et al, 2008; Kim et al, 2010; Shaikh and Teague, 2012; Yaqoob, 2004; Colas et al 2014). The n-6 PUFA arachidonic acid (AA) is the precursor to pro-inflammatory signalling molecules, eicosanoids (Calder et al, 1992). Whereas eicosanoids derived from the n-3 PUFAs EPA and DHA are and pro-resolving (Kinsella and Lokesh, 1990). EPA is a precursor for E-series resolvins and DHA is a precursor for D-series resolvins, known to coordinate down regulation of inflammation and exert protection in experimental animal models of inflammatory disease (Hong et al, 2003; Hudert et al, 2006). Fish oil supplementation also increases E- and D- series resolvins in humans (Mas et al, 2012). As confirmed by our results supplementation with n-3 PUFAs like EPA and DHA results in an alteration of the

whole blood lipid order EPA and DHA are incorporated into the cell membrane at the expense of AA (Calder et al, 1994; Kew et al, 2004), likely resulting in a decrease in the precursor for pro-inflammatory eicosanoid production and an increase in the precursor for pro-resolving mediator production (Bagga et al, 2003; Wada and Tango, 2007).

In the current study we observed no effect of PUFA or SFA supplementation on the T-lymphocyte surface expression of CD69. CD69 is the earliest marker of activation expressed on the surface of an activated T-lymphocyte (Marzio et al, 1999). Previously no effect of supplementation with DHA alone has been observed on lymphocyte proliferation (Kelley et al, 1998; Thies et al, 2001) but a lower expression of CD69 indicating a lower level of activation was observed after 4 weeks of supplementation with 4.9g/day of DHA, 2004 (Kew et al, 2004). DHA disrupts lipid raft molecular organisation (Chapkin et al, 2008; Turk et al, 2013) indicating a possible role of DHA in alteration of lymphocyte function but not proliferation. In the present study, we observed no effect of an n-3 PUFA supplement that contained both EPA and DHA on surface T-lymphocyte expression of CD69 suggesting the function of DHA is different in the presence of EPA, or the DHA dose of 1.5g/day was too low to observe a change given the dose was 4.9g/day in the Kew study (Kew et al, 2004). We observed a higher level of CD69 activation in our samples than Kew et al (2004). One potential explanation could be that Kew used healthy but not trained participants. Our results indicated, an increase in activated $CD4^+CD69^+$ and $CD8^+CD69^+$ T-lymphocytes at the post exercise time point in both PUFA and SFA supplemented groups. This would suggest that higher level of CD69 activation at rest in our samples could be a result of regular exercise that regularly activates $CD4^+$ and $CD8^+$ T-lymphocytes. Thus, regular mobilisation could result in a higher level of CD69 activation at rest, however, a full explanation is not immediately apparent and follow-up analysis does not indicate that this was a result of freezing our cells (see appendix). Latent CMV virus was a significant covariate in the activated $CD4^+CD69^+$ T-lymphocytes but not the $CD8^+CD69^+$ T-lymphocytes. Suggesting

CMV infection results in an increased number and proportion of activated $CD4^+$ T-lymphocytes but not activated $CD8^+$ T-lymphocytes. This observation is contrary to what would be expected from previous literature, in which the CMV effect was greater in the $CD8^+$ T-lymphocyte population (Turner et al, 2011). However, the study was not designed to investigate CMV infection so the number of individuals that were CMV positive and CMV negative were not equal or matched, therefore I think there could be a power issue in the analysis.

Increased EPA and DHA in the cell membrane reputedly supresses inflammatory signalling via nuclear factor kappa-light-chain (NF-kB) (Lo et al, 1999; Novak et al, 2003; Zhao et al, 2004). Located in the cytosol of T-lymphocytes, NF-kB controls gene transcription (Gilmore and Herscovitch, 2006). Activation of the T-lymphocyte by the T-cell receptor and membrane associated recognition receptors like Toll-like receptors (TLRs) trigger a cascade of phosphorylation events resulting in activated NF-kB entering the nucleus to upregulate genes involved in T-lymphocyte development, maturation, proliferation and Th1 and Th2 cytokine production (Perkins, 2007). Recent studies suggest three mechanisms by which EPA and DHA exert anti-inflammatory properties through suppression of signalling via NF-kB. These are interfering with early membrane events involved in activation of NF-kB via TLR-4 (Wong et al, 2010), activation of the nuclear receptor PPARy which physically interacts with NF-kB preventing nuclear translocation (Kong et al, 2010; Zapata-Gonzalez et al, 2008) and action via G-protein coupled receptor GPR120 which initiates an anti-inflammatory signalling cascade that inhibits activation of NF-kB (Oh et al, 2010). However, Zeyda et al observed unaltered CD69 expression after supplementation with PUFA similar to the results of the current study suggesting the altered signalling mechanisms previously observed are independent of CD69 expression (Zeyda et al, 2003). Alterations in the fatty acid content of membranes suggest that an increased content of SFAs decreases the functional activity of immune cells, but an increased content of PUFAs increases their activity

(Kew et al, 2004). However, in the current study we observed no evidence of a difference between the SFA and PUFA groups for early T-lymphocyte activation marker despite whole blood lipid composition change over the 4 week supplementation period.

Supplementation with both PUFA and SFA resulted in an increase in the resting plasma concentrations of Th1 cytokines TNFa and IL-2 and Th2 cytokines IL-6 and IL-4 with a trend towards significance in Th1 cytokine IFN- γ and Th2 cytokine IL-10. The effect of PUFA supplementation on in vitro cytokine production has provided contrasting results ((Molvig et al, 1991; Soyland et al, 1994; Yaqoob et al, 2000). Similar to the current study, Trebble et al (2003) observed an increase in both the Th1 cytokine IFN- γ and a trend towards an increase in the Th2 cytokine IL-4 after supplementation with (up to 2g/d) of EPA and DHA (Trebble et al, 2003). However this was PBMC cytokine production in vitro and was not assessed in vivo through analysis of plasma cytokine concentrations. The current study observed no difference in the plasma concentrations of Th1 or Th2 cytokines in response to the exercise protocol. Similarly, Gray et al observed no difference in the Th2 cytokine IL-6 immediately and up to 3 hours after 1-hour of cycling at 70% VO_{2max} and no effect of supplementation with 3g/day of EPA and DHA on plasma IL-6 (Grey et al, 2012). Contrastingly, (Toft et al, 2000) observed an increase in the plasma concentration of the Th1 cytokine TNF-α and the Th2 cytokine IL-6 in response to a marathon but no effect of 6 weeks supplementation with 6g/day of EPA and DHA. These observations suggest that exercise intensity and duration rather than an alteration in cell lipid composition drives the release of these cytokines. Supplementation with 2.4g/day of EPA and DHA for 6 weeks had no effect on plasma Th1 and Th2 cytokine concentrations in response to 3 days of intense exercise (Nieman et al, 2009). It would appear that the longer duration of exercise in the Toft study is the reason for the increase in plasma TNF-α and IL-6 observed post exercise and supported by previous studies (Drenth et al, 1995; Northoff and Berg, 1991; Ostrowski et al, 1998; Ostrowski et al, 1999; Pedersen et al, 1998). Differences in dose between studies

could also be responsible for the inconsistent findings (Caughey et al, 1996; Yaqoob, 2003). It has been previously speculated that the relationship between nutrients and immune function is 'bell shaped' (Chandra, 1991) suggesting increased ingestion of PUFAs due to habitual dietary intake increases immune cell function by altering the plasma membrane thus altering the Th1 and Th2 cytokine production. Intakes of n-3 PUFAs higher than habitual intake decrease immune cell function by producing plasma membranes that are too fluid (Gonzalez et al, 2000). Alterations in eicosanoid profiles (Caughey et al, 1996) and effects on cell signalling (Denys et al, 2001; Miles and Calder, 1998) will also alter the genes which are upregulated, and will subsequently alter cytokine production. The high dose of both PUFA and short chain SFA supplementation used in this study, much higher than habitual intake, could be masking any differences between the PUFA and SFA supplemented Th1 and Th2 cytokine responses. The general increase in plasma cytokine concentration is not specific to Th1 or Th2 cytokines, or to the PUFA or SFA supplementation, suggesting that the increase could simply be the response to an overall dose of PUFA and SFA that is just too high.

The increase in plasma concentrations of both Th1 and Th2 cytokines after supplementation for 4 weeks with both SFA and PUFA can, therefore, not be fully explained. Previous studies that have investigated cytokine concentrations have such contrasting levels it makes it difficult for a comparison to be made or they have measured a single cytokine. The natural variation between participants (Calder and Kew, 2002) means for a difference to be significant it would have to be very large. Latent CMV infection was a significant covariate for all the Th1 and Th2 cytokines measured in this study suggesting CMV is related to the Th1 and Th2 cytokine concentrations, but not to any one specific cytokine or family of cytokines in particular. Given that the changes observed in this study are not specific to a single cytokine or a family of cytokines, and that they are not specific to the PUFA or the SFA supplementation and CMV infection is accounted for in the analysis, it would seem that the

changes are a result of natural variation in plasma cytokine levels combined with a supplementation of a PUFA or SFA at a dose that is higher than habitual intake. It is possible that the high dose masked any differences between the fatty acid supplementation groups. The lack of clear evidence of cytokine responses reflects the lack of changes in lymphocyte subset proportions observed in Chapter 4, and suggests that supplementation with PUFA or SFA has no beneficial effects on T-lymphocyte proportions, T-lymphocyte activation, or cytokine release. These observations highlight that supplementation with PUFA and SFA at the dose used in the present study is not recommended in a young, healthy, trained, male population for immune function purposes. However, future dose-response investigations would seem prudent before ruling out any possible beneficial effects of these fatty acid supplements.

5.6 References

Bagga, D., Wang, L., Farias-Eisner, R., Glaspy, J.A., Reddy, S.T., 18-2-2003. Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc. Natl. Acad. Sci. U. S. A 100, 1751-1756.

Banchereau, J., Pascual, V., O'Garra, A., 2012. From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. Nat. Immunol. 13, 925-931.

Betz, M. , Fox, B.S., 1-1-1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J. Immunol. 146, 108-113.

Bladbjerg, E.M., Larsen, T.M., Due, A., Stender, S., Astrup, A., Jespersen, J., 2011. Effects on markers of inflammation and endothelial cell function of three ad libitum diets differing in type and amount of fat and carbohydrate: a 6-month randomised study in obese individuals. Br. J. Nutr. 106, 123-129.

Calder, P.C., 1997. n-3 polyunsaturated fatty acids and cytokine production in health and disease. Ann. Nutr. Metab 41, 203-234.

Calder, P.C., 1998. Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Braz. J. Med. Biol. Res. 31, 467-490.

Calder, P.C., 2006. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot. Essent. Fatty Acids 75, 197-202.

