MARKERS OF IMMUNOSENESCENCE AND OXIDATIVE STRESS IN HEALTHY ADULTS

by

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ABSTRACT

This thesis investigated markers of oxidative stress and immunosenescence in healthy adults. The results presented represent several novel findings which support the notion that oxidative stress and infection with micro-organisms shape our biology and can accelerate aspects of ageing. Acute exercise of high intensity was shown to cause alterations in the cellular composition of blood, which was most pronounced in lymphocyte sub-populations important for immunosurveillance. This exercise also resulted in increased markers of oxidative stress in lymphocytes, and resulted in a whole body oxidative stress, which was more pronounced and prolonged, following ultra-endurance exercise. Studies also showed that infection with a highly prevalent and asymptomatic herpes virus, Cytomegalovirus (CMV), shapes our immune biology in two significant ways. First, CMV amplified the magnitude and kinetics of lymphocyte responses to exercise, which could potentially facilitate immune surveillance, or aggravate inflammatory processes. Second, CMV was seen to drive the development of an 'Immune Risk Profile' in young adults, characterised by increased inflammatory activity and smaller responses to vaccination. These outcomes are associated with frailty, cognitive decline, and mortality in the elderly.

ACKNOWLEDGEMENTS

LIST OF PUBLICATIONS

This thesis incorporates four papers, each corresponding to one of the four empirical studies:

1) Turner, J.E., Aldred, S., Witard, O., Drayson, M.T., Moss, P.M., Bosch, J.A. (2010) Latent Cytomegalovirus infection amplifies CD8 T-lymphyocyte mobilisation and egress in response to exercise. **Brain Behav Immun,** 24 1362-1370.

2) Turner, J.E., Bosch, J.A., Drayson, M. T., Aldred, S. (in review) Assessment of oxidative stress in lymphocytes following exercise. **J Appl Physiol,**

3) Turner, J.E., Hodges, N.J., Bosch, J.A., Aldred, S. (In press) Prolonged depletion of antioxidant capacity following ultra-endurance exercise. **Med Sci Sports Exerc,**

4) Turner, J.E., Campbell, J.P., Edwards, K.E., Howarth, L.J., Aldred, S.A., Moss, P.M., Drayson, M.T., Burns, V.E., Bosch, J.A. (in preparation) A mild Immune Risk Profile in CMV seropositive healthy young adults. **Mech Ageing Dev,**

In addition, the following presentations arose from material in this thesis:

- Preferential lymphocytosis and lymphocytopenia of effector-memory CD8 T cells with intensive exercise: effects of latent Cytomegalovirus infection. *An Integrated Approach to Research in Infectious Diseases. University of Birmingham.* April 2010.*

* Awarded second prize oral presentation

During the period of postgraduate study at the University of Birmingham, the following papers and presentations were produced:

Publications

- Thompson, D., Markovitch, D., Betts, J.A., Mazzatti, D., Turner, J.E., Tyrrell, R.M. (2010) Time course of changes in inflammatory markers during a 6-mo exercise intervention in sedentary middle-aged men: a randomized-controlled trial. **J Appl Physiol**, 108, (4): 769-779.

- Turner, J.E., Markovitch, D., Betts, J.A., Thompson, D. (2010) Non-prescribed physical activity energy expenditure is maintained with structured exercise and implicates a compensatory increase in energy intake. **Am J Clin Nutr,** 95 (5): 1009-1016. *

- Witard, O.C., Jackman, S.R., Turner, J.E., Bosch, J.A., Jeukendrup, A.E., Kies, A.K., Tipton, K.D. (in preparation) High-intensity exercise training impairs lymphocytosis of CD8$^+$ effector cells without compromising lymphocytopenia. **Brain Behav Immun,**

Poster presentations

- Differential mobilisation of stem cell subsets in response to exhaustive exercise. *British Microcirculation Society Meeting. University of Birmingham.* March 2009.

* This article was selected and evaluated for Faculty 1000 (F1000) placing this work in the F1000 library of the top 2% of published articles in biology and medicine.

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
BMI	Body mass index
CD	Cluster of differentiation
CMV	Cytomegalovirus
CM	Central memory T lymphocyte
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked-immunosorbent-assay
EM	Effector memory T lymphocyte
EMRA	Revertant effector memory T lymphocyte
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FPG	Formamidopyrimidine DNA glycosylase
γ-GCS	γ-glutamylcysteine synthetase
GPx	Glutathione peroxidase
GSH	Reduced glutathione
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL-6	Interleukin-6
IPAQ	International physical activity questionnaire
IRP	Immune risk profile
LTPA	Leisure time physical activity
MBB	Monobromobimane
MFI	Median fluorescence intensity
PE	Phycoerythrin
PerCP	Peridinin chlorophyll A protein
ROS	Reactive oxygen species
RPE	Rating of perceived exertion

CHAPTER ONE

1.0. INTRODUCTION

1.1. PERSPECTIVES AND OVERVIEW

The immune system is a multi-level defence network consisting of molecules, cells, tissues, and organs. These defences have evolved to provide protection against viruses, bacteria, fungi and parasites. In addition, the immune system offers protection against the effects of harmful self mutations as seen in cancer cells. The cells of this system are subject to alterations with ageing. Such alterations can potentially manifest in whole body impairment and functional decline (Wikby et al., 2005; Wikby et al., 2006). One of the most widely accepted theories of ageing addresses changes at the cellular level; the 'free radical theory of ageing' (Harman, 1956). This theory proposes that changes arise from an imbalance between reactive species or free radicals, which are key to cellular respiration and signalling, and antioxidants, which quench and buffer such molecules (Beckman and Ames, 1998). When imbalance ensues, oxidative stress is observed, which affects nearly all systems and processes in the human body, including the immune system. While certain immune cells elicit their protective effects by releasing reactive radical species, causing local oxidative stress, oxidative stress can in turn alter the function of immune cells. The research in this thesis assessed markers of an ageing immune system (immunosenescence) and oxidative stress in healthy adults.

1.2. A CONCISE INTRODUCTION TO THE IMMUNE SYSTEM

The immune system can be broadly categorised into two sub-components; innate immunity, and adaptive immunity.

1.2.1. Innate immunity

Innate immunity is a rapid-acting first line of defence against microbial infection. This aspect of immunity is non-specific because it makes use of general pattern recognition receptors to detect and interact with potential pathogens. In addition, innate immunity does not develop 'memory'; responses are similar each time a pathogen is encountered. The innate immune system includes anatomical, physiological, cellular and inflammatory defences, which act together providing protection against a broad range of pathogens. For example the low pH of skin and normal body temperature deter the entry and growth of microbes. Likewise, cells of the innate immune system help to rid the body of infection; both tissue-residing cells (e.g., macrophages and dendritic cells) and 'mobile' cells (e.g., neutrophils, eosinophils and monocytes) recognise specific molecular patterns associated with pathogens, and initiate effector mechanisms. These functions include the internalisation and destruction of pathogens, and the secretion of chemokines and cytokines to recruit inflammatory cells and plasma proteins, culminating in 'inflammation'. The series of events leading to inflammation include enhanced capillary permeability and blood flow, local influx of plasma proteins, and increased local temperature, and tissue swelling. These changes often result in local discomfort and loss of function. At the site of inflammation, local antigen presenting cells such as dendritic cells, that have captured antigen, become activated and express co-stimulatory molecules. Upon activation, these cells travel to the secondary lymphoid organs (e.g., lymph nodes) to present antigen to cells of the adaptive immune system.

1.2.2. Adaptive immunity

Adaptive immunity provides a second line of defence, and although initially slower than innate responses, adaptive responses can engage pathogens with a much higher specificity. Most

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importantly, adaptive immunity develops an immunological memory, by building a repository of long lived antigen-experienced cells ('memory cells'). As a result, when pathogens are re-encountered, the adaptive immune response is initiated much faster such that inflammation and disease does not develop. Adaptive immunity is conveyed by most lymphocytes, which can be divided into three main populations; B cells, T cells, which in turn can be sub-divided into 'helper' or 'cytotoxic' cells, and natural killer cells (see Figure 1.1). Natural killer cells are the only lymphocytes that belong to innate immunity, and are discussed later.

B cells develop in the bone marrow and express a B cell receptor (BCR) which, different from T cell receptors, is able to interact with soluble antigen and recognise antigens in their native state. Upon antigen encounter, B cells differentiate into memory B cells, or effector B cells called plasma cells. Plasma cells home back to the bone marrow where they produce high volumes of soluble receptors, which they secrete as immunoglobulins (also called antibodies). Traditionally, it was thought that these soluble receptors were unable to cross cell membranes (with the exception of S-IgA) and thus provide extracellular immunity by binding to antigens that hereby become neutralised (e.g., preventing viruses docking on host cells). However, it has recently been shown that humoral immunity is able to provide intracellular protection, whereby antibodies cross cell membranes attached to viruses, and facilitate intracellular pathogen degradation (Mallery et al., 2010). Other functions of immunoglobulins involve facilitation of phagocytosis and activation the complement system (Moser and Leo, 2010).

All nucleated cells express major-histocompatibility-class I molecules (MHC class-I) that display intracellular proteins on the cell surface. This allows cytotoxic T cells, which sample MHC class-

I, to detect anomalous cell products such as viral and cancer-specific proteins (Moser and Leo, 2010). Cytotoxic T cells lyse target cells by initiating effector mechanisms, such as perforin and granzyme B release, or interaction with the 'death receptor' Fas-Ligand, resulting in apoptosis of the cell. Perforin is membraneolytic protein, and granzyme B is a serine proteinase, which are both found in cytoplasmic granules of cytotoxic T cells and natural killer cells (van Lier et al., 2003). The major role of helper T cells is to orchestrate the immune response by secretion of cytokines, and by expression of co-stimulatory molecules which activate other cells (Moser and Leo, 2010).