Calder, P.C., 2007. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot. Essent. Fatty Acids 77, 327-335.

Calder, P.C., 2010. Omega-3 fatty acids and inflammatory processes. Nutrients. 2, 355-374.

Calder, P.C., 2011. Fatty acids and inflammation: the cutting edge between food and pharma. Eur. J. Pharmacol. 668 Suppl 1, S50-S58.

Calder, P.C., 2012a. Mechanisms of action of (n-3) fatty acids. J. Nutr. 142, 592S-599S.

Calder, P.C., 2012b. The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability. Mol. Nutr. Food Res. 56, 1073-1080.

Calder, P.C., 2013. n-3 fatty acids, inflammation and immunity: new mechanisms to explain old actions. Proc. Nutr. Soc. 72, 326-336.

Calder, P.C., 2013. Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology? Br. J. Clin. Pharmacol. 75, 645-662.

Calder, P.C., Bevan, S.J., Newsholme, E.A., 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. Immunology 75, 108-115.

Calder, P.C. , Deckelbaum, R.J., 2014. Dietary fatty acids in health and disease: greater controversy, greater interest. Curr. Opin. Clin. Nutr. Metab Care 17, 111-115.

Calder, P.C. , Kew, S., 2002. The immune system: a target for functional foods? Br. J. Nutr. 88 Suppl 2, S165-S177.

Calder, P.C. , Yaqoob, P., 2009a. Omega-3 polyunsaturated fatty acids and human health outcomes. Biofactors 35, 266-272.

Calder, P.C. , Yaqoob, P., 2009b. Understanding omega-3 polyunsaturated fatty acids. Postgrad. Med. 121, 148-157.

Calder, P.C., Yaqoob, P., Harvey, D.J., Watts, A., Newsholme, E.A., 1-6-1994. Incorporation of fatty acids by concanavalin A-stimulated lymphocytes and the effect on fatty acid composition and membrane fluidity. Biochem. J. 300 (Pt 2), 509-518.

Caughey, G.E., Mantzioris, E., Gibson, R.A., Cleland, L.G., James, M.J., 1996. The effect on human tumor necrosis factor alpha and interleukin 1 beta production of diets enriched in n-3 fatty acids from vegetable oil or fish oil. Am. J. Clin. Nutr. 63, 116-122.

Chandra, R.K., 1991. Interactions between early nutrition and the immune system. Ciba Found. Symp. 156, 77-89.

Chapkin, R.S., McMurray, D.N., Davidson, L.A., Patil, B.S., Fan, Y.Y., Lupton, J.R., 2008. Bioactive dietary long-chain fatty acids: emerging mechanisms of action. Br. J. Nutr. 100, 1152-1157.

Colas RA, Shinohara M, Dalli J, Chiang N, Serhan CN., 2014. Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. Am J Physiol Cell Physiol. 1;307(1):C39-54

Denys, A., Hichami, A., Khan, N.A., 2001. Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase (ERK1/ERK2) signaling in human T cells. J. Lipid Res. 42, 2015-2020.

Drenth, J.P., Van Uum, S.H., Van, D.M., Pesman, G.J., Van, d., V, Van der Meer, J.W., 1995. Endurance run increases circulating IL-6 and IL-1ra but downregulates ex vivo TNF-alpha and IL-1 beta production. J. Appl. Physiol (1985.) 79, 1497-1503.

Fedyk, E.R., Brown, D.M., Phipps, R.P., 1997. PGE2 regulation of B lymphocytes and T helper 1 and T helper 2 cells: induction of inflammatory versus allergic responses. Adv. Exp. Med. Biol. 407, 237-242.

Ferrucci, L., Cherubini, A., Bandinelli, S., Bartali, B., Corsi, A., Lauretani, F., Martin, A., ndres-Lacueva, C., Senin, U., Guralnik, J.M., 2006. Relationship of plasma polyunsaturated fatty acids to circulating inflammatory markers. J. Clin. Endocrinol. Metab 91, 439-446.

Galli, C. , Calder, P.C., 2009. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. Ann. Nutr. Metab 55, 123-139.

Gilmore, T.D. , Herscovitch, M., 30-10-2006. Inhibitors of NF-kappaB signaling: 785 and counting. Oncogene 25, 6887-6899.

Gonzalez, B., Iturralde, M., Alava, M.A., Anel, A., Pineiro, A., 2000. Metabolism of n -9, n -6 and n -3 fatty acids in hepatoma Morris 7777 cells. Preferential accumulation of linoleic acid in cardiolipin. Prostaglandins Leukot. Essent. Fatty Acids 62, 299-306.

Goodwin, J.S., Bankhurst, A.D., Messner, R.P., 1-12-1977. Suppression of human T-cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J. Exp. Med. 146, 1719-1734.

Gray, P., Gabriel, B., Thies, F., Gray, S.R., 2012. Fish oil supplementation augments post-exercise immune function in young males. Brain Behav. Immun. 26, 1265-1272.

Hilkens, C., Snijders, A., Vermeulen, H., van der, M.P., Wierenga, E., Kapsenberg, M., 31-10-1996. Accessory cell-derived interleukin-12 and prostaglandin E2 determine the level of interferon-gamma produced by activated human CD4+ T cells. Ann. N. Y. Acad. Sci. 795, 349-350.

Hong, S., Gronert, K., Devchand, P.R., Moussignac, R.L., Serhan, C.N., 25-4-2003. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. J. Biol. Chem. 278, 14677-14687.

Hudert, C.A., Weylandt, K.H., Lu, Y., Wang, J., Hong, S., Dignass, A., Serhan, C.N., Kang, J.X., 25-7-2006. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. Proc. Natl. Acad. Sci. U. S. A 103, 11276-11281.

Kelley, D.S., Taylor, P.C., Nelson, G.J., Mackey, B.E., 1998. Dietary docosahexaenoic acid and immunocompetence in young healthy men. Lipids 33, 559-566.

Kew, S., Mesa, M.D., Tricon, S., Buckley, R., Minihane, A.M., Yaqoob, P., 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am. J. Clin. Nutr. 79, 674-681.

Kim, W., Khan, N.A., McMurray, D.N., Prior, I.A., Wang, N., Chapkin, R.S., 2010. Regulatory activity of polyunsaturated fatty acids in T-cell signaling. Prog. Lipid Res. 49, 250-261.

Kinsella, J.E. , Lokesh, B., 1990. Dietary lipids, eicosanoids, and the immune system. Crit Care Med. 18, S94-113.

Kong, W., Yen, J.H., Vassiliou, E., Adhikary, S., Toscano, M.G., Ganea, D., 2010. Docosahexaenoic acid prevents dendritic cell maturation and in vitro and in vivo expression of the IL-12 cytokine family. Lipids Health Dis. 9, 12-

LaVoy, E.C., Bosch, J.A., Lowder, T.W., Simpson, R.J., 2013. Acute aerobic exercise in humans increases cytokine expression in CD27(-) but not CD27(+) CD8(+) T-cells. Brain Behav. Immun. 27, 54-62.

Lo, C.J., Chiu, K.C., Fu, M., Lo, R., Helton, S., 1999. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF kappa B activity. J. Surg. Res. 82, 216-221.

Marzio, R., Mauel, J., Betz-Corradin, S., 1999. CD69 and regulation of the immune function. Immunopharmacol. Immunotoxicol. 21, 565-582.

Mas, E., Croft, K.D., Zahra, P., Barden, A., Mori, T.A., 2012. Resolvins D1, D2, and other mediators of self-limited resolution of inflammation in human blood following n-3 fatty acid supplementation. Clin. Chem. 58, 1476-1484.

Meydani, S.N., Endres, S., Woods, M.M., Goldin, B.R., Soo, C., Morrill-Labrode, A., Dinarello, C.A., Gorbach, S.L., 1991. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J. Nutr. 121, 547-555.

Miles, E.A., Banerjee, T., Wells, S.J., Calder, P.C., 2006. Limited effect of eicosapentaenoic acid on T-lymphocyte and natural killer cell numbers and functions in healthy young males. Nutrition 22, 512-519.

Miles, E.A. , Calder, P.C., 1998. Modulation of immune function by dietary fatty acids. Proc. Nutr. Soc. 57, 277-292.

Molvig, J., Pociot, F., Worsaae, H., Wogensen, L.D., Baek, L., Christensen, P., Mandrup-Poulsen, T., Andersen, K., Madsen, P., Dyerberg, J., ., 1991. Dietary supplementation with omega-3-polyunsaturated fatty acids decreases mononuclear cell proliferation and interleukin-1 beta content but not monokine secretion in healthy and insulin-dependent diabetic individuals. Scand. J. Immunol. 34, 399-410.

Nieman, D.C., Henson, D.A., McAnulty, S.R., Jin, F., Maxwell, K.R., 2009. n-3 polyunsaturated fatty acids do not alter immune and inflammation measures in endurance athletes. Int. J. Sport Nutr. Exerc. Metab 19, 536-546.

Northoff, H. , Berg, A., 1991. Immunologic mediators as parameters of the reaction to strenuous exercise. Int. J. Sports Med. 12 Suppl 1, S9-15.

Novak, T.E., Babcock, T.A., Jho, D.H., Helton, W.S., Espat, N.J., 2003. NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription. Am. J. Physiol Lung Cell Mol. Physiol 284, L84-L89.

Oh, D.Y., Talukdar, S., Bae, E.J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W.J., Watkins, S.M., Olefsky, J.M., 3-9-2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell 142, 687-698.

Ostrowski, K., Hermann, C., Bangash, A., Schjerling, P., Nielsen, J.N., Pedersen, B.K., 15-12-1998. A trauma-like elevation of plasma cytokines in humans in response to treadmill running. J. Physiol 513 (Pt 3), 889-894.

Ostrowski, K., Rohde, T., Asp, S., Schjerling, P., Pedersen, B.K., 15-2-1999. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J. Physiol 515 (Pt 1), 287-291.

Pedersen, B.K., Ostrowski, K., Rohde, T., Bruunsgaard, H., 1998. The cytokine response to strenuous exercise. Can. J. Physiol Pharmacol. 76, 505-511.

Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat. Rev. Mol. Cell Biol. 8, 49-62.

Shaikh, S.R. , Teague, H., 2012. N-3 fatty acids and membrane microdomains: from model membranes to lymphocyte function. Prostaglandins Leukot. Essent. Fatty Acids 87, 205-208.

Simopoulos, A.P., 2007. Omega-3 fatty acids and athletics. Curr. Sports Med. Rep. 6, 230-236.

Simpson, R.J., Florida-James, G.D., Whyte, G.P., Guy, K., 2006. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur. J. Appl. Physiol 97, 109-121.

Soyland, E., Lea, T., Sandstad, B., Drevon, A., 1994. Dietary supplementation with very long-chain n-3 fatty acids in man decreases expression of the interleukin-2 receptor (CD25) on mitogen-stimulated lymphocytes from patients with inflammatory skin diseases. Eur. J. Clin. Invest 24, 236-242.

Thies, F., Miles, E.A., Nebe-von-Caron, G., Powell, J.R., Hurst, T.L., Newsholme, E.A., Calder, P.C., 2001. Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids 36, 1183-1193.

Toft, A.D., Thorn, M., Ostrowski, K., Asp, S., Moller, K., Iversen, S., Hermann, C., Sondergaard, S.R., Pedersen, B.K., 2000. N-3 polyunsaturated fatty acids do not affect cytokine response to strenuous exercise. J. Appl. Physiol (1985.) 89, 2401-2406.

Trebble, T.M., Wootton, S.A., Miles, E.A., Mullee, M., Arden, N.K., Ballinger, A.B., Stroud, M.A., Burdge, G.C., Calder, P.C., 2003. Prostaglandin E2 production and T cell function after fish-oil supplementation: response to antioxidant cosupplementation. Am. J. Clin. Nutr. 78, 376-382.

Turk, H.F., Monk, J.M., Fan, Y.Y., Callaway, E.S., Weeks, B., Chapkin, R.S., 1-5-2013. Inhibitory effects of omega-3 fatty acids on injury-induced epidermal growth factor receptor transactivation contribute to delayed wound healing. Am. J. Physiol Cell Physiol 304, C905-C917.

Wada, M. , Tango, T., 2007. [Development of a new estimation method of ingestion time to suspected food in food-poisoning outbreaks]. Shokuhin Eiseigaku Zasshi 48, 27-34.

Wong, C.K., Wong, P.T., Tam, L.S., Li, E.K., Chen, D.P., Lam, C.W., 2010. Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus. Clin. Exp. Immunol. 159, 11-22.

Yaqoob, P., 2003. Lipids and the immune response: from molecular mechanisms to clinical applications. Curr. Opin. Clin. Nutr. Metab Care 6, 133-150.

Yaqoob, P., 2004. Fatty acids and the immune system: from basic science to clinical applications. Proc. Nutr. Soc. 63, 89-104.

Yaqoob, P., Pala, H.S., Cortina-Borja, M., Newsholme, E.A., Calder, P.C., 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur. J. Clin. Invest 30, 260-274.

Zapata-Gonzalez, F., Rueda, F., Petriz, J., Domingo, P., Villarroya, F., az-Delfin, J., de Madariaga, M.A., Domingo, J.C., 2008. Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly through PPAR(gamma):RXR heterodimers: comparison with other polyunsaturated fatty acids. J. Leukoc. Biol. 84, 1172-1182.

Zeyda, M., Szekeres, A.B., Saemann, M.D., Geyeregger, R., Stockinger, H., Zlabinger, G.J., Waldhausl, W., Stulnig, T.M., 15-6-2003. Suppression of T cell signaling by polyunsaturated fatty acids: selectivity in inhibition of mitogen-activated protein kinase and nuclear factor activation. J. Immunol. 170, 6033-6039.

Zhao, Y., Joshi-Barve, S., Barve, S., Chen, L.H., 2004. Eicosapentaenoic acid prevents LPS-induced TNF-alpha expression by preventing NF-kappaB activation. J. Am. Coll. Nutr. 23, 71-78.

CHAPTER 6

CHAPTER 6: General Discussion

6.1 Introduction

The resting T-lymphocyte pool is known to be influenced by many factors: infection (Mallia et al, 2014) age; sleep; baseline fitness; macronutrient intake; training volume; body fat; and sex. However, the effects of these factors, and the T-lymphocyte populations mobilised in response to exercise, are not fully understood. Additionally, the effect of different fatty acid supplementation protocols (saturated fats vs. polyunsaturated fats) on the T-lymphocyte pool and response to exercise is currently unknown. The novel data in this thesis may enhance our understanding of different T-lymphocyte subsets at rest, and their response to acute exercise. By investigating factors that could impact on the T-lymphocyte population responses, like training status, sex, increased training volume, PUFA supplementation and latent CMV infection, the thesis provides the framework to guide further investigations of the adaptive immune response in male and female exercisers.

Chapter 2 investigated the effect of training status and sex on senescent T-lymphocytes at rest and in response to acute maximal exercise. A training status difference was observed with the untrained group with a higher proportion of senescent and a lower proportion of naïve $CD8^+$ T-lymphocytes than the trained group. A sex effect was also observed with the male participants having a higher proportion of senescent and a lower proportion of naïve T-lymphocytes than the females. It also appeared that the untrained male population was driving both the training status and sex differences observed in this study. Speculatively, the sex differences observed may be due to the protective effect of oestrogen against telomere shortening, with less senescent T-lymphocytes observed in females. The training status differences observed are likely due to a more regular mobilisation of senescent T-lymphocytes in the trained compared to the untrained, resulting in a lower resting population of senescent T-lymphocytes in trained individuals.

Chapter 3 compared the T-lymphocyte composition changes over time in a trained group that trained hard, versus an untrained group that did not train hard, and investigated the impact of increasing training volume in the trained group. It was observed that the increase in training volume over a 2 week period had no effect on T-lymphocyte populations in trained females. However, there were differences in the proportions of $\gamma\delta^+$, $CD4^+$ and the ratio of $CD4^+$:$CD8^+$ T-lymphocytes between the trained and untrained groups. Although the study was not designed to manipulate diet, we observed increased macronutrient intake, above normal, in the trained group during the increased training volume period. The lack of change in T-lymphocyte populations in the trained group after a period of increased training volume indicates that macronutrient intake was likely sufficient for optimal recovery or that they're used to training.

Chapter 4 assessed the effect of 4 weeks supplementation with short chain SFA versus n-3 PUFA on T-lymphocyte populations at rest and in response to acute exercise. A difference between groups was evident for the proportion and number of $\gamma\delta^+$ T-lymphocytes but there was no conclusive evidence to suggest that the difference was related to the supplementation period.

Chapter 5 continuing on from Chapter 4 examined the effect of 4 weeks of supplementation with short chain SFA versus n-3 PUFA on the early T-lymphocyte activation marker CD69 and the plasma cytokine concentrations of Th1 and Th2 cytokines. No differences in CD69 expression were observed between the supplemented groups. Plasma concentrations of both Th1 and Th2 cytokines increased after supplementation in both groups possibly highlighting the impact of high dose supplementation on cytokine response.

From the body of work as a whole what can we conclude about acute exercise responses, training status, increases in training volume, diet manipulation, gender, and CMV serostatus? What does the thesis contribute to the literature?

Two theories exist surrounding the senescent T-lymphocyte response to exercise. These theories are not opposing, they could exist alongside each other, but they both focus on a slightly different aspect of the response. The first model proposed by Dhabhar et al (2012) suggests that the preferential influx of cytotoxic senescent $CD8^+$ T-lymphocytes to the peripheral tissues with retained effector killing functions following exercise (Campbell et al, 2008; Simpson et al, 2007; Simpson et al, 2008; Turner et al, 2010) is important for immunosurveillance (Dhabhar et al, 2012). The mechanisms of the model suggest that in response to a stressor (i.e. exercise) naïve T-lymphocytes traffic to the lymph nodes where they come into contact with novel antigens. Concomitantly, antigen-experienced effector and memory T-lymphocytes traffic to peripheral tissues like the skin, lung or mucosal lining of the gut where they encounter familiar antigens. In agreement with this model Bosch et al (2003) observed a selective mobilisation of T-lymphocytes primed for inflammation in response to an acute stressor (Bosch et al, 2003). The data presented in this thesis support this theory. We observed a decrease in naïve T-lymphocyte proportions immediately post exercise suggesting that naïve T-lymphocytes have trafficked to the lymph nodes during the exercise bout with a return to baseline in the 1 hour post exercise. We also observed an increase in senescent T-lymphocytes during exercise suggesting they are mobilised into the blood stream during exercise, and in the observed egress in the 1 hour post exercise they have trafficked to the peripheral tissues (Figure 1).

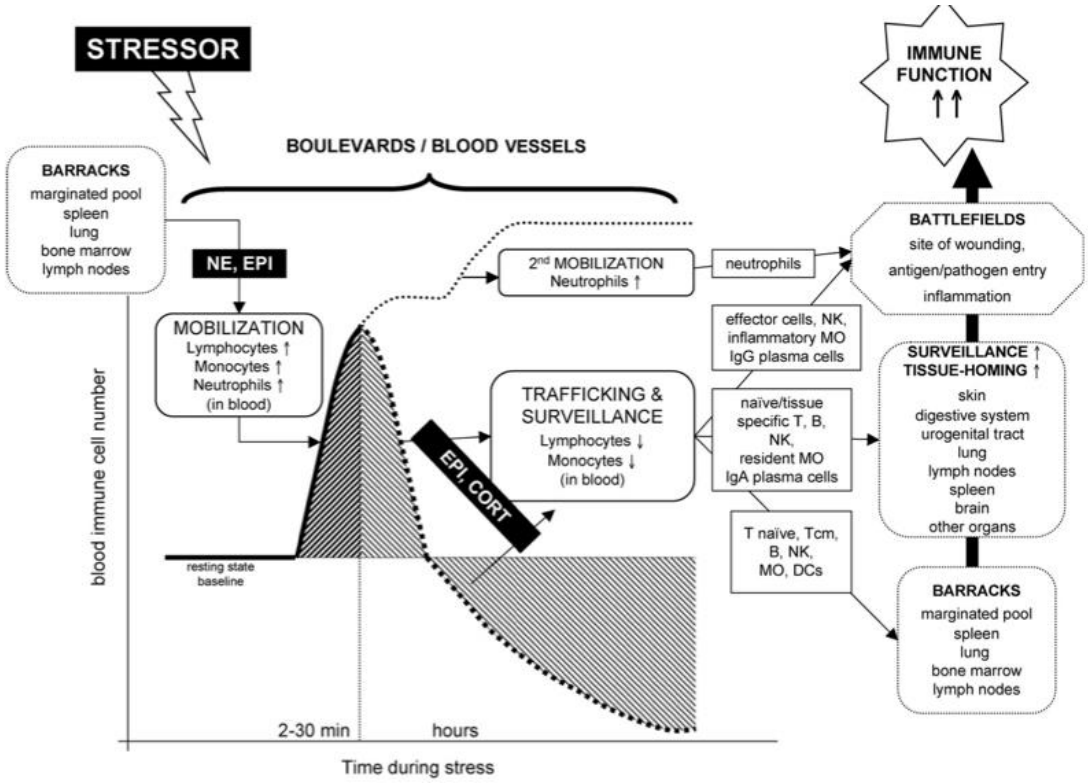

Fig 1. Model of stress-induced changes in leukocyte distribution from Dhabhar et al 2012. It is based on data presented in the literature (Dhabhar, 2009; Dhabhar et al, 2012a; Dhabhar and McEwen, 1996; Dhabhar and McEwen, 1997; Dhabhar and Viswanathan, 2005; Rosenberger et al, 2009; Viswanathan and Dhabhar, 2005). Norepinephrine (NE), epinephrine (EPI), and corticosterone (CORT), monocyte (MO).