Natural killer cells are large granular lymphocytes which provide protection against viruses, bacteria, parasites and tumours. Natural killer cells produce immunoregulatory cytokines such as interferon-γ (IFN-γ) and directly kill target cells via perforin and granzyme B release. As some of their actions are not antigen dependent, these cells are categorised as innate immune cells. Recently however, it has been shown that natural killer cells express receptors which recognise MHC class-I antigen expression, and also possess immunological memory as with cells of the adaptive immune system (Cooper and Yokoyama, 2010).

1.3. IMMUNE CELL PHENOTYPING

Lymphocytes can be enumerated and typed using flow cytometry. Flow cytometry is a technique whereby individual cells are forced to pass a laser, which reveals distinct characteristics in terms of size (forwards scatter; FSC) and internal complexity/granularity (sideways scatter; SSC). Lymphocytes, fairly small, round and mostly non-granular cells can be identified by their

location on a plot of FSC *versus* SSC (see Figure 1.1). The laser light serves a second function: excitement of fluorescently conjugated monoclonal antibodies against identifying cellular molecules. These cell surface proteins are often identified by cluster of differentiation (CD) nomenclature. For example, helper-T cells are identified by co-expression of both CD3 and CD4 ($CD3^+CD4^+$). Likewise, cytotoxic T cells are $CD3^+CD8^+$, B cells are $CD3^-CD19^+$ and natural killer cells are $CD3^-CD56^+$ (see Figure 1.1).

1.4. PHENOTYPES AND CHARACTERISTICS OF T CELL MEMORY POPULATIONS

$CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells can be further categorised into sub-populations based upon (surface) expression of additional CD molecules. This can be useful when various cell phenotypes are associated with specific functional characteristics. This thesis used two established nomenclatures for the identification of T lymphocyte sub-populations (see Figures 1.2 and 1.3, and Table 2.1; chapter two). The first nomenclature utilises the expression of CD27 in combination with CD45RA (Hamann et al., 1997; Sallusto et al., 2004; Sallusto et al., 1999), and is illustrated in Figure 1.2. This strategy yields one population of antigen inexperienced cells; the naïve subset ($CD27^-CD45RA^+$), and three memory populations; central memory (CM; $CD27^+$ $CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$), and 'revertant' memory cells which have re-expressed the 'naïve' cell marker CD45RA (EMRA; $CD27^-CD45RA^+$) (Hamann et al., 1997; Sallusto et al., 2004; Sallusto et al., 1999). The latter population is sometimes also referred to as 'terminally differentiated' or 'effector' cells. CD45RA is one of the multiple isoforms of the leukocyte surface protein CD45. This RA isoform is present on naïve T cells and replaced by the RO isoform after antigenic recognition. $CD27^-CD45RA^+$ cells are known to exhibit a strong

FIGURE 1.1.
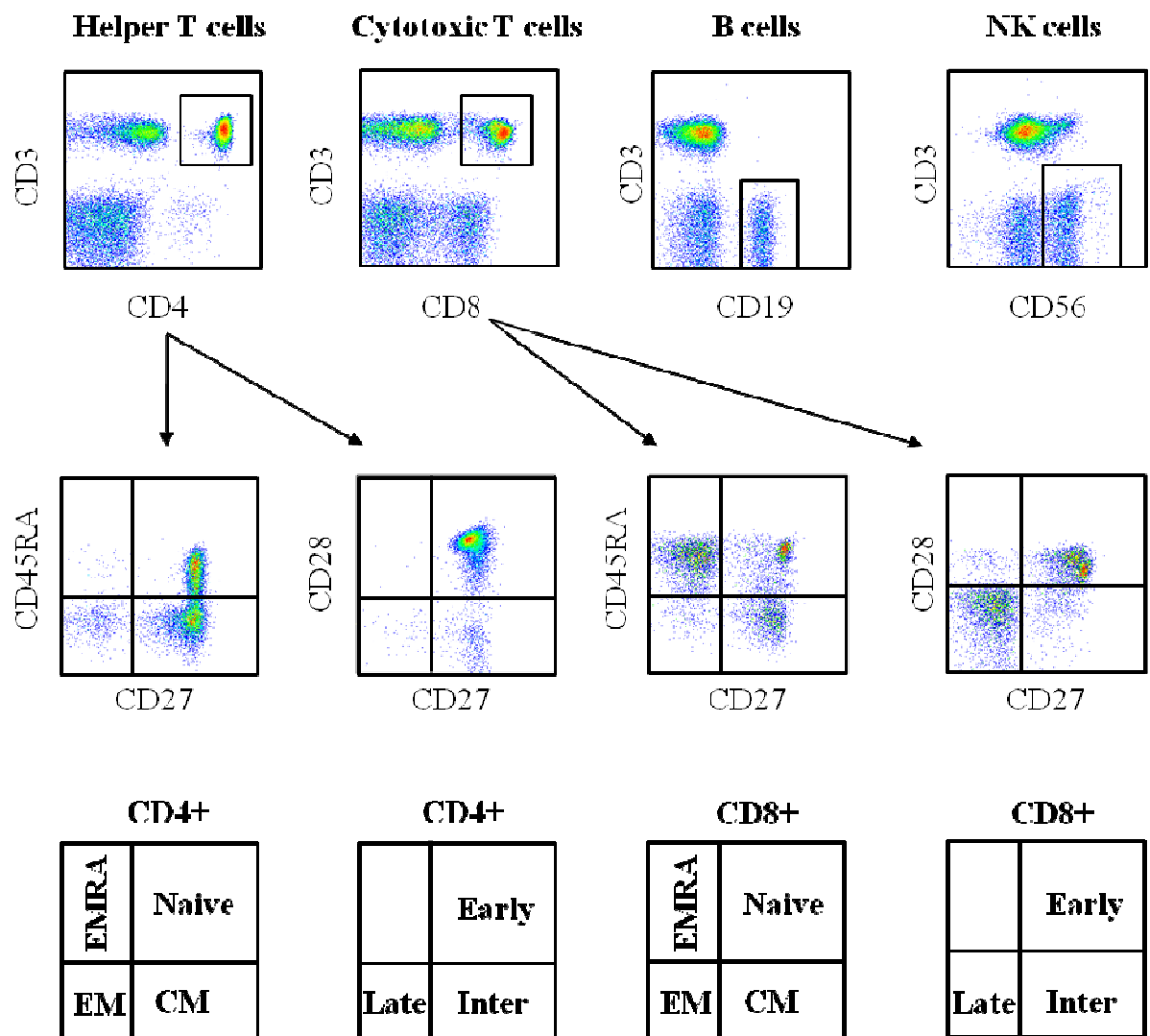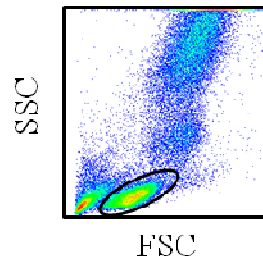
effector potential (such as the ability for rapid target killing, inflammatory cytokine production, and tissue migration, see Figure 1.2 and Table 2.1; chapter two). Other groups have utilised different combinations of surface molecules to discern the naïve, CM, EM and EMRA sub-populations. For example, instead of CD27, the co-stimulatory molecule CD28 or the chemokine/lymphoid homing receptor CCR7, are used in combination with CD45RA (Appay et al., 2008; Sallusto et al., 2004; Sallusto et al., 1999; van Lier et al., 2003).

The second nomenclature used in this thesis that identifies T lymphocyte subpopulations analyses the combined expression of CD27 and CD28 (see Figure 1.3 and Table 2.1; chapter two). Identifying cells on this basis yields three distinct subsets which differ along a continuum of differentiation stage; early stage (CD27$^+$CD28$^+$), intermediate stage (CD27$^+$CD28$^-$), and late stage differentiated cells (CD27$^-$CD28$^-$) (Appay et al., 2002; Appay et al., 2008). This nomenclature was originally developed to distinguish between memory populations, and does not make a clear distinction between naïve and memory cells (Appay et al., 2002; Appay et al., 2008). Due to the overlapping loss and expression of CD27, CD28, and CD45RA with cellular differentiation and antigenic stimulation, several of the sub-populations described share similar functional characteristics (Appay et al., 2008). Thus, naïve, CM and early-stage differentiated cells show a propensity to migrate towards the secondary lymphoid organs (e.g., lymph nodes). Here, upon interaction with antigen presenting cells, rapid proliferation occurs, producing antigen-specific effector cells which readily migrate to inflamed tissue (Sallusto et al., 2004; Sallusto et al., 1999).

FIGURE 1.2.

Revertant effector memory (EMRA)	CD27− CD45RA+
Effector memory cells which re-express CD45RA, have extensive replicative history, and express the highest levels of cytotoxic effector molecules, such as granzyme and perforin. Many of these cells are CMV specific.	

Naïve	CD27+ CD45RA+
Cells which have not experienced cognate antigen and circulate between the blood and the lymph nodes. These cells do not have immediate cytotoxic effector functions.	

Effector memory (EM)	CD27− CD45RA−
Differentiated cells which can exert immediate effector function without a need for differentiation. These cells down-regulate most lymph node homing receptors, so migrate to inflamed tissue.	

Central memory (CM)	CD27+ CD45RA−
Antigen-experienced cells which circulate between the blood and the lymph nodes, and lack immediate effector function. Upon re-exposure, there is rapid development into effector memory cells.	

FIGURE 1.3.

Early-stage differentiated cells	CD27+ CD28+

Cells with the lowest effector potential, with a short replicative history, slow turnover, and limited cell renewal. These cells have similar functional characteristics to the naïve population.

Intermediate-stage differentiated cells	CD27+ CD28–

Cells at the intermediate stage of differentiation which no longer express the co-stimulatory molecule CD28. These cells have shorter telomeres than early-stage cells, and share similar functional characteristics to the EM population.

Late-stage differentiated cells	CD27 – CD28–

Cells at the latest stage of differentiation, with extensive replicative history. These cells have short telomeres, and possess functional characteristics similar to the EM and EMRA population.