The second theory proposed by Simpson et al (2011) relates to the acute response to exercise but also the effect of regular training on the senescent T-lymphocyte population. In response to exercise, senescent T-lymphocytes are mobilised and subsequently egress to the peripheral tissues where they experience a proapoptotic environment (Kruger et al, 2008). Simpson et al (2011) propose a negative feedback loop that controls the peripheral T-lymphocyte numbers. Hence, when senescent T-lymphocytes undergo apoptosis, peripheral T-lymphocyte numbers are low. The subsequent feedback loop increases the output of naïve T-lymphocytes from the thymus and thus restores the peripheral T-lymphocyte pool. If the feedback loop were to be apparent, it would occur more frequently in trained participants, who undergo regular bouts of exercise. While this is not a widely accepted theory and further investigation is warranted, our observation of a blunted senescent T-lymphocyte response,

together with an increased naïve T-lymphocyte response to exercise in trained compared to untrained, lend support to the theory that regular exercise training mobilises senescent T-lymphocytes and thus frees up 'immune space' for naïve T-lymphocytes. From the work of the current thesis this model seems to apply to males but possibly not females (Figure 2).

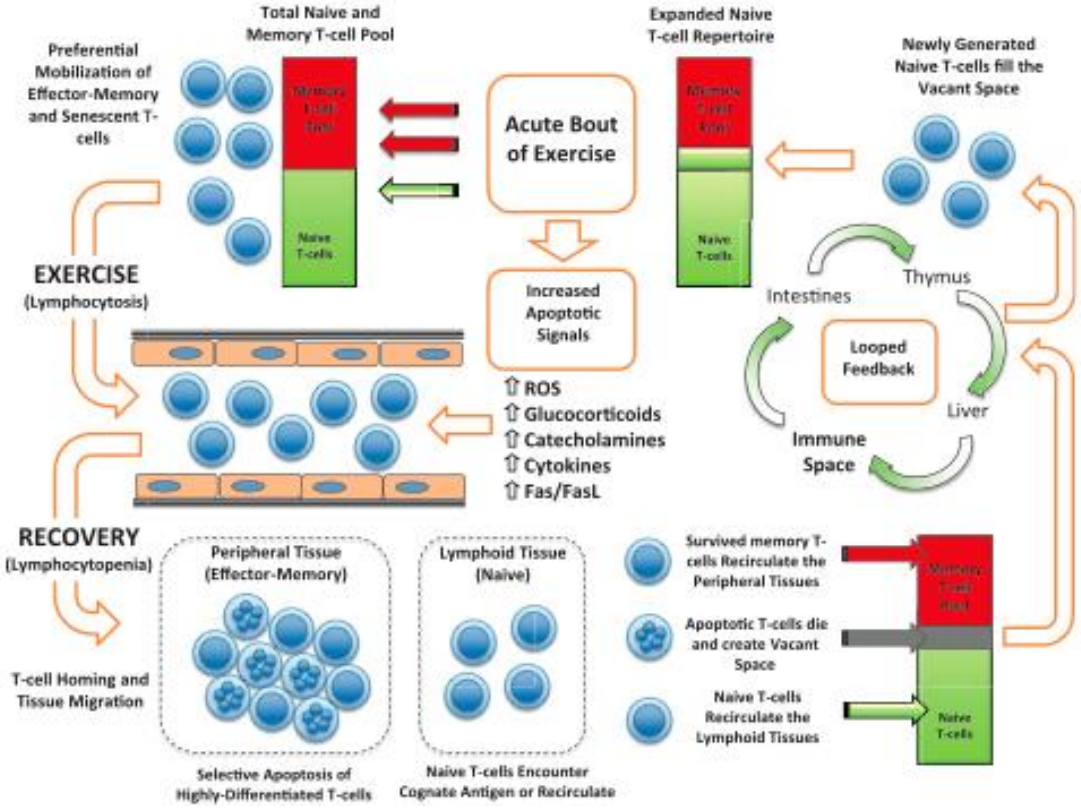

Fig 2. A model taken from Simpson et al (2011) demonstrating the effect of repeated bouts of acute exercise on the immune space.

Both of the above theories suggest that the mobilisation and subsequent egress of senescent T-lymphocytes in response to exercise is a beneficial response. Accumulation of the senescent T-lymphocyte population, and a diminished naïve T-lymphocyte population at rest, has been linked to the increase in infection susceptibility in older adults. This has not been tested experimentally but it is assumed to be due to the lesser ability of senescent T-lymphocytes at recognising new infections. Both of these theories highlight the importance of a balance in the T-lymphocyte populations within the T-lymphocyte pool.

To really answer these questions in the future we need to understand exactly where the senescent T-lymphocytes are coming from into the blood in response to an exercise

stimulus, and where they end up after exercise. This could be done by obtaining tissue samples in human participants. Animal studies have found that lymphocytes migrate to the lungs and intestinal Peyer patches after exercise (Kruger et al, 2008; Kruger et al, 2009). At this stage experiments like that are not possible in humans. Even a muscle tissue biopsy itself would cause a significant inflammatory response thus making it difficult to differentiate what was a result of exercise and what was a response to the muscle insult.

A slight limitation that could have provided a more in depth insight into the training status differences observed is hormone measurements. Current thinking suggests that stress hormones influence the differential redistribution of T-lymphocytes (Landmann, 1992). Due to the descriptive nature of Chapter 2, no measurements of stress hormones (i.e., epinephrine and cortisol) concentrations in blood were collected. Previous work has demonstrated a smaller epinephrine response to submaximal exercise in trained compared to untrained individuals (Hong et al, 2005; Kjaer et al, 1985; Vinten and Galbo, 1983). A lower epinephrine response in trained would be expected to be associated with a lower redistribution of senescent cells into the blood. However, at maximal exercise intensity, we would expect trained participants to have a higher epinephrine response (Kjaer et al, 1985). Thus, the present data imply, albeit indirectly, that training status may override the impact of stress hormones on the redistribution of senescent cells in response to exercise. Thus, measurement of hormones would have allowed us to directly compare the training status vs stress hormone response on the senescent T-lymphocyte response and identify if training status overrides the stress hormone impact.

One unexplored explanation for the differences in T-lymphocytes observed between trained and untrained populations is the role of diet composition. Previous work has observed carbohydrate ingestion during exercise results in a lower post-exercise lymphocytosis

(Henson et al, 1999; Lancaster et al, 2003; Nieman et al, 1997; Nieman et al, 2004; Timmons et al, 2004). However, this has not been confirmed by other groups (Bishop et al, 2005; Green et al, 2003; Henson et al, 2004; McFarlin et al, 2004; Nieman and Bishop, 2006; Peake et al, 2008). During the increased training volume study reported in Chapter 3 dietary intake was monitored. It was observed that the trained consumed sufficient carbohydrate to prevent any impaired mobilisation of T-lymphocytes. An impaired mobilisation has previously only been observed elsewhere in a study that fixed carbohydrate intake at a low level during high intensity training (Witard et al, 2012). A further study by these same authors demonstrated that increased protein intake restored impaired T-lymphocyte mobilisation during high intensity training when energy balance was matched between high and low intensity exercise groups through an increase in dietary fat (Witard et al, 2013). We observed in chapter 3 that our trained group consumed significantly more protein, monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids during the high intensity period of increased volume training than the habitual diet of an untrained group. Of the dietary PUFA's, omega-3 (*n*-3) fatty acids, that consist primarily of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), are abundant in oily fish sources. These n-3 PUFAs are also known to exhibit strong anti-inflammatory properties (Thies et al, 2003) leading to dietary fish oils being implicated in neurological and chronic inflammatory diseases, such as Alzheimer's (Samieri et al, 2008b; Samieri et al, 2008a), diabetes mellitus (Nettleton and Katz, 2005) and cardiovascular disease (Lemaitre et al, 2003). PUFA supplementation studies have observed contrasting results of immune/inflammatory markers and T-lymphocyte function in both supplementation alone and supplementation combined with exercise studies. The contrasting results could be explained by variations in the experimental design of the various studies. Dose, duration and placebo used differ between studies, some studies use a single cell model and others use a whole body model. A suitable placebo for comparisons with *n*-3 PUFA must be a fatty acid that does not alter the *n*-6:*n*-3 ratio. Corn oil is used in many studies as a placebo, however, corn oil is an *n*-6 fatty acid therefore it will alter the *n*-6:*n*-3 ratio and observations from studies using corn oil as a

placebo should therefore be interpreted with caution. Olive oil is a suitable placebo, it is a *n*-9 PUFA therefore would not alter the *n*-3:*n*-6 ratio and it is believed that the coconut oil used in Chapters 4 and 5 is also a suitable placebo. Coconut oil is comprised of short chain saturated fatty acids therefore does not alter the *n*-3:*n*-6 ratio. However, it is worth mentioning at this point that recent health related benefits have been associated with coconut oil (Amarasiri and Dissanayake, 2006). One recent study observed antioxidant and anti-inflammatory effects of coconut oil on adjuvant induced arthritis in rats (Vysakh et al, 2014). Although their study used an animal model and has not been investigated in humans, it could highlight a reason for the coconut and fish oil groups similar cytokine response to the supplementation period observed in Chapter 5.

Various studies have used different experimental models for investigating the effects of PUFA on immune function. Often single cell models are used to identify the mechanisms but it can be difficult to replicate the findings in whole body models and vice versa. In Chapters 4 and 5 we used a whole body model to observe the outcome of supplementation with n-3 PUFA or SFA. Therefore, we can only speculate about the mechanisms using the outcomes observed. In future n-3 PUFA supplementation studies due care should be applied to the dose, duration, placebo and experimental model used. A combination of isolated single cell in vitro mechanistic work in combination with the in vivo whole body response in the same participants is worth investigating. The immune system is so diverse that any future supplementation studies should look to combine more than one supplement, potentially in a carbohydrate beverage (Bakker et al, 2010) as this would probably work better than one supplement on its own.

Speculatively, the sex-difference in T-lymphocyte redistribution could be explained by hormonal differences between sexes, namely oestrogen concentration. T-lymphocytes are

known to express oestrogen receptors and the $CD8^+$ T-lymphocyte subset binds oestrogen with high affinity (Cutolo et al, 1995). Indeed, cell culture studies reveal that in the presence of 17 B-estradiol (E2), the ratio of activated $CD4^+/CD8^+$ human T-lymphocytes is decreased (Athreya et al, 1993). Moreover, the promoter region of interferon gamma (IFN y) appears to be positively modulated by oestrogen (Betz and Fox, 1991). In turn, IFN-y is produced by senescent T-lymphocytes and has been reported to up-regulate the enzyme telomerase in lymphoid cell lines (Xu et al, 2000). The hormonal milieu is known to change cyclically with menstrual cycle phase (Oertelt-Prigione, 2012). Reductions in $CD4^+$ T-lymphocyte number have been observed in the luteal compared to early follicular phase of the cycle (Lee et al, 2010). It is unknown what influence a changing hormonal milieu will have on senescent T-lymphocytes. Interestingly, we observed no difference in the redistribution of senescent T-lymphocytes in response to exercise between trained and untrained females, whereas a difference was observed between trained and untrained males. This observation suggests a sex-specific effect of training status on the redistribution of senescent T-lymphocytes. Our observations also support the notion that oestrogen may provide a preventative mechanism against T-lymphocyte telomere shortening in females that could possibly have contributed to the lower proportion of senescent $CD4^+$ and $CD8^+$ T-lymphocytes at rest and in response to exercise. However, menstrual cycle phase was not controlled for and no hormone measurements were collected. Therefore, to fully understand the sex effect observed in Chapter 2 further investigation into the effect of exercise on the T-lymphocyte population at rest and in response to exercise at different phases of the menstrual cycle is important.

In older people infected with CMV a large proportion of their memory T-lymphocyte pool are specific for CMV virus resulting in a higher proportion of senescent T-lymphocytes and a lower proportion of naïve T-lymphocytes (Simpson, 2011). The accumulation of CMV viral specific T-lymphocytes ‘squeezes’ the memory T-lymphocytes for other antigens out of the memory pool increasing the risk of infection with a new antigen, but also with a previously encountered antigen that the memory T-lymphocytes have been squeezed out (Figure 3).

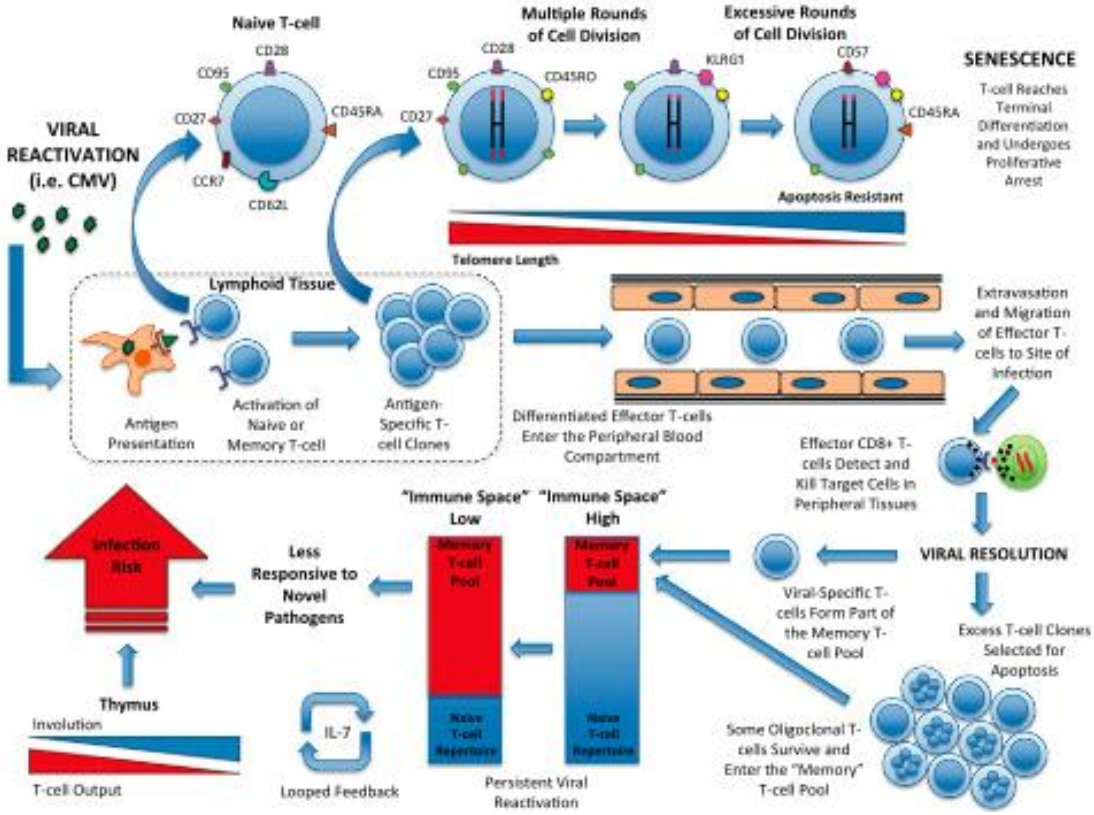

Fig 3. Latent viral infection (CMV) alters the immune space and subsequent infection risk. Taken from Simpson (2011).

Early signs of this immunosenescence known to affect the elderly (Pawelec et al, 2010), have recently been observed in a younger population (Turner et al, 2014). Senescent T-lymphocyte proportions are therefore greater in CMV seropositive individuals at rest. In response to exercise the senescent T-lymphocyte mobilisation and egress is amplified (Turner et al, 2010). Despite the previously discussed beneficial immunosurveillance of senescent T-lymphocytes, the amplified response of senescent T-lymphocytes as a result of CMV infection increases the mobilisation of senescent T-lymphocytes to the tissues and areas of atherosclerotic plaques, increasing inflammatory damage in tissues (Bosch et al, 2003). An interesting observation in both Chapter 2 and 3 was the higher per cent of CMV positive individuals in the trained population compared to the untrained population. This difference could be explained by living in close quarters together and sharing water bottles during training camp environments. Throughout this thesis CMV was never a main factor in any of the study designs i.e. we did not recruit specifically for CMV positive or CMV negative individuals because it was not our main aim in any of the studies. However, due to the

previously discussed known influence of CMV on T-lymphocyte populations it was always included as a covariate in the data analysis in the studies. A study design similar to the Turner et al (2014) comparing large numbers of CMV positive to CMV negative participants in response to exercise could be an enlightening future direction.

A number of limitations exist within this body of work. Firstly a lack of hormonal measures, the measurement of both stress and sex hormones would have provided further insight to the reasons for the differences between the trained and untrained groups and the males and females. In addition controlling for the menstrual cycle, would have removed menstrual cycle phase as a variable. However, the trained female participants all lived together so their menstrual cycles could have been in sync, but without measurements this is speculative. Secondly the anatomical location of where senescent T-lymphocytes are mobilised from and egressed too is not fully understood, therefore it is unknown if the response is beneficial or detrimental. Finally, CMV infection influences the T-lymphocyte pool and the response to exercise but it was never investigated as a factor on its own.

The main findings from this thesis that warrant further work are; males had higher proportions of senescent T-lymphocytes compared to females. An untrained group had higher proportions of senescent T-lymphocytes compared to a trained group. CMV infection appears to be more prevalent in trained populations than untrained populations. Increasing the volume of training in a trained female group for 2 weeks has no effect on the resting T-lymphocyte pool. Four weeks of PUFA or SFA supplementation has no effect on the T-lymphocyte pool at rest or in response to exercise in a group of trained males.

Considering both the limitations and the main findings of this thesis, future studies should be designed to examine the effect of exercise on the T-lymphocyte pool at different phases of the menstrual cycle. This would fully identify the influence of sex hormones on the response

in females. The differential influence of stress hormones in response to exercise on the T-lymphocyte pool in both trained and untrained populations requires closer examination. Exact anatomical locations of where senescent T-lymphocytes are mobilised from and egress to could be investigated by bio mathematical modelling of the response or more invasive techniques like skin, muscle or fat biopsies this could provide a more in depth insight into the senescent T-lymphocyte response to exercise. Studies in which large numbers of CMV positive and CMV negative participants are matched would allow for the effect of CMV infection on the T-lymphocyte response to exercise to be fully elucidated. Future research should consider moving into clinical populations. By investigating populations in which immune system dysfunction exists, and the mechanisms of the dysfunction are understood, would allow questions about effects of exercise, periods of increased training volume, or fatty acid supplementation / diet to be explored. It would also be of interest to determine whether sex differences in immune response remain in these populations, such as those known to exist in autoimmune diseases like rheumatoid arthritis. This approach would allow for whole body analysis to be made and the overall outcome of the immune response investigated. This would be an important step for exercise immunology research due to the nature of the multifactorial immune response.

6.2 References

Amarasiri, W.A. , Dissanayake, A.S., 2006. Coconut fats. Ceylon Med. J. 51, 47-51.

Athreya, B.H., Pletcher, J., Zulian, F., Weiner, D.B., Williams, W.V., 1993. Subset-specific effects of sex hormones and pituitary gonadotropins on human lymphocyte proliferation in vitro. Clin. Immunol. Immunopathol. 66, 201-211.

Betz, M. , Fox, B.S., 1-1-1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J. Immunol. 146, 108-113.

Bishop, N.C., Walker, G.J., Bowley, L.A., Evans, K.F., Molyneux, K., Wallace, F.A., Smith, A.C., 2005. Lymphocyte responses to influenza and tetanus toxoid in vitro following intensive exercise and carbohydrate ingestion on consecutive days. J. Appl. Physiol 99, 1327-1335.

Bosch, J.A., Berntson, G.G., Cacioppo, J.T., Dhabhar, F.S., Marucha, P.T., 2003. Acute stress evokes selective mobilization of T cells that differ in chemokine receptor expression: a potential pathway linking immunologic reactivity to cardiovascular disease. Brain Behav. Immun. 17, 251-259.

Campbell, J.P., Guy, K., Cosgrove, C., Florida-James, G.D., Simpson, R.J., 2008. Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human peripheral blood following an acute bout of high-intensity exercise. Brain Behav. Immun. 22, 375-380.

Cutolo, M., Sulli, A., Seriolo, B., Accardo, S., Masi, A.T., 1995. Estrogens, the immune response and autoimmunity. Clin. Exp. Rheumatol. 13, 217-226.

Dhabhar, F.S., 2009. A hassle a day may keep the pathogens away: The fight-or-flight stress response and the augmentation of immune function. Integr. Comp Biol. 49, 215-236.

Dhabhar, F.S., Malarkey, W.B., Neri, E., McEwen, B.S., 2012. Stress-induced redistribution of immune cells--from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner. Psychoneuroendocrinology 37, 1345-1368.

Dhabhar, F.S. , McEwen, B.S., 1-4-1996. Stress-induced enhancement of antigen-specific cell-mediated immunity. J. Immunol. 156, 2608-2615.

Dhabhar, F.S. , McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav. Immun. 11, 286-306.