CM cells which have previously responded to infection, produce effector cells more quickly than antigen inexperienced naïve cells (Sallusto et al., 2004; Sallusto et al., 1999). The EM and EMRA sub-populations strongly overlap with the intermediate and late stage differentiated cells. These subsets preferentially migrate to peripheral tissue (e.g., the skin), express cytotoxic effector molecules (e.g., perforin and granzyme B) and readily produce inflammatory cytokines (e.g., IFN-γ) (Appay et al., 2002; Lanzavecchia and Sallusto, 2005; Takata and Takiguchi, 2006).

1.5. EXERCISE AND LYMPHOCYTE MIGRATION

Exercise results in an immediate mobilisation of lymphocytes into peripheral blood (Gleeson and Bishop, 2005). This lymphocytosis is driven by a preferential mobilisation of specific lymphocyte sub-populations: $CD8^+$ cytotoxic T cells mobilise to a greater extent than $CD4^+$ helper T cells (Anane et al., 2009; Campbell et al., 2009). Within these subsets, cells characterised by increased effector potential and homing to peripheral tissue (e.g., $CD8^+CD27^-CD45RA^+$; EMRA) show the largest changes with exercise (Campbell et al., 2009). It is now clear that lymphocytosis is largely driven by beta-adrenergic mechanisms, although non-specific mechanisms, such as shear forces, may also play a role. The cells selectively mobilised with exercise exhibit a high beta-adrenergic receptor density and sensitivity, which in turn, regulates their detachment from vascular endothelium and release into the blood (Benschop et al., 1994; Dimitrov et al., 2010; Elenkov et al., 2000; Karaszewski et al., 1991). Indeed, these effects are replicated under other conditions of adrenergic stimulation (e.g., acute psychological stress) and almost identical effects are observed with adrenaline or isoproteronol infusion (Anane et al., 2009; Dimitrov et al., 2010). This mobilisation of cells with exercise is thought to represent

an adaptive response, possibly reflecting enhanced immunosurveillance under conditions where infection is more likely (Dhabhar et al, 1996; Kruger and Mooren, 2007).

One to two hours after strenuous and prolonged exercise, lymphocytosis is followed by a post-exercise decline in the number of circulating lymphocytes to below baseline levels (lymphocytopenia) (Gleeson and Bishop, 2005). Much less is known about the mechanisms underlying this process. It has previously been suggested that lymphocytopenia is a result of post-exercise apoptosis, or might be mediated by neuro-endocrine mechanisms, in particular cortisol (Mars et al., 1998; McCarthy and Dale, 1988). More recent research suggests that apoptotic mechanisms are unlikely (Dimitrov et al., 2009; Simpson et al., 2007; Tanimura et al., 2008). Instead, animal studies show that lymphocytopenia reflects extravasation of lymphocytes to peripheral tissues, potentially facilitating the detection and elimination of antigen (Dhabhar, 2000, 2002; Krüger and Mooren, 2007). This hypothesis is appealing considering the phenotype of cells which are preferentially mobilised with exercise: these cells are characterised by ability for immediate cytotoxicity and homing to peripheral tissue (Appay et al., 2002; Campbell et al., 2009). It is unknown whether the T cells that comprise exercise lymphocytosis also drive lymphocytopenia post-exercise.

1.6. SENESCENCE OF THE IMMUNE SYSTEM

The term 'immunosenescence' was first used by Roy Walford in 1969, who hypothesised that normal ageing in humans and animals is related to faulty immune processes (Walford 1969 cited in Effros, 2005). The majority of research into immunosenescence is focussed on adaptive

immunity, as it was once thought that innate immunity is better preserved with ageing (Franceschi et al., 2000a). It is now emerging however that age-related changes are detectable in nearly all cells and processes of the immune system, including innate immunity. For example, ageing is associated with decreased natural killer cell function and altered neutrophil migration (Gomez et al., 2008; Panda et al., 2009). It is beyond the scope of this thesis to describe immunosenescence in the innate immune compartment therefore the reader is directed towards some excellent reviews on this topic (Gomez et al., 2008; Panda et al., 2009).

An aged adaptive immune system is characterised by increased sensitivity and susceptibility to infection, which in turn, is believed to promote frailty, accelerate cognitive decline, and increase mortality (Akbar et al., 2004; Larbi et al., 2008; Wikby et al., 2005). These outcomes form a final phase of a process that starts as early as adolescence (Akbar and Fletcher, 2005; Nikolich-Zugich, 2008). Around that time the thymus, an organ essential for the maturation of T lymphocytes, begins to shrink, resulting in a gradual decline in the output of naïve T cells. Consequently immunity becomes increasingly dependent on the existing pool of memory T cells (Woodland and Blackman, 2006). Compensating for the loss of naïve cells, this memory pool steadily expands, whereby specifically the number and proportion 'senescent' memory T cells with a late-differentiated phenotype (e.g., $CD27^-CD28^-/CD45RA^+$) have been found to increase. These phenotypic changes are particularly prominent in the cytotoxic T cell pool, and the accumulation of $CD8^+CD27^-CD28^-/CD45RA^+$ cells is considered a hallmark of immunosenescence (Akbar and Fletcher, 2005; Bosch et al., 2009; Hadrup et al., 2006; Pawelec, 2006). Consequently, compared to young adults, the elderly exhibit marked alterations in the T cell repertoire (Chidrawar et al., 2009; Weinberger et al., 2007), and it is likely that thymic involution plays a

significant role in this process. For example, young adults thymectomised in the first few years of life, exhibit reduced numbers and proportions of naïve T lymphocytes, and sometimes increased numbers of cytotoxic T cells (Eysteinsdottir et al., 2004; Sauce et al., 2009; Torfadottir et al., 2006). These immune profiles are qualitatively and quantitatively similar to those of middle-aged and elderly adults, who also show little or no thymic output (Eysteinsdottir et al., 2004; Sauce et al., 2009; Torfadottir et al., 2006). However, it seems that reduced thymic output is only one cause of immunosenescence. For example, the investigation by Sauce *et al* (2009) shows that young adults with no thymus, who were infected with Cytomegalovirus (CMV), exhibited more severe alterations in the T cell repertoire compared to those who were free of infection (Sauce et al., 2009). This combination of little or no thymic output and selective expansion/maintenance of cytotoxic T cell populations leads to a gradual 'filling of immunological space' with $CD8^+$ T cells that that have a highly restricted ('oligoclonal') T cell repertoire of only a few immuno-dominant antigens (Akbar and Fletcher, 2005; Brunner et al., 2010; Sauce et al., 2009; van Lier et al., 2003).

1.6.1. Infection history and the T cell repertoire

Acute viral infection results in differentiation of naïve T lymphocytes and elimination of virally infected self cells (van Lier et al., 2003). Most effector cells undergo apoptosis after this acute episode, but approximately 10% remain as long lived memory cells, which can rapidly employ effector functions upon re-infection (van Lier et al., 2003). The exact phenotype of these memory cells appears to be dependent on the type of virus they are directed towards, and might be governed by the frequency of antigenic stimulation, or by homeostatic regulation via cytokines such as IL-7 and IL-15 (Kaech et al., 2003; Schluns and Lefrancois, 2003; Stowe et al., 2007;

Weng et al., 2002). Infection with Epstein-Barr virus (EBV) results in an accumulation of CM-like cells ($CD27^+CD28^+/CD45RA^-$) (Appay et al., 2002), so that approximately 1-2% of the total $CD8^+$ T cell pool becomes specific for EBV epitopes (Appay et al., 2002; Ouyang et al., 2003; Vescovini et al., 2004). CMV infection is far more immunodominant, and this might be related to continual low level viral reactivation (Stowe et al., 2007; Zanghellini et al., 1999). Infection with CMV causes a large accumulation of late stage EMRA cells ($CD27^-CD28^-/CD45RA^+$) (Appay et al., 2002). These changes are often modest in the $CD4^+$ pool, but can be very dramatic among $CD8^+$ subsets (Chidrawar et al., 2009; Weinberger et al., 2007). Up to 23% of the $CD8^+$ T cell pool has been reported to become specific for just one of the many CMV epitopes (Appay et al., 2002; Khan et al., 2002). Although once assumed a consequence of chronological ageing, the accumulation of $CD8^+CD27^-CD28^-/CD45RA^+$ cells, identified as a hallmark of immunosenescence, is a direct result of CMV infection (Derhovanessian et al., 2009; Pawelec and Derhovanessian, 2010; Pawelec et al., 2009a; Pawelec et al., 2009b).

CMV is a highly prevalent beta herpes virus; approximately 35% of young children and 90% of the elderly are seropositive (Staras et al., 2006). Overall sero-prevalence in western societies approaches 60% (Staras et al., 2006). Individuals free from infection at birth will likely become infected early in life: post-natal or childhood CMV infection is common, and caused by exchange of bodily fluids, including breast-milk (Kenneson and Cannon, 2007; van der Meer et al., 1996). Primary infection and reactivation of CMV in immunocompetent hosts has been associated with malaise, fever and sweats, but is more typically asymptomatic (Wreghitt et al., 2003; Zanghellini et al., 1999). It has now become clear that this once assumed inconsequential virus, is the driving force behind immunosenescence (Moss, 2010; Pawelec et al., 2004; Pawelec and

Derhovanessian, 2010; Pawelec et al., 2009a). CMV is associated with age-related conditions such as cognitive decline, and is implicated in the pathogenesis and severity of cardiovascular disease (Aiello et al., 2006; Michaelis et al., 2009; Soderberg-Naucler, 2006). In addition, the extent of CMV infection, measured by immunoglobulin G (IgG) antibodies to CMV, independently predicts mortality (Roberts et al., 2010; Strandberg et al., 2009).

1.6.2. The Immune Risk Profile (IRP)

Many of the age-associated alterations in immunity are a result of infection with CMV, and this latent infection in turn is subsumed under the Immune Risk Profile (IRP) (Olsson et al., 2000; Pawelec et al., 2002; Wikby et al., 2002). The IRP is a collection of immunological risk factors which has been found to predict mortality in two distinct populations of community-dwelling adults in Sweden; the so called OCTO-immune (i.e., octogenarians) and NONA-immune (i.e., nonagenarians) longitudinal studies (Nilsson et al., 2003; Wikby et al., 1994). OCTO subjects were in exceptionally good health, defined by modified SENIEUR criteria, whereas NONA subjects were representative of the typical elderly; only 10% were in excellent health.