Dhabhar, F.S. , Viswanathan, K., 2005. Short-term stress experienced at time of immunization induces a long-lasting increase in immunologic memory. Am. J. Physiol Regul. Integr. Comp Physiol 289, R738-R744.

Green, K.J., Croaker, S.J., Rowbottom, D.G., 2003. Carbohydrate supplementation and exercise-induced changes in T-lymphocyte function. J. Appl. Physiol (1985.) 95, 1216-1223.

Henson, D.A., Nieman, D.C., Blodgett, A.D., Butterworth, D.E., Utter, A., Davis, J.M., Sonnenfeld, G., Morton, D.S., Fagoaga, O.R., Nehlsen-Cannarella, S.L., 1999. Influence of exercise mode and carbohydrate on the immune response to prolonged exercise. Int. J. Sport Nutr. 9, 213-228.

Henson, D.A., Nieman, D.C., Pistilli, E.E., Schilling, B., Colacino, A., Utter, A.C., Fagoaga, O.R., Vinci, D.M., Nehlsen-Cannarella, S.L., 2004. Influence of carbohydrate and age on lymphocyte function following a marathon. Int. J. Sport Nutr. Exerc. Metab 14, 308-322.

Hong, S., Johnson, T.A., Farag, N.H., Guy, H.J., Matthews, S.C., Ziegler, M.G., Mills, P.J., 2005. Attenuation of T-lymphocyte demargination and adhesion molecule expression in response to moderate exercise in physically fit individuals. J. Appl. Physiol 98, 1057-1063.

Kjaer, M., Christensen, N.J., Sonne, B., Richter, E.A., Galbo, H., 1985. Effect of exercise on epinephrine turnover in trained and untrained male subjects. J. Appl. Physiol 59, 1061-1067.

Kruger, K., Frost, S., Most, E., Volker, K., Pallauf, J., Mooren, F.C., 2009. Exercise affects tissue lymphocyte apoptosis via redox-sensitive and Fas-dependent signaling pathways. Am. J. Physiol Regul. Integr. Comp Physiol 296, R1518-R1527.

Kruger, K., Lechtermann, A., Fobker, M., Volker, K., Mooren, F.C., 2008. Exercise-induced redistribution of T lymphocytes is regulated by adrenergic mechanisms. Brain Behav. Immun. 22, 324-338.

Lancaster, G.I., Jentjens, R.L., Moseley, L., Jeukendrup, A.E., Gleeson, M., 2003. Effect of pre-exercise carbohydrate ingestion on plasma cytokine, stress hormone, and neutrophil degranulation responses to continuous, high-intensity exercise. Int. J. Sport Nutr. Exerc. Metab 13, 436-453.

Landmann, R., 1992. Beta-adrenergic receptors in human leukocyte subpopulations. Eur. J. Clin. Invest 22 Suppl 1, 30-36.

Lee, S., Kim, J., Jang, B., Hur, S., Jung, U., Kil, K., Na, B., Lee, M., Choi, Y., Fukui, A., Gilman-Sachs, A., Kwak-Kim, J.Y., 1-7-2010. Fluctuation of peripheral blood T, B, and NK cells during a menstrual cycle of normal healthy women. J. Immunol. 185, 756-762.

Lemaitre, R.N., King, I.B., Mozaffarian, D., Kuller, L.H., Tracy, R.P., Siscovick, D.S., 2003. n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study. Am. J. Clin. Nutr. 77, 319-325.

Mallia, P., Message, S.D., Contoli, M., Gray, K., Telcain, A., Laza-Stanca, V., Papi, A., Stanciu, L.A., Elkin, S., Kon, O.M., Johnson, M., Johnstona, S.L., 2014. Lymphocyte subsets in experimental rhinovirus infection in chronic obstructive pulmonary disease. Respir Med. 108(1): 78-85.

McFarlin, B.K., Flynn, M.G., Stewart, L.K., Timmerman, K.L., 2004. Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2. J. Appl. Physiol (1985.) 96, 271-275.

Nettleton, J.A. , Katz, R., 2005. n-3 long-chain polyunsaturated fatty acids in type 2 diabetes: a review. J. Am. Diet. Assoc. 105, 428-440.

Nieman, D.C. , Bishop, N.C., 2006. Nutritional strategies to counter stress to the immune system in athletes, with special reference to football. J. Sports Sci. 24, 763-772.

Nieman, D.C., Davis, J.M., Brown, V.A., Henson, D.A., Dumke, C.L., Utter, A.C., Vinci, D.M., Downs, M.F., Smith, J.C., Carson, J., Brown, A., McAnulty, S.R., McAnulty, L.S., 2004. Influence of carbohydrate ingestion on immune changes after 2 h of intensive resistance training. J. Appl. Physiol 96, 1292-1298.

Nieman, D.C., Fagoaga, O.R., Butterworth, D.E., Warren, B.J., Utter, A., Davis, J.M., Henson, D.A., Nehlsen-Cannarella, S.L., 1997. Carbohydrate supplementation affects blood granulocyte and monocyte trafficking but not function after 2.5 h or running. Am. J. Clin. Nutr. 66, 153-159.

Oertelt-Prigione, S., 2012. Immunology and the menstrual cycle. Autoimmun. Rev. 11, A486-A492.

Pawelec, G., Larbi, A., Derhovanessian, E., 2010. Senescence of the human immune system. J. Comp Pathol. 142 Suppl 1, S39-S44.

Peake, J., Peiffer, J.J., Abbiss, C.R., Nosaka, K., Laursen, P.B., Suzuki, K., 2008. Carbohydrate gel ingestion and immunoendocrine responses to cycling in temperate and hot conditions. Int. J. Sport Nutr. Exerc. Metab 18, 229-246.

Rosenberger, P.H., Ickovics, J.R., Epel, E., Nadler, E., Jokl, P., Fulkerson, J.P., Tillie, J.M., Dhabhar, F.S., 2009. Surgical stress-induced immune cell redistribution profiles predict short-term and long-term postsurgical recovery. A prospective study. J. Bone Joint Surg. Am. 91, 2783-2794.

Samieri, C., Feart, C., Letenneur, L., Dartigues, J.F., Peres, K., Auriacombe, S., Peuchant, E., Delcourt, C., Barberger-Gateau, P., 2008a. Low plasma eicosapentaenoic acid and depressive symptomatology are independent predictors of dementia risk. Am. J. Clin. Nutr. 88, 714-721.

Samieri, C., Jutand, M.A., Feart, C., Capuron, L., Letenneur, L., Barberger-Gateau, P., 2008b. Dietary patterns derived by hybrid clustering method in older people: association with cognition, mood, and self-rated health. J. Am. Diet. Assoc. 108, 1461-1471.

Simpson, R.J., 2011. Aging, persistent viral infections, and immunosenescence: can exercise "make space"? Exerc. Sport Sci. Rev. 39, 23-33.

Simpson, R.J., Cosgrove, C., Ingram, L.A., Florida-James, G.D., Whyte, G.P., Pircher, H., Guy, K., 2008. Senescent T-lymphocytes are mobilised into the peripheral blood

compartment in young and older humans after exhaustive exercise. Brain Behav. Immun. 22, 544-551.

Simpson, R.J., Florida-James, G.D., Cosgrove, C., Whyte, G.P., Macrae, S., Pircher, H., Guy, K., 2007. High-intensity exercise elicits the mobilization of senescent T lymphocytes into the peripheral blood compartment in human subjects. J. Appl. Physiol 103, 396-401.

Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C., Grimble, R.F., 8-2-2003. Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet 361, 477-485.

Timmons, B.W., Tarnopolsky, M.A., Bar-Or, O., 2004. Immune responses to strenuous exercise and carbohydrate intake in boys and men. Pediatr. Res. 56, 227-234.

Turner, J.E., Aldred, S., Witard, O.C., Drayson, M.T., Moss, P.M., Bosch, J.A., 2010. Latent cytomegalovirus infection amplifies CD8 T-lymphocyte mobilisation and egress in response to exercise. Brain Behav. Immun. 24, 1362-1370.

Turner, J.E., Campbell, J.P., Edwards, K.M., Howarth, L.J., Pawelec, G., Aldred, S., Moss, P., Drayson, M.T., Burns, V.E., Bosch, J.A., 2014. Rudimentary signs of immunosenescence in Cytomegalovirus-seropositive healthy young adults. Age (Dordr.) 36, 287-297.

Vinten, J. , Galbo, H., 1983. Effect of physical training on transport and metabolism of glucose in adipocytes. Am. J. Physiol 244, E129-E134.

Viswanathan, K. , Dhabhar, F.S., 19-4-2005. Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. Proc. Natl. Acad. Sci. U. S. A 102, 5808-5813.

Vysakh, A., Ratheesh, M., Rajmohanan, T.P., Pramod, C., Premlal, S., Girish, K.B., Sibi, P.I., 6-3-2014. Polyphenolics isolated from virgin coconut oil inhibits adjuvant induced arthritis in rats through antioxidant and anti-inflammatory action. Int. Immunopharmacol. 20, 124-130.

Witard, O.C., Turner, J.E., Jackman, S.R., Kies, A.K., Jeukendrup, A.E., Bosch, J.A., Tipton, K.D., 10-10-2013. High dietary protein restores overreaching induced impairments in leukocyte trafficking and reduces the incidence of upper respiratory tract infection in elite cyclists. Brain Behav. Immun.

Witard, O.C., Turner, J.E., Jackman, S.R., Tipton, K.D., Jeukendrup, A.E., Kies, A.K., Bosch, J.A., 2012. High-intensity training reduces CD8+ T-cell redistribution in response to exercise. Med. Sci. Sports Exerc. 44, 1689-1697.

Xu, D., Erickson, S., Szeps, M., Gruber, A., Sangfelt, O., Einhorn, S., Pisa, P., Grander, D., 15-12-2000. Interferon alpha down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. Blood 96, 4313-4318.

APPENDIX

Introduction

As mentioned in chapter 5 we observed a higher level of T-lymphocyte $CD69^+$ activation when compared to previous studies (Kew et al, 2004). To investigate this discrepancy, we carried out some retrospective further analyses. The main difference between the analysis conducted in Chapter 5 and that of previous work was that we froze and stored our samples before batch analysing, for logistical reasons. Therefore, to investigate if the freezing procedure has any effect on the $CD69^+$ expression we performed two small experiments. Initially, we obtained blood from participants and examined $CD69^+$ expression in fresh vs. frozen PBMC's. We then performed a second experiment in which cultured PBMC's were treated fresh and frozen (from the same participant) with a mitogen (PHA) at a low ($2.5ug/10^6$cells) medium ($5ug/10^6$cells) and high ($7.5ug/10^6$cells) concentration.

Methods

Participants

For the first experiment n=6 and for the second n=5 participants were recruited. A blood sample was taken from a forearm vein and the sample was equally split into two, one half for analysis of the fresh sample the other half was frozen and stored then analysed.