One of the most significant immunological risk factors for mortality was found to be an inverted CD4:CD8 ratio < 1.0, which appears largely driven by an expansion of $CD8^+CD27^-CD28^-/CD45RA^+$ T cells (Olsson et al., 2000; Wikby et al., 2002; Wikby et al., 2006). This accumulation of late-differentiated cells is driven by CMV, and is a second component of the IRP (Hadrup et al., 2006; Olsson et al., 2000). Elevated low-grade inflammation, as measured by plasma interleukin-6 (IL-6), was also one of the predictors of two-year mortality among OCTO and NONA individuals (Wikby et al., 2006). This relationship

appeared independent of other inflammatory markers (e.g., C-reactive protein; CRP) and several disease states associated with inflammation (e.g., cardiovascular disease, type-II diabetes, and Alzheimer's disease) (Wikby et al., 2006). Although the OCTO/NONA studies primarily examined whether immunological risk factors were predictive of mortality, it is noteworthy that some of these parameters are also associated with increased frailty and declining immune function throughout life. For example, both increased levels of IL-6 and expanded numbers of $CD8^+CD28^-$ cells are associated with cognitive decline and smaller antibody responses to vaccination in the elderly (Larbi et al., 2009; Saurwein-Teissl et al., 2002; Trzonkowski et al., 2009; Wikby et al., 2005).

1.7. OXIDATIVE STRESS

In 1956, the 'free radical theory of ageing' was proposed as a mechanism for cellular ageing (Harman, 1956). Free radicals are pro-oxidant molecules with one or more un-paired electrons (e.g., the superoxide ion; $O_2^{\bullet-}$). Together with non-radical species (e.g., hydrogen peroxide; H_2O_2), free radicals are classified under the umbrella term; reactive oxygen species (ROS). ROS are produced as a consequence of normal bodily processes. Sources include the mitochondrial electron transport chain, peroxisomes, endothelial or hepatic xanthine oxidase, and leukocytes (Beckman and Ames, 1998; Sachdev and Davies, 2008). ROS have a variety of roles in normal homeostasis, including respiration, cellular signalling and immune defence. As these molecules are highly reactive and can potentially oxidise bodily molecules, cells are equipped with antioxidant defences which are normally at balance with ROS production. An antioxidant can be defined as a substance that, when present at low concentrations compared to those of an

'oxidisable' substrate, significantly delays or prevents the oxidation of that substrate (Halliwell and Gutteridge, 1999). When an abnormally large amount of ROS are produced, as with certain disease states and vigorous exercise, proteins, lipids and nucleic acids can be oxidised (Bermejo et al., 2008; Neubauer et al., 2008). Oxidative stress is defined as a state whereby ROS overcome anti-oxidant defences resulting in oxidation of body molecules (Halliwell and Gutteridge, 1999). It has been argued that the age-associated increase in oxidative stress is a result of three different factors; an increase in ROS production; a decline in antioxidant defences; and an impaired ability to repair or remove damaged molecules (Sohal and Weindruch, 1996).

1.7.1. Measurement of oxidative stress

Free radicals have very short half-lives therefore their direct quantification in humans is often impractical and expensive (Sachdev and Davies, 2008; Vollaard et al., 2005). Instead, oxidative stress is most commonly assessed indirectly through the measurement of oxidative-modifications to bodily molecules and via the assessment of antioxidant capacity (Sachdev and Davies, 2008; Vollaard et al., 2005). Biomarkers are usually measured in peripheral blood, and have been shown to correlate well with similar measurements in tissues (Veskoukis et al., 2009). Thus, markers of oxidative stress in peripheral blood can provide an indication of whole body oxidative stress (Veskoukis et al., 2009). Proteins are targets of free-radical mediated oxidation, and the amino acids cysteine and methionine are highly susceptible to oxidation (Berlett and Stadtman, 1997). Interaction with ROS leads to protein carbonylation, which is a robust, stable, and general marker of oxidative stress (Dalle-Donne et al., 2003). Indeed, increased protein carbonylation can be detected with ageing, cognitive decline, Alzheimer's disease, cancer and auto-immune conditions (Bermejo et al., 2008; Calabrese et al., 2007; Mecocci et al., 1999; Zhang et al., 2010;

Zipprich et al., 2009). Likewise, oxidation of lipids provides another general measure of whole body oxidative stress. Lipid peroxidation likely represents interaction of ROS with membrane lipids or fatty acids, and has been shown to disturb membrane integrity, structure and function (Finaud et al., 2006; Niki, 2009). Lipid peroxides are a common measure of oxidative stress, and increased lipid peroxidation is associated with cardiovascular disease (Schwedhelm et al., 2004). Damage to DNA in peripheral blood lymphocytes is a highly sensitive and specific marker of oxidative stress (Collins et al., 1993; Singh et al., 1988). The use of DNA repair endonucleases (e.g., formamidopyrimidine glycosylase; FPG) allows the direct quantification of damage caused specifically by oxidative stress (Collins et al., 1997; Collins et al., 1993). Free radicals, and in particular the hydroxyl radical ($OH^{\bullet}$) cause sugar and base modifications, strand breaks and DNA-protein cross links (Dizdaroglu et al., 2002). Damage to DNA has been directly implicated in the risk and pathogenesis of cardiovascular disease, since elevated levels of DNA damage have been found in atherosclerotic plaques (Martinet et al., 2002; Wu et al., 2004). Complimenting the assessment of oxidative-modifications to bodily molecules, measures of antioxidant capacity are often conducted on blood plasma (Bartosz, 2010). Plasma 'antioxidant power' is largely attributable to uric acid, vitamins A, C, and E, thiols, bilirubin, and albumin. Thus, measures of total antioxidant capacity correspond to the sum of all antioxidants present in the fluid examined (Bartosz, 2010; Finaud et al., 2006). In turn, this measure is partly influenced by diet and antioxidant compounds in blood cells and the vascular endothelium (Bartosz, 2010; Cao and Prior, 2000; Finaud et al., 2006). Assessment of thiol groups such as reduced glutathione (GSH), or the ratio of GSH to oxidised glutathione (GSSG), is also frequently used to assess antioxidant capacity of blood. Intracellular thiols are important indicators of oxidative stress, and show a decline in concentration under prolonged conditions of ROS production, such as ultra-endurance

exercise (Finaud et al., 2006). In addition, this measure of antioxidant capacity appears to be less susceptible to alterations with diet (Ibrahim et al., 2008; Shin et al., 2010).

1.7.2. Exercise and oxidative stress

Exercise is associated with increased ROS production, and mitochondria are often cited to be the major source of ROS during exercise (Powers and Jackson, 2008). Due to original estimates of 2-5% of total oxygen consumed leading to $O_2^{\bullet-}$ production, coupled with the 50-100 fold increase in respiration with exercise, it is often assumed that exercise-induced ROS production is 50-100 times more than during rest (Powers and Jackson, 2008). However, more recent estimates for the amount of oxygen used by mitochondria resulting in $O_2^{\bullet-}$ production is much less, only 0.15% (St-Pierre et al., 2002). Thus, the magnitude of ROS production with exercise is likely to be much smaller than originally thought, and more likely only 15 times above basal values (Powers and Jackson, 2008).

Oxidative stress has been examined following various modes, intensities and durations of exercise. For example, increased markers of oxidative stress have been reported after lower-body resistance exercise (Rietjens et al., 2007), intermittent team sport activities (Ascensão et al., 2008), and short duration rock-climbing (Magalhaes et al., 2007). The majority of studies have investigated running or cycling exercise, ranging in duration from relatively short; < 60 minutes (e.g., Michaildis et al., 2007), to moderate; 6-7 hours (e.g., Mastaloudis et al., 2001), and finally long duration; 13-33 hours of continuous exercise (e.g., Nieman et al., 2003; Skenderi et al., 2008). In addition to exercise performed largely in one continuous bout, other studies have examined several consecutive days of prolonged endurance activity, including periods of rest and

recovery (e.g., Hattori et al., 2009; Radák et al., 2003). In general, oxidative stress can be detected immediately after exercise, and with longer and more intense bouts of exercise, can persist for up to 72 hours depending on the biomarker examined (Machefer et al., 2004; Neubauer et al., 2008; Skenderi et al., 2008; Tauler et al., 2006). More prolonged effects are observed when antioxidant activity is the primary measurement. For example, decreased levels of enzymatic antioxidants have been reported 19 days after an ironman triathlon competition (Neubauer et al., 2008). It is unclear however whether these prolonged changes are a possible 'de-training' effect caused by the typical period of physical inactivity in the recovery from such events.

The effects and consequences of exercise-induced oxidative stress are subject to speculation and debate. It was first assumed that exercise, due to increased oxygen consumption and concomitant increased ROS production, would induce deleterious changes to proteins, lipids and DNA (Packer, 1997). However, more recent research has indicated that ROS produced during exercise are key to adaptive processes (Fisher-Wellman and Bloomer, 2009; Ji, 2008; McArdle et al., 2001). Indeed, it has been suggested that at least some of the health benefits derived from regular physical activity are mediated through redox-sensitive signalling (Kesaniemi et al., 2001; Radak et al., 2005, 2008). Considering that markers of oxidative stress are causally related to ageing, and the development of a number of diseases associated with ageing (Schwedhelm et al., 2004; Wu et al., 2004; Yeh et al., 2010) there has been substantial debate as to whether the health benefits of moderate intensity physical activity extend to more extreme forms of exercise which result in prolonged oxidative stress (Kesaniemi et al., 2001; Knez et al., 2006). For example, epidemiological studies show that individuals taking part in an exceptionally large amount of physical activity have impaired cardiovascular health (Knez et al., 2006). In addition, it appears

that athletes undertaking very strenuous exercise, either in the form of increased exercise training loads (sometimes referred to as 'overtraining') or following very extreme forms of exercise (e.g., ultra-endurance running) might be at increased risk of infection (Gleeson, 2007; Malm, 2006; Nieman, 1995; Peters and Bateman, 1983). It is possible that altered immune function with extreme exercise is a consequence of oxidative stress.

1.8. OXIDATIVE STRESS AND SENESCENCE OF THE IMMUNE SYSTEM

There are several lines of evidence to suggest that oxidative stress and immunosenescence are linked. First, in line with Harman's theory (Harman, 1956) that oxidative stress is a cause of cellular ageing, 'aged' immune cells are also characterised by increased oxidative damage. Scarpaci and colleagues (2003) showed that DNA damage in memory (CD45RO$^+$) cells was increased compared to naïve (CD45RA$^+$) cells, and damage was greater in elderly individuals compared to young controls. In addition, cells from the elderly had minimal repair mechanisms. Thus, it seems that at a theoretical level, the oxidative stress theory of ageing generalises to cells of the immune system.

Second, anecdotal observations provide evidence for associations between oxidative stress and immunosenescence. Rheumatoid arthritis, Multiple sclerosis, Alzheimer's disease, and Systemic Lupus Erythematosus, are conditions of heightened oxidative stress and also result in an accumulation of CD8$^+$CD28$^-$ cells (Filaci et al., 2005; Larbi et al., 2009; Markovic-Plese et al., 2001; Schmidt et al., 1996; Sun et al., 2008). Extensive research shows that oxidative stress and inflammation are intrinsically linked (Reuter et al., 2010). ROS activate a variety of transcription

factors (e.g., nuclear-factor κ B; NFκB), which can lead to expression of hundreds of genes, including those of inflammatory cytokines (Reuter et al., 2010). Oxidative stress and inflammatory flare ups in Systemic Lupus Erythematosus disease are associated with CMV reactivation (Su et al., 2007), and the role of inflammation dubbed 'inflammageing' is central to immunosenescence (De Martinis et al., 2005; Franceschi et al., 2000a; Franceschi et al., 2000b).

Finally, more direct evidence implicates oxidative stress in immunosenescence in the context of latent viral reaction. Human immunodeficiency virus (HIV), has been proven to reactivate with oxidative stress via NFκB (Baruchel and Wainberg, 1992). Viral reactivation also occurs due to cellular depletion of antioxidant molecules (e.g., GSH) by ROS (Droge et al., 1994; Staal et al., 1990). Depleted GSH prevents lymphocyte proliferation, IL-2 production, and reduces cytolytic activity (Liang et al., 1991; Smyth, 1991; Suthanthiran et al., 1990; Yamauchi and Bloom, 1993). Thus, reduction of GSH by ROS and the resulting oxidative stress might accelerate immunosenescence by inhibiting immune function and reactivating latent herpes viruses.

1.9. SUMMARY AND OVERVIEW OF THESIS

This thesis consists of four experimental chapters. The first two studies (chapters two and three) used acute high intensity exercise to study the effects of exercise on markers of oxidative stress and T cell subsets that become expanded in immunosenescence. It was already established that these cells become mobilised during exercise (Campbell et al., 2009), and chapter two examined whether these cells also show the largest egress during post-exercise lymphocytopenia. Considering the dramatic effects of CMV infection on the expansion of differentiated memory

cells, it was also investigated whether infection with CMV would be associated with larger mobilisation and egress in response to exercise. The third study (chapter four) examined whether ultra-endurance exercise is associated with long term (up to one month) oxidative stress. The final study (chapter five) examined whether the rudimentary features of immunosenescence can be detected in CMV seropositive 'healthy' young adults.

The major questions investigated in this thesis were:

1) Is lymphocytopenia following exercise driven by an extravasation of the same 'senescent' cytotoxic T cells that are mobilised into the blood during exercise?

2) Does CMV infection, which causes marked alterations in the T cell memory pool, influence the magnitude of T cell mobilisation and egress with exercise?

3) Does exercise cause oxidative damage in immune cells, and are measurements of oxidative stress in lymphocytes with exercise affected by changes in the cellular composition of blood?

4) Does ultra-endurance exercise result in long term (up to one month) alterations in our capacity to withstand oxidative stress?

5) Do young adults latently infected with CMV exhibit an IRP, suggesting an early onset of immunosenescence?

1.10. REFERENCE LIST FOR CHAPTER ONE

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CHAPTER TWO

2.0. LATENT CYTOMEGALOVIRUS INFECTION AMPLIFIES CD8 T-LYMPHOCYTE MOBILISATION AND EGRESS IN RESPONSE TO EXERCISE

2.1. ABSTRACT

Exercise induces a mobilisation of $CD8^+$ T lymphocytes (CD8TL) into peripheral blood. This response is largely confined to effector-memory CD8TLs: antigen experienced cells which have a strong tissue-homing and effector potential. This study investigated whether T cell memory phenotype also accounts for the CD8TL egress from peripheral blood following exercise. As latent Cytomegalovirus (CMV) infection is associated with a robust expansion in the number and proportion of effector-memory CD8TLs, we also investigated if CMV serostatus was a determinant of the CD8TL responses to exercise. Fourteen males (Mean age 35, SD ± 14 yrs), half of whom were CMV seropositive (CMV^+), ran on a treadmill for 60 minutes at 80% $\dot{V}O_2$ max. Blood was collected at baseline (Pre), during the final minute of exercise (Ex60), and 15 (Post15) and 60 (Post60) minutes thereafter. CD8TL memory subsets were characterised by flow cytometry, using the cell-surface markers CD27, CD28 and CD45RA. The results confirmed that CD8TLs with an effector-memory phenotype ($CD27^-CD28^-/CD45RA^+$) exhibited the largest increase during exercise (+200% to +250%), and further showed that these cells exhibited the largest egress from blood 60 minutes post-exercise (down to 40% of baseline values). Strikingly, the mobilisation and subsequent egress of total CD8TLs was nearly twice as large in CMV^+ individuals. This effect appeared specific to CD8TLs, and was not seen for $CD4^+$ T lymphocytes or total lymphocytes. This effect of CMV was largely driven by higher numbers of exercise-responsive effector-memory CD8TLs in the CMV^+ participants. This is the first study to demonstrate that infection history is a determinant of immune responses to exercise.

2.2. INTRODUCTION

All mammals, including humans, have a remarkable capacity to acutely change the cellular composition of peripheral blood in response to psychological and physiological stressors (Dhabhar, 2000). For example, strenuous exercise causes a near-immediate mobilisation of lymphocytes into the blood, a process referred to as lymphocytosis (Gleeson and Bishop, 2005; McCarthy and Dale, 1988). This phenomenon is particularly marked for lymphocytes with a cytotoxic potential, such as Natural Killer cells, $\gamma\delta$ T lymphocytes, and CD8$^+$ T lymphocytes (CD8TLs) (Anane et al., 2009). These subsets exhibit a high adrenergic receptor density and sensitivity, which in turn, regulates their detachment from vascular endothelium and release into the blood (Anane et al., 2009; Benschop et al., 1994; Dimitrov et al., 2010). Indeed, there is now conclusive evidence that stress and exercise-induced lymphocytosis is under the control of the sympathetic nervous system and the concomitant release of catecholamines (Benschop et al., 1994; Dimitrov et al., 2010; Elenkov et al., 2000).

One to two hours after high-intensity exercise, the lymphocytosis is followed by a lymphocytopenia whereby the numbers of circulating lymphocytes fall below baseline level (Gleeson and Bishop, 2005; McCarthy and Dale, 1988). Experimental studies in rodents suggest that this lymphocytopenia reflects extravasation of lymphocytes to selected peripheral tissues, such as the lungs, presumably part of enhanced immunosurveillance in front-line tissues (Krüger et al., 2008; Krüger and Mooren, 2007). Consistent with this model, recent studies in humans have shown that exercise preferentially mobilises a subset of memory CD8TLs that have a strong tissue migrating potential and the capacity to induce rapid effector responses (e.g., target killing)

(Campbell et al., 2009; Simpson et al., 2008; Simpson et al., 2007a). It might therefore be predicted that these tissue-migrating memory cells would preferentially egress from peripheral blood post-exercise, but there is currently no data to support this contention.

Subsets of CD8TLs have been identified on the basis of the combined expression of cell surface markers such as CD27, CD28, and CD45RA (Appay et al., 2002; Appay et al., 2008; Hamann et al., 1997; Romero et al., 2007) (see Table 2.1). In our study we used two phenotypic profiles to define effector memory subpopulations; comparing CD27 and CD45RA expression, or CD27 and CD28 expression (see Table 2.1). CD45RA is one of the isoforms of the leukocyte surface protein CD45. This CD45RA isoform is present on naïve T cells and replaced by the isoform CD45RO after antigenic recognition. However, CD45RA is re-expressed on a subset of so-called 'revertant' memory cells, and this $CD27^-CD45RA^+$ phenotype is known to exhibit strong effector potential (such as the ability for rapid target killing, inflammatory cytokine production, and tissue migration, see Table 2.1). CD27 and CD28 are co-stimulatory and survival molecules that are co-expressed on naïve and early memory populations. However, during differentiation (i.e., a process characterised by the accumulation of effector functions), these markers are gradually lost and a $CD27^-CD28^-$ phenotype defines a population of late differentiated effector memory cells. $CD27^-CD45RA^+$ cells are themselves largely $CD28^-$ and there is therefore considerable overlap between these phenotypic profiles (Appay et al., 2008; Hamann et al., 1997; van Lier et al., 2003)

Individual differences in the proportions and numbers of the various CD8TL memory subsets are to a considerable extent determined by infection history (Chidrawar et al., 2009; Khan et al., 2002). For example, infection with Cytomegalovirus (CMV) causes a robust accumulation of late

TABLE 2.1.