Peripheral blood mononuclear cells (PBMC) isolation

The methods used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood have been described elsewhere (Simpson et al, 2006). Briefly whole blood was mixed with an equal volume of 0.9% NaCl and layered over 3ml of Lymphoprep (Axis-Shield, Oslo, Norway). Samples were centrifuged at 2000rpm for 30mins at 20˚C. After centrifugation, the PBMCs at the sample/medium interface were removed and washed twice for 10 mins with 0.9% NaCl then once for 10 mins with RPMI-1640 medium (Sigma-Aldrich, Ltd, UK).

The isolated PBMCs were then analysed fresh, cultured or stored in 70% (700µl) fetal bovine serum (Sigma-Aldrich, Ltd, UK), 20% (200µl) RPMI-1640 growth medium (Sigma-Aldrich, Ltd, UK) and 10% (100µl) DMSO (Sigma-Aldrich, Ltd, UK) that was added to the samples on ice. Samples were then snap frozen in liquid nitrogen and stored in liquid nitrogen until later analysis.

Cell culture

Isolated PBMC's were cultured fresh or thawed and resuspended in 10ml of RPMI-1640 medium (Sigma-Aldrich, Ltd, UK), supplemented with 5% heat inactivated fetal bovine serum (Sigma-Aldrich, Ltd, UK), and 1% sodium azide. Cells were counted using a haemocytometer to determine the appropriate PHA concentrations to be added, 2.5ml of the cell suspension was then distributed into 6 well plates (Thermo Scientific). Cells were then incubated at 37°C with 5% CO_2 with or without different concentrations (0, 2.5, 5 and 7ug/10^6 cells) of PHA (Sigma-Aldrich, Ltd, UK) for 6h. After incubation supernatant was removed and stored for further analysis. Cells were washed for 10mins at 300g (CR 312) with 1ml of PBS (Sigma-Aldrich, Ltd, UK), supplemented with 0.1% BSA and 0.1% sodium azide. Fastimmune kit (BD biosciences San Diego CA) combining antibodies CD8 FITC CD69 PE and CD3 perCP was added according to the manufacturers instructions. Cells were washed again for 10mins at 300g with 1ml of PBS. After the wash cells were fixed with 400ul of 0.5% formaldehyde and stored at 4°C until acquisition on the flow cytometer.

Flow Cytometry

Fluorescence of the directly conjugated mAbs bound to the cell surface was detected on a FACSCalibur (Becton Dickinson, Oxford, UK) flow cytometer equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter mode using CellQuest Pro software. Fluorescence triggering in the FL3 channel to gate on CD3+ was used for acquisition. Data was then displayed as two-colour dot plots (FL1 versus FL2) to determine the proportion of activated lymphocyte subsets expressing CD69. Following acquisition, FCS files were transferred to a third party software programme for analysis by FlowJo version 7.6.3.

Data presentation and statistical analysis

All data are presented as means ± standard deviation (SD), unless otherwise stated. Statistical analysis was performed using Mini-tab v-16 statistical software. Repeated measures analysis of variance (ANOVA) was used to compare between fresh and frozen at the different mitogen concentrations. Post-hoc analysis used the Tukey test and 95% confidence interval. Main effects and interactions were accepted as statistically significant at the $P < 0.05$ level.

Results

In resting samples with no mitogen stimulation there was no significant difference between the fresh and frozen samples for CD69 cell number and proportion of $CD69^+$ in the $CD4^+$ and $CD8^+$ T-lymphocyte populations (Figure 1).

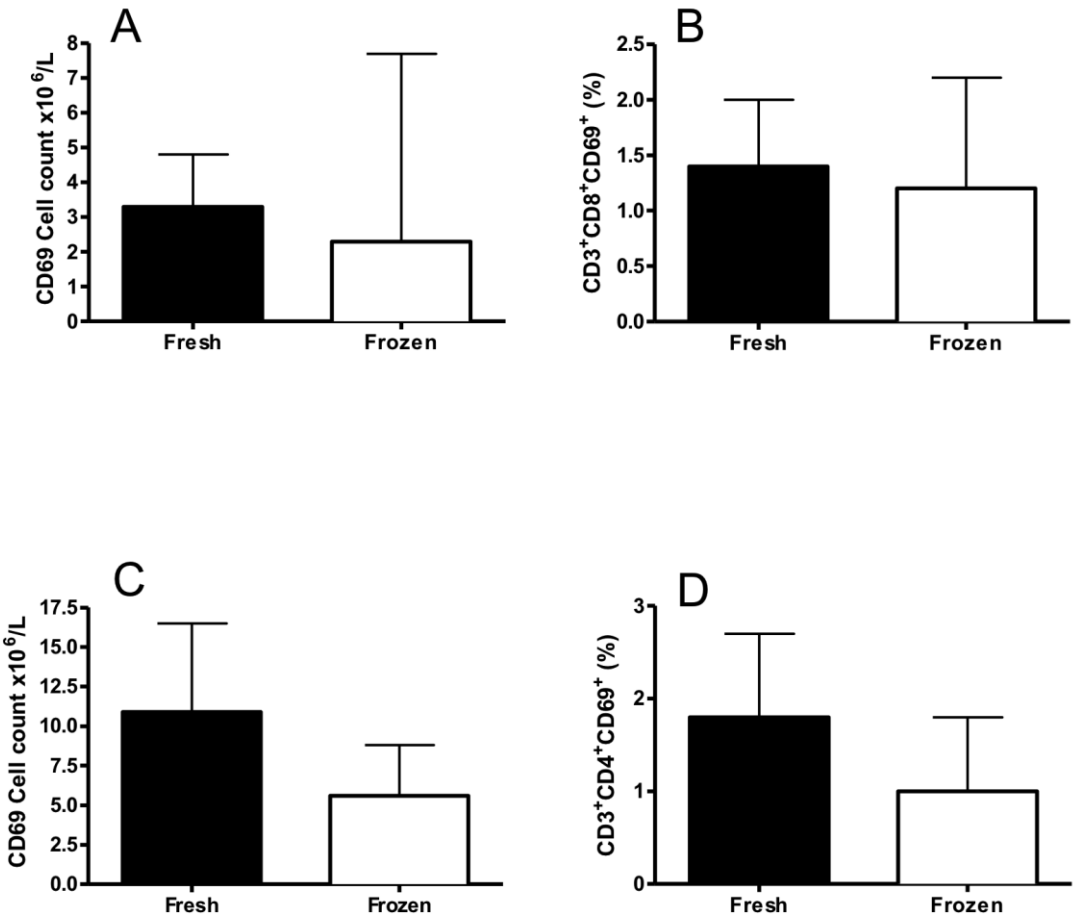

Fig 1. Values are expressed as mean ± SD the cell count $x10^6$/L of T-lymphocytes expressing the phenotype $CD3^+CD8^+CD69^+$ (A) and $CD3^+CD4^+CD69^+$ (C). And the mean percent ± SD of the T-lymphocytes expressing the phenotype $CD3^+CD8^+CD69^+$ (B) $CD3^+CD4^+CD69^+$ (D). Samples were analysed fresh n=6 and frozen and stored prior to being thawed for analysis n=6.

A significant main effect of the freezing process was observed (Figure 2A,C) whereby the samples assessed when fresh had a higher proportion of activated T-lymphocytes than the samples assessed after freezing. There was no significant main effect of mitogen concentration or a mitogen-by-freezing interaction observed in the $CD8^+$ T-lymphocytes,

indicating the mitogen was not activating either the fresh or frozen samples. However, when the data is expressed as % change at the different mitogen concentrations (Figure 2B) it does appear that the mitogen is activating the fresh but not frozen samples. A significant main effect of mitogen and a mitogen-by-freezing procedure interaction was observed on the $CD4^+$ T-lymphocytes (Figure 2C) indicating the mitogen is activating the fresh but not the frozen samples in a dose dependent manner. The % change further supports this observation (Figure 2D).

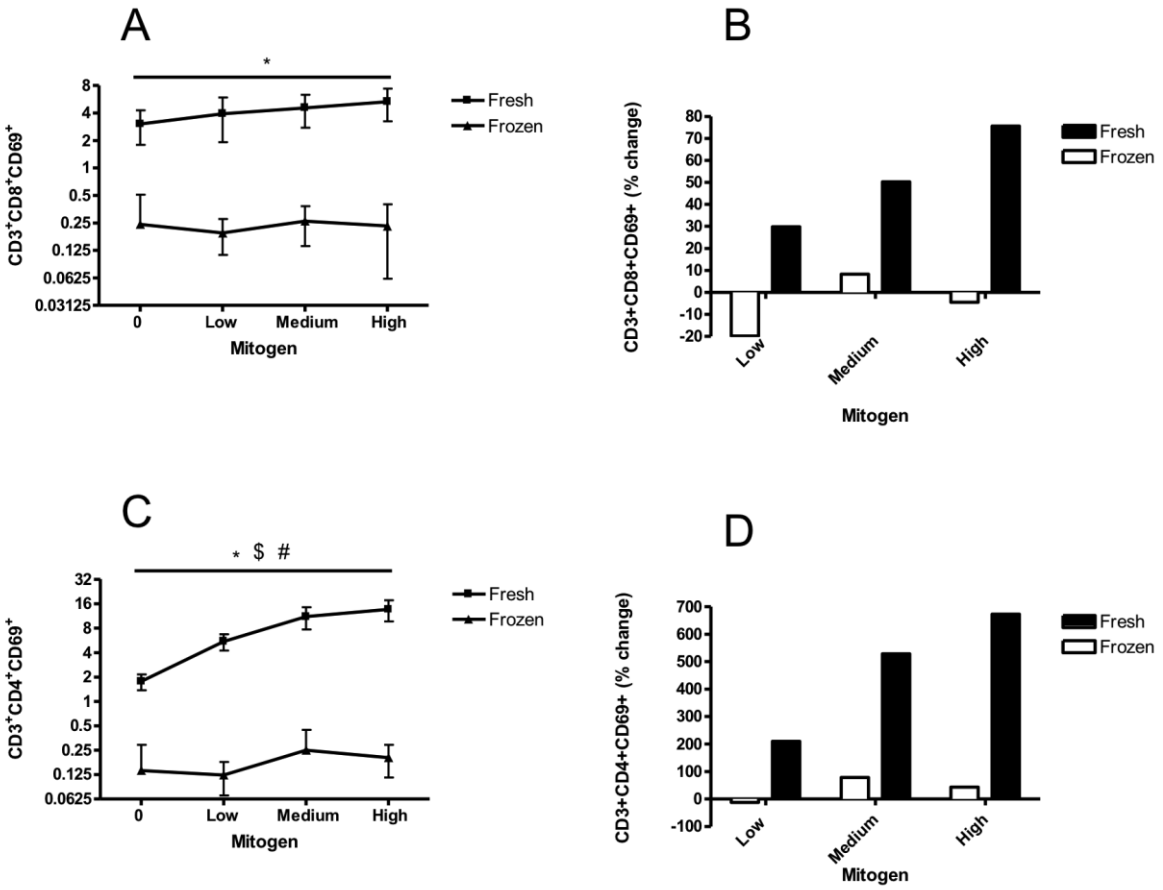

Fig 2. Values are expressed as mean ± SD percent of total $CD3^+$ T-lymphocytes expressing $CD8^+CD69^+$ (A) and $CD4^+CD69^+$ (C) at low (2.5ug/10^6cells) medium (5ug/10^6cells) and high (7.5ug/10^6cells) concentration of PHA mitogen. And the percentage change of $CD8^+CD69^+$ (B) and $CD4^+CD69^+$ (D) from 0 mitogen. Samples were treated fresh n=5 and frozen n=5. * indicates a significant main effect of the freezing procedure ($P<0.05$). $ indicates a significant main effect of mitogen ($P<0.05$). # indicates a signficant mitogen-by-freezing procudure interaction ($P<0.05$).