Phenotypic identification and functional properties of CD8TL (CD3$^+$CD8$^+$) subsets

Cell description	Identification	Migration preference	Effector potential	Reference
Naïve	CD27$^+$CD45RA$^+$	Lymphoid tissue	−	Hamann et al. 1997; Romero et al 2007
Central memory (CM)	CD27$^-$CD45RA$^+$	Lymphoid tissue	−	Hamann et al. 1997; Romero et al 2007
Effector memory (EM)	CD27$^-$CD45RA$^-$	Peripheral tissue	+	Hamann et al. 1997; Romero et al 2007
CD45RA$^+$ effector memory (EMRA)	CD27$^-$CD45RA$^+$	Peripheral tissue	++	Hamann et al. 1997; Romero et al 2007
Early stage (early)	CD27$^+$CD28$^+$	Lymphoid tissue	−	Appay et al. 2002
Intermediate stage (inter)	CD27$^+$CD28$^-$	Peripheral tissue	+	Appay et al. 2002
Late stage (late)	CD27$^-$CD28$^-$	Peripheral tissue	++	Appay et al. 2002

differentiated effector-memory CD8TLs, which are characterised by loss of CD27 and CD28 expression, as well as frequent 'reversion' to expression of the naïve CD45RA isoform (Appay et al., 2002; van Lier et al., 2003). CMV is an endemic herpes virus which infects approximately 60% of western populations (Lubeck et al., 2010; Staras et al., 2006). In healthy individuals infection with CMV is usually asymptomatic, although some individuals develop symptoms of malaise and fever upon primary infection (van der Meer et al., 1996). CMV remains latent in the body, but is believed to undergo intermittent reactivation (Stowe et al., 2007; Zanghellini et al., 1999). In view of research showing a selective mobilisation of effector memory CD8TLs with exercise (Campbell et al., 2009), we investigated if CMV seropositive individuals demonstrated an exaggerated CD8TL exercise-response as a consequence of the accumulation of late-differentiated/effector-memory CD8TLs.

The aim of the current study was therefore to determine the pattern of egress within discrete CD8TL memory subsets following recovery from exercise and to compare this to the profile of mobilisation during exercise. Secondly, we aimed to determine if infection history is a determinant of this immune cell redistribution with a specific investigation of how CMV serostatus influences the magnitude and kinetics of CD8TL mobilisation.

2.3. METHODS

2.3.1. Participants

Participants were fourteen healthy non-smoking men (mean age = 35.1 years, SD ± 14.6; BMI; 24 ± 3 $kg.m^{-2}$, cardio-respiratory fitness ($\dot{V}O_2$ max) 58.0 ± 4.3 $ml.kg^{-1}.min^{-1}$), who were accustomed to vigorous endurance exercise and had a $\dot{V}O_2$ max within the 90th percentile for their age (Whaley et al., 2006). Participants provided informed consent, and the study was approved by the School of Sport and Exercise Sciences research ethics committee, of the University of Birmingham.

2.3.2. Procedures

Participants visited the laboratory to undergo two graded exercise tests on a treadmill. The first exercise test measured $\dot{V}O_2$ max by running to volitional exhaustion, to enable the intensity of the exercise trial to be prescribed relative to the fitness of each participant (i.e., 80% of $\dot{V}O_2$ max). A second test assessed the relationship between oxygen consumption ($\dot{V}O_2$) and four sub-maximal running speeds (range 8.2 -11.6 $km \cdot h^{-1}$, gradient 2%) to calculate the speed to elicit 80% of $\dot{V}O_2$ max, using linear regression. Breath-by-breath measurements were recorded every 5 seconds throughout both tests (Oxycon Pro, Jaegar, Germany), with heart rate monitored (RS200, Polar, Finland) and ratings of perceived exertion (RPE) (Borg, 1973) recorded during the final minute of each stage. In addition, participants had leisure time physical activity assessed using the international physical activity questionnaire (IPAQ) (Craig et al., 2003). IPAQ data was

expressed as MET-minutes per week, where 1 MET (metabolic equivalent) is equal to resting metabolic rate (Ainsworth et al., 1993).

2.3.3. Exercise trial

One week after the preliminary tests, participants visited the laboratory for an exercise trial. Participants were instructed to refrain from exercising and drinking alcohol or caffeine during the day prior to the trial. After an overnight fast, participants reported to the laboratory between 06:00 and 07:00. Following a 15 minute rest, a baseline blood sample (Pre) was collected from forearm vein by an indwelling catheter, which was kept patent by flushing regularly with saline.

The baseline sample was followed by the exercise trial, which consisted of treadmill running at a speed to elicit 80% of $\dot{V}O_2$ max for 60 minutes. Treadmill gradient was maintained at a 2% incline. Breath-by-breath measurements, heart rate and RPE were recorded for periods of 5 minutes at regular intervals. Blood samples were collected during the final minute of exercise (Ex60), and again 15 minutes (Post15) and 60 minutes (Post60) post-exercise. Pilot work showed that the nadir in the blood lymphocyte count occurred 60-minutes post exercise (data not shown). To address possible confounding of exercise results by diurnal variation in lymphocyte counts, seven participants also completed a control trial which involved sitting in the same room for 2 hours, with all measures collected in an identical manner to the exercise trial. The order of the two trials was counterbalanced.

2.3.4. Flow cytometry

Blood was collected in ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Oxford, UK) and samples were prepared within 3 hours of collection. Briefly, whole blood was incubated with the following monoclonal antibodies for 20 minutes at room temperature; CD45RA FITC, CD27 PE (Pharmingen, San Diego, USA), CD3 PerCP, CD4 APC, CD8 APC-cy7 (BD Biosciences, San Jose, USA) and CD28 PE-cy7 (eBioscience, Hatfield, UK). Erythrocytes were lysed with FACS lysing solution (BD Biosciences, San Jose, USA) according to manufacturer instructions, and then centrifuged at $250 \times g$ for 7 minutes. Cells were re-suspended in 2% paraformaldehyde phosphate-buffered saline solution and stored in the dark at 4°C. Cells were read within 24 h on a six colour flow cytometer (BD FACS CANTO II, BD Biosciences), collecting 25,000 gated lymphocytes. The flow cytometer was regularly calibrated using Calibrite beads (BD Biosciences, San Jose, USA) and compensation adjustments were made prior to each run using single labelled antibody tubes. Data were analysed using FlowJo7 (Tree Star, Inc., Ashland, OR), and lymphocytes enumerated using a Coulter ACT[diff] haematology analyser (Beckman-Coulter, High Wycombe, UK).

The lymphocyte population was gated on the forward *versus* side-scatter and further identified by CD8 expression in combination with CD3 (Campbell et al., 2008). CD8TL expression of CD27 and CD45RA was examined to identify naïve, central memory, effector memory, and CD45RA^{+} effector memory sub-populations as described elsewhere (Campbell et al., 2009; Hamann et al., 1997; Romero et al., 2007). CD8TLs were also examined for CD27 and CD28 expression to identify early, intermediate and late stage sub-populations, as described by Appay et al. (2002).

2.3.5. CMV serostatus

Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) (Biocheck, Inc. CA, USA) according to manufacturer instructions.

2.3.6. Statistical analyses

Data were inspected for normal distribution using the Kolmogrov Smirnov test. Non-normally distributed data were transformed logarithmically. Responses to exercise were examined using repeated-measures Analyses of Variance (ANOVAs), with age and baseline cell count included as covariates where appropriate (Jennings and Stine, 2007). Differences between individual time points were examined using post-hoc paired samples *t*-tests. To confirm our parametric analyses, equivalent non-parametric tests were used. Statistical significance was accepted at the $p < .05$ level. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated. Data were analysed using SPSS statistical package version 18.0 for Windows (SPSS Inc, USA).

2.4. RESULTS

All participants completed the exercise trial successfully. Table 2.2 compares $\dot{V}O_2$, energy expenditure, heart rate, and perceived exertion during treadmill running with seated rest during the control trial.

2.4.1. Leukocyte subsets

Table 2.3 shows the summary data for leukocyte subsets. Exercise was associated with an acute leukocytosis, whereby total leukocyte count increased by +85% during exercise and remained elevated +22% above baseline values at 60 minutes post-exercise. The leukocytosis during exercise was mainly driven by a mobilisation of lymphocytes (+113%) and granulocytes (+66%), while 60 minutes after the exercise bout, lymphocyte numbers fell below baseline levels (−25%), accompanied by a secondary mobilisation of granulocytes (+67%) (See Table 2.3). The biphasic lymphocyte response was replicated in both CD4$^+$ T lymphocytes (CD4TLs) and CD8TLs, although it was more marked for the latter (see Table 2.3).

2.4.2. CD8TL subsets during exercise

Replicating previous findings (Campbell et al., 2009), exercise resulted in a significantly larger mobilisation of CD8TL effector-memory (+207%) and CD45RA$^+$ effector-memory cells (+239%), as compared to central memory (+108%) or naïve CD8TLs (+58%) (paired samples t-tests, all $t_{(13)} > 4.4$, $p < .01$; see Figure 2.1A). Identifying CD8TL subsets on the basis of CD27 and CD28 expression (yielding early, intermediate, and late differentiated cells, see Table 2.1), showed that phenotypically late-differentiated cells exhibited a larger increase (+265%) as

TABLE 2.2.

Physiological demands of the exercise and control trials (mean ± SD)

	Exercise $n = 14$	Control $n = 7$
$\dot{V}O_2$ (ml·kg^{-1}·min^{-1})	45.6 ± 3.3***	3.6 ± 0.5
$\dot{V}O_2$ (% $\dot{V}O_2$max)	78.7 ± 1.9***	6.4 ± 1.1
Energy expenditure (kcal)	1011.0 ± 85.4***	80.4 ± 13.8
Heart rate (bpm)	162 ± 13***	51 ± 7
Heart rate (% max)	88.0 ± 4.4***	28.9 ± 3.4
RPE	14 ± 2***	6 ± 0

*** $p < .001$ Comparing exercise with control trial (independent samples t-test)
RPE; ratings of perceived exertion

TABLE 2.3.

Leukocyte subsets before (Pre), after 60 minutes of exercise (EX60), and 15 minutes (POST15) and 60 minutes (POST60) post exercise (mean ± SD)

Cells x 10^9/L	Pre	Ex 60	Post 15	Post 60	Main effects of time #
Leukocytes	6.29 ± 1.18	11.66 ± 3.13***	8.19 ± 3.01***	7.73 ± 3.51***	$F_{(3, 33)}$ = 15.8; p<.001
Lymphocytes	2.59 ± 0.59	5.51 ± 1.82***	2.94 ± 1.20	1.93 ± 0.94***	$F_{(3, 33)}$ = 150.0; p<.001
Monocytes	0.53 ± 0.19	0.89 ± 0.41	0.54 ± 0.25	0.51 ± 0.23	$F_{(3, 33)}$ = 0.4; p=NS
Granulocytes	3.17 ± 0.92	5.26 ± 2.10***	4.71 ± 1.63***	5.29 ± 2.15**	$F_{(3, 33)}$ = 16.9; p<.001
CD4TLs	1.09 ± 0.36	1.65 ± 0.81***	1.15 ± 0.63	0.86 ± 0.47***	$F_{(3, 33)}$ = 57.9; p<.001
CD8TLs	0.63 ± 0.24	1.33 ± 0.60***	0.71 ± 0.29*	0.40 ± 0.16***	$F_{(3, 33)}$ = 62.2; p<.001

*** p< .001, ** p< .01, * p< .05, compared to Pre (paired samples t-test)

Age as a covariate

compared to both intermediate (+154%) and early differentiated cells (+67%). Intermediate differentiated cells, in turn, showed a larger increase than CD8TL with an early differentiated phenotype (paired samples t-tests, all t (13) > 5.0, p <.01; see Figure 2.1C).

2.4.3. CD8TL subsets post-exercise

Figure 2.1B and 2.1D show that the pattern of CD8TL mobilisation seen during exercise was broadly mirrored for the egress 60 minutes post-exercise. Specifically, $CD45RA^+$ effector memory cells showed a significantly larger decrease (% decrease relative to pre-exercise values; −60%) than effector memory cells (−38%), which both showed a larger decrease than central memory (−30%) and naïve CD8TLs (−29%) (paired samples t-tests, all $t_{(13)}$ > 2.4, p <.03). Identifying CD8TL subsets on the basis of CD27 and CD28 expression (see Table 2.1) revealed that late differentiated cells had the greatest fall in numbers (−60%) compared to cells with an intermediate (−52%) or early (−28%) phenotype (paired samples t-tests, all $t_{(13)}$ > 4.7, p <.001; see Figure 2.1D).

2.4.4. Control trial

Participants who took part in the control trial ($n = 7$, condition-order counterbalanced) did not exhibit any significant changes in CD8TL subpopulations or any other leukocyte subset (all pair-wise comparisons p >.10).

FIGURE 2.1.
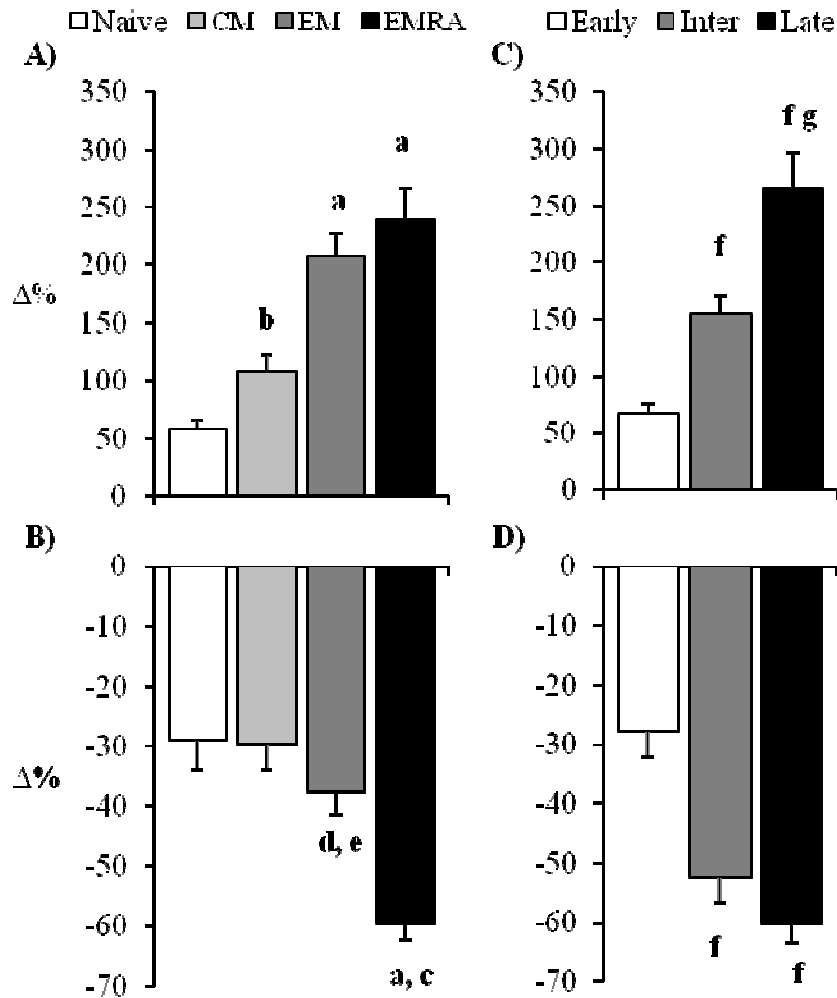
Percentage change ($\Delta\%$) in cell numbers of CD8 TLs sub-populations, during exercise (Ex60; A and C), and at 1-hour post-exercise (Post60; B and D). Panel A and B show subsets identified by CD27 and CD45RA expression. Panel C and D show subsets identified by CD27 and CD28 expression. Naïve CD27^{+}CD45RA^{+}, Central memory (CM) CD27^{-}CD45RA^{+}. Effector memory (EM) CD27^{-}CD45RA^{-}. CD45RA^{+} Effector memory (RAEM) CD27^{-}CD45RA^{+}. Early CD27^{+}CD28^{-}. Inter CD27^{+}CD28^{-}. Late CD27^{-}CD28^{-}. [a] $p<.001$ compared to naïve and CM. [b] $p<.01$ compared naïve. [c] compared to EM. [d] compared to naïve, $p<.03$. [e] compared to CM $p<.01$. [f] compared to early $p<.001$. [g] compared to inter $p<.01$ (paired samples t-tests). Data are Means ± SEM.

2.4.5. CMV serostatus and lymphocyte responses to exercise

Repeated measures ANOVA yielded a significant time (Pre, Ex60, Post15, Post60) by serostatus interaction ($F_{(3, 33)} = 6.3$, $p < .05$, $\eta^2 = 0.37$, adjusted for age) for CD8TL responses (Figure 2.2 A). Post-hoc analyses revealed that this effect was largely driven by a ~2-fold larger increase in CD8TLs during exercise ($F_{(1, 11)} = 5.3$, $p < .05$, $\eta^2 = 0.33$), as well as a ~2-fold larger post-exercise egress ($F_{(1, 11)} = 5.2$, $p < .05$, $\eta^2 = 0.32$) in CMV$^+$ individuals (Figure 2.2C and 2.2D; all analyses adjusted for age). Comparable results were obtained when using non-parametric statistical tests (Wilcoxon signed-rank test). As shown in Table 2.4, CMV serostatus was unrelated to age, BMI, leisure time physical activity, fitness, maximum heart rate, or any of the measures of exercise performance. Adjustment for these parameters statistically did not attenuate the observed difference in CD8TL mobilisation. CMV serostatus was unrelated to CD4TL responses (Figure 2.2B) or total lymphocyte responses (data not shown) (serostatus by time interactions for CD4TL and lymphocytes respectively; $F_{(3, 33)} = 0.4$, $p > .7$ and $F_{(3, 33)} = 1.8$, $p > .17$, analyses adjusted for age).

FIGURE 2.2.
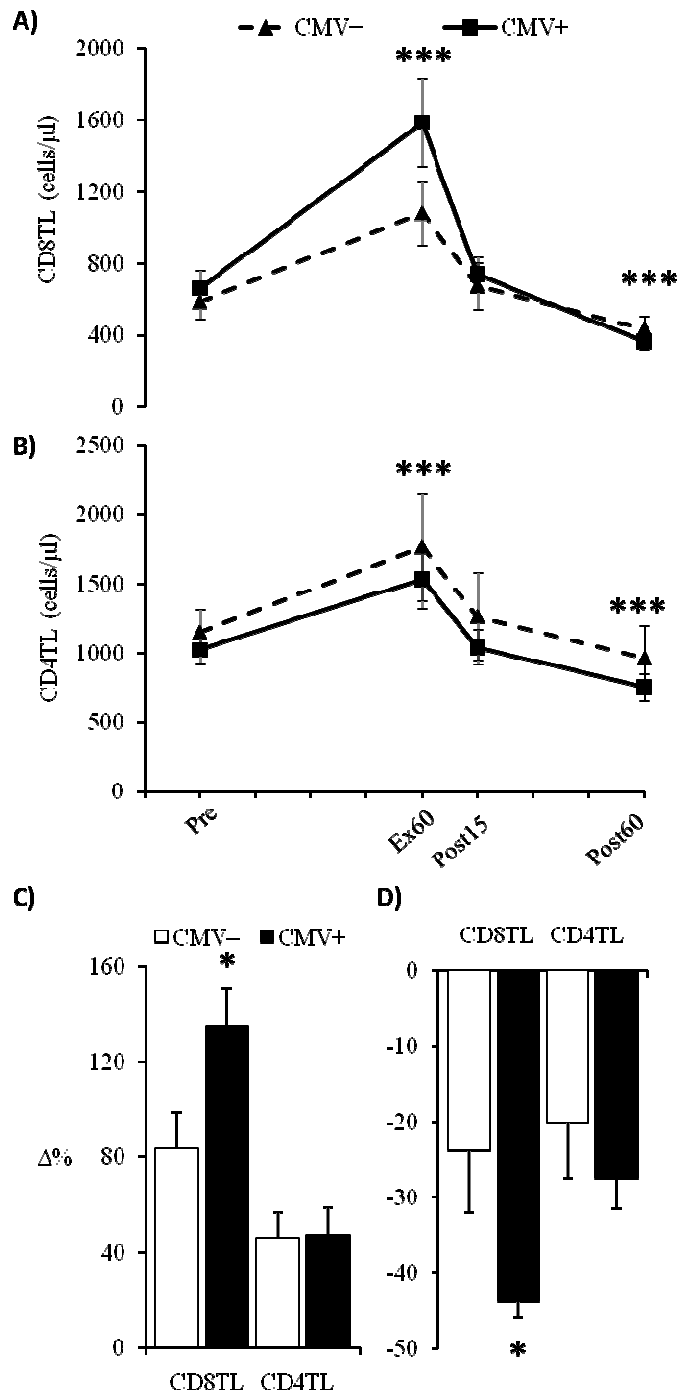
Changes in **A)** CD8TLs (cells/μl). **B)** CD4TLs (cells/μl). **C)** Percentage change (Δ%) in CD8TL and CD4TL numbers during exercise (Ex60) **D)** Percentage change (Δ%) in CD8TL and CD4TL numbers 60-minutes post-exercise (Post60).