To further support the observed mitogen activation in the fresh but not the frozen (Figure 2B, C and D) the raw flow cytometry plots below demonstrate that there is still an effect of mitogen in the fresh samples (Figure 4) but not the frozen (Figure 3).

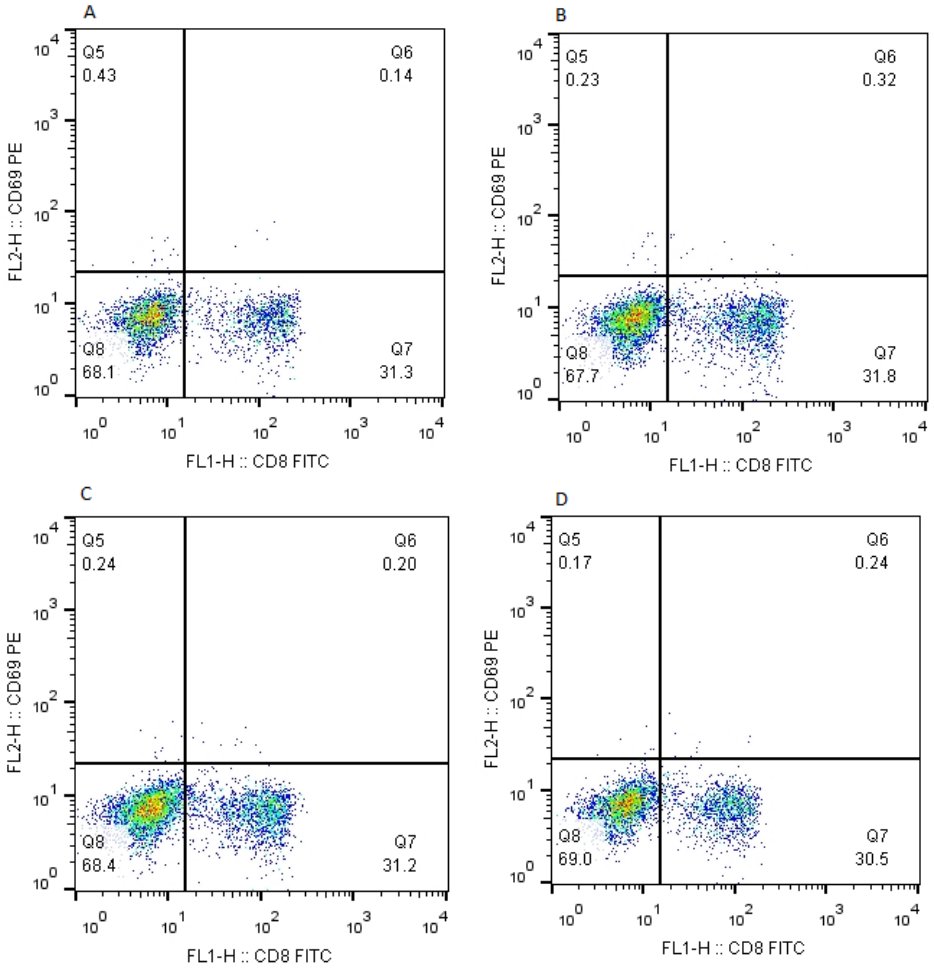

Fig 3. Flow cytometry plots of $CD69^+$ expression in the $CD3^+$ population at 0 (A) low (B) medium (C) and high (D) concentrations of PHA mitogen. These plots are from samples that were frozen and stored prior to being thawed for analysis.

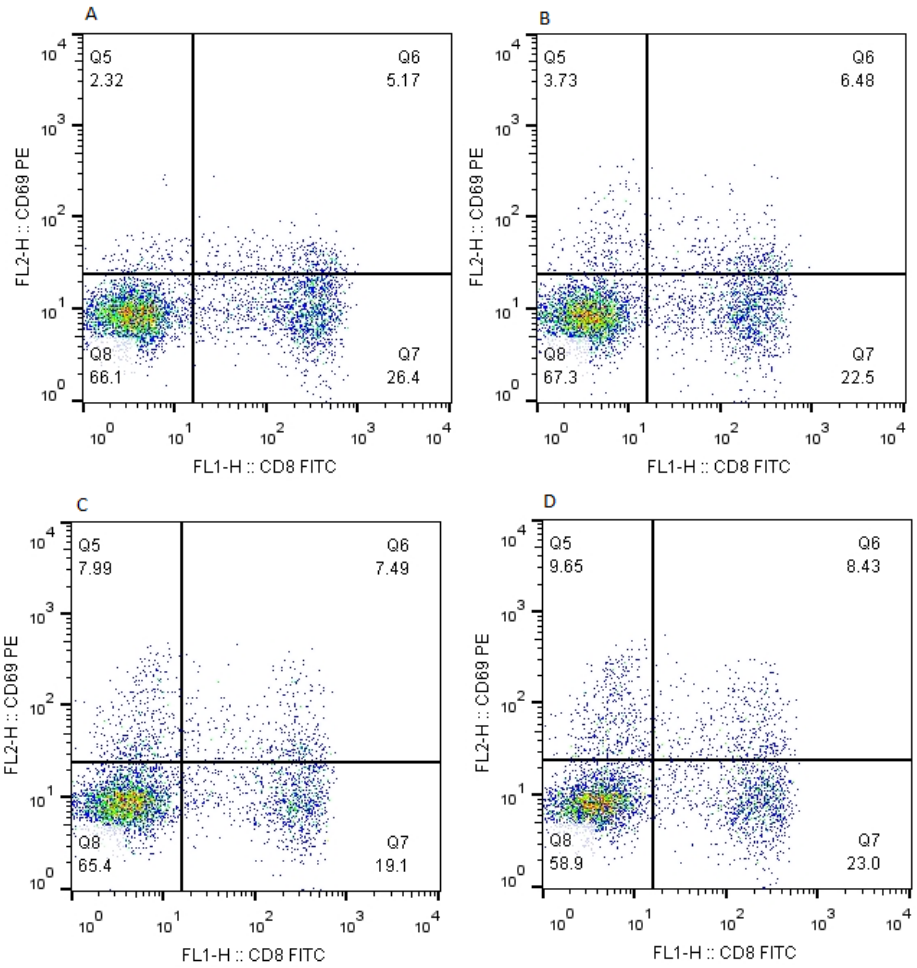

Fig 4. Flow cytometry plots of $CD69^{+}$ expression in the $CD3^{+}$ population at 0 (A) low (B) medium (C) and high (D) concentrations of PHA mitogen. These plots are all from the same participant as (Figure 2) and the samples were cultured fresh.

Discussion

The aim of these retrospective analyses was to identify if freezing PBMC's after isolation influenced the activation and therefore expression of $CD69^+$. No significant difference was observed between the fresh and frozen resting samples with no mitogen (Figure 1). However, the SD in the frozen samples suggests when treated frozen the samples become more variable. Although there was not a statistically significant difference between the fresh and frozen, the $CD4^+CD69^+$ T-lymphocytes do seem to be higher in the fresh samples. This was a small study using only 6 participants, with more participants this difference could become more apparent. From the current analyses it is clear that the freezing procedure blunts the number and proportion of $CD8^+CD69^+$ T-lymphocytes observed in response to a PHA mitogen (Figure 1). These data suggest that the freezing procedure damages the cells to the point where the early activation marker on the cell surface is lost, and that the cells are unable to express this marker upon exposure to the mitogen (PHA). When the data is plotted as percentage change at the different mitogen concentrations (Figure 2) and the individual responses are observed within the same participant between the fresh and frozen sample it is quite clear (Figure 3 and 4) that the freezing procedure is preventing the expression of $CD69^+$. This data suggests the freezing procedure increases the variability of the cell count and proportions of activated T-lymphocytes at rest. It does not result in a statistically significant difference from the proportions and counts of activated T-lymphocytes in fresh samples at rest. But it was carried out in a small subject number suggesting if repeated with more participants the difference between fresh and frozen samples could become more apparent. However, from this data the freezing procedure cannot be held responsible for the higher proportions of activated T-lymphocytes observed in Chapter 5 compared to previous studies (Kew et al, 2004), the resting values in Chapter 5 were even higher than the data from the frozen samples presented here.

An interesting observation from the second of these small studies is the blunted mitogen activation observed in the frozen $CD4^+$ T-lymphocyte samples, while this did not reach

statistical significance in the $CD8^+$ T-lymphocytes the likely explanation is low power from the small participant cohort. A possible explanation for the blunted mitogen activation lies in the experimental protocol, after the PMBC's were defrosted they were not rested for 24 hours as a very recent paper published since I have done all of this analysis suggests (Mata et al, 2014) while this study did not investigate CD69 expression, it investigated other functional markers in PBMC's and suggested after thaw the PBMC's should be rested for 24 hours. Highlighting the importance of redoing the whole procedural experiments using frozen cells that are rested for 24 hours after thawing. Only this would confirm if there is a real reduction in retention or expression of the CD69 surface marker in response to the freezing procedure.

References

Kew, S., Mesa, M.D., Tricon, S., Buckley, R., Minihane, A.M., Yaqoob, P., 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am. J. Clin. Nutr. 79, 674-681.

Mata, M.M., Mahmood, F., Sowell, R.T., Baum, L.L., 20-2-2014. Effects of cryopreservation on effector cells for antibody dependent cell-mediated cytotoxicity (ADCC) and natural killer (NK) cell activity in Cr-release and CD107a assays. J. Immunol. Methods

Simpson, R.J., Florida-James, G.D., Whyte, G.P., Guy, K., 2006. The effects of intensive, moderate and downhill treadmill running on human blood lymphocytes expressing the adhesion/activation molecules CD54 (ICAM-1), CD18 (beta2 integrin) and CD53. Eur. J. Appl. Physiol 97, 109-121.