*** $p < .001$ comparison with baseline values. * $p < .05$, comparing CMV⁻ with CMV⁺ (ANOVA). Data are Means ± SEM.

TABLE 2.4.

Characteristics of CMV seropositive and seronegative participants (mean ± SD). No significant group differences were observed ($p >.10$, independent samples t-test).

	CMV$^+$ *n = 7*	CMV$^-$ *n = 7*
Subject characteristics at baseline		
Age (years)	34.5 ± 12.0	33.7 ± 15.9
BMI (kg·m^{-2})	24.1 ± 3.6	23.3 ± 1.4
LTPA[a] (Median MET·min·week^{-1})[b]	3960 (2853-4850)	2880 (1080-7290)
Measured $\dot{V}O_2$ max	58.2 ± 5.1	57.8 ± 3.8
Maximum heart rate at $\dot{V}O_2$ max (bpm)	181 ± 10	188 ± 9
Exercise trial results		
$\dot{V}O_2$ (ml·kg^{-1}·min^{-1})	46.0 ± 3.6	45.1 ± 3.2
Energy expenditure (kcal)	1040 ± 82	982 ± 84
Heart rate (bpm)	159 ± 11	166 ± 14
RPE	13 ± 1	14 ± 2

[a] Leisure Time Physical Activity, as assessed by the international physical activity questionnaire (IPAQ).

[b] Data are medians (min-max), differences assessed using a Mann-Whitney U Test.

2.4.6. CMV serostatus and CD8TL subset responses to exercise

The differential CD8TL response described above was anticipated because positive CMV serostatus is associated with higher numbers of exercise-responsive late-differentiated/effector-memory subsets (van Lier et al., 2003). This observation was replicated in the current study: compared to CMV⁻ individuals, those who were CMV⁺ had a ~6-fold greater number of CD45RA⁺ effector memory ($t_{(12)} = -3.3$, $p < .01$) and a ~6-fold greater number of late differentiated CD8TLs ($t_{(12)} = -3.4$, $p < .01$; independent samples t-tests).

Figure 2.3 displays the response of individual CD8TL subpopulations, as identified on the basis of CD27 and CD45RA expression (see Table 2.1). Repeated measures ANOVA confirmed that serostatus was associated with a larger increase in effector memory cells (CD27⁻CD45RA⁻) (serostatus by time interaction; $F_{(1, 11)} = 5.3$, $p < .05$, $\eta^2 = 0.33$) and CD45RA⁺ effector memory cells (CD27⁻ CD45RA⁺) (serostatus by time interaction; $F_{(1, 11)}$ 15.2, $p < .01$, $\eta^2 = 0.58$) (all analyses adjusted for age). The magnitude of post-exercise lymphocytopenia of these subsets was similarly moderated by CMV serostatus (serostatus by time interactions: for effector memory cells; $F_{(1, 11)} = 8.5$, $p < .05$, $\eta^2 = 0.43$; for CD45RA⁺ effector memory cells; $F_{(1, 11)} = 8.5$, $p < .05$, $\eta^2 = 0.43$, both analyses adjusted for age). CMV serostatus was not associated with differential mobilisation or egress of naïve or central memory cells (serostatus by time interactions $F_{(1, 11)} < 3.5$, $p > .2$, adjusted for age).

FIGURE 2.3.
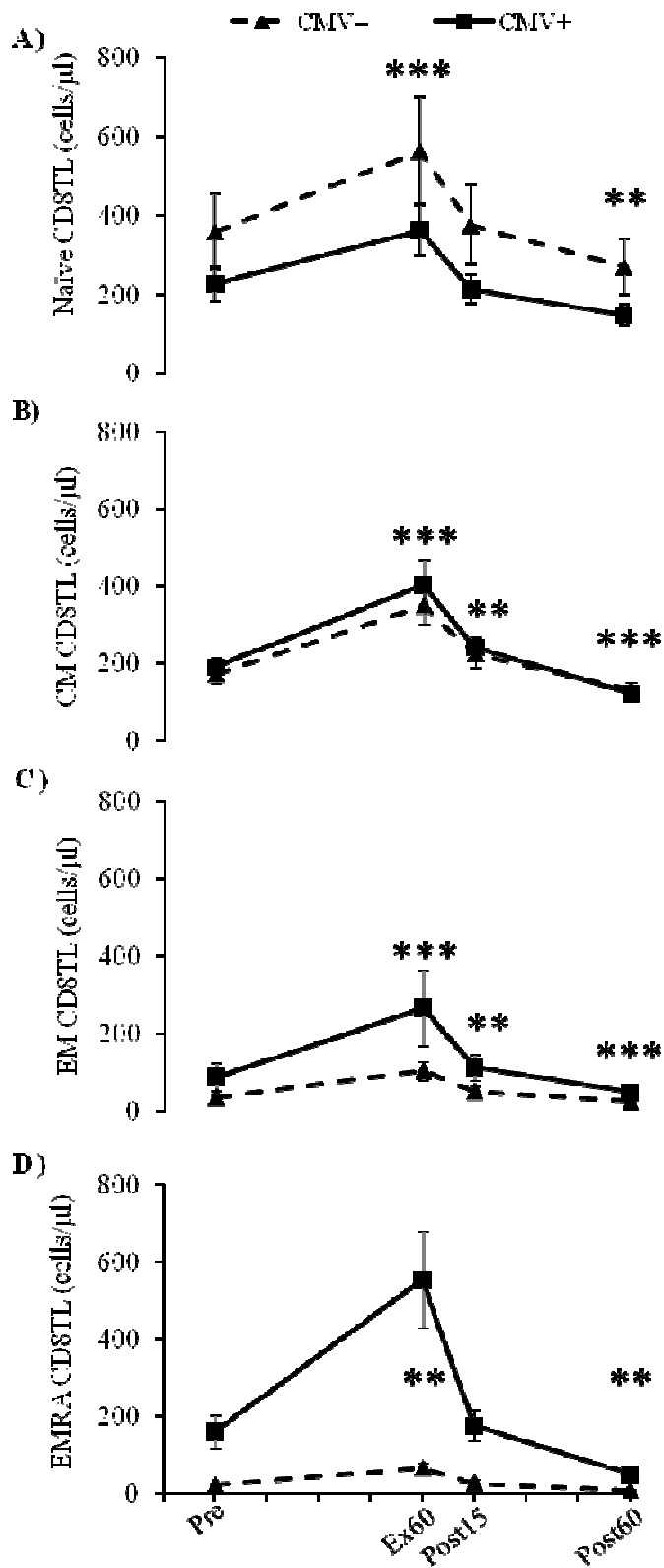
Changes in CD8TL sub-populations (cells/μl) identified by CD45RA and CD27 expression. A) Naïve CD8TLs (CD27⁺CD45RA⁺). B) Central memory CD8TLs (CM; CD27⁻CD45RA⁺). C) Effector memory CD8TLs (EM; CD27⁻CD45RA⁻). D) CD45RA⁺ effector memory CD8TLs (EMRA; CD27⁻CD45RA⁺).

*** $p < .001$; ** $p < .01$; comparison with baseline values. *See text for 'time by serostatus' interactions.* Data are Means ± SEM.

To test if the larger memory CD8TL changes in CMV$^+$ individuals were explained by higher basal numbers of these cells, the above analyses were repeated with statistical adjustment for initial values (i.e., entering baseline cell count as a covariate in ANOVAs). After this correction, the interaction effects for effector memory and CD45RA$^+$ effector memory cells during exercise disappeared ($F_{(1, 10)} < 4.5$, $p > .06$). Similarly, the augmented post-exercise decrease in CD45RA$^+$ effector memory cells was no longer statistically different between CMV$^+$ and CMV$^-$ individuals after adjusting for baseline cell count ($F_{(1, 10)} = 3.0$, $p = .11$, adjusted for age and initial values), although the larger post-exercise decrease in effector memory cells seen for CMV$^+$ individuals remained significant after adjustment ($F_{(1, 10)} = 6.2$, $p < .05$, $\eta^2 = 0.38$, adjusted for age and initial values).

Shown in Figure 2.4, a comparable pattern of results was found when memory subsets were identified on the basis on CD27 and CD28 expression (see Table 2.1), demonstrating a significantly larger mobilisation of late differentiated cells in CMV$^+$ individuals (serostatus by time interaction; $F_{(1, 11)} = 6.9$, $p < .05$, $\eta^2 = 0.39$, adjusted for age), which was still apparent after controlling for baseline cell count ($F_{(1, 10)}$ 5.5, $p < .05$, $\eta^2 = 0.355$), although the post-exercise egress did not reach statistical significance (serostatus by time interaction; $F_{(1, 11)} = 2.7$, $p = .13$, $\eta^2 = 0.20$, adjusted for age). CMV serostatus did not significantly moderate the responses of intermediate or early differentiated cells (serostatus by time interactions; $F_{(1, 11)} < 3.8$, $p > .08$; all analyses adjusted for age).

FIGURE 2.4.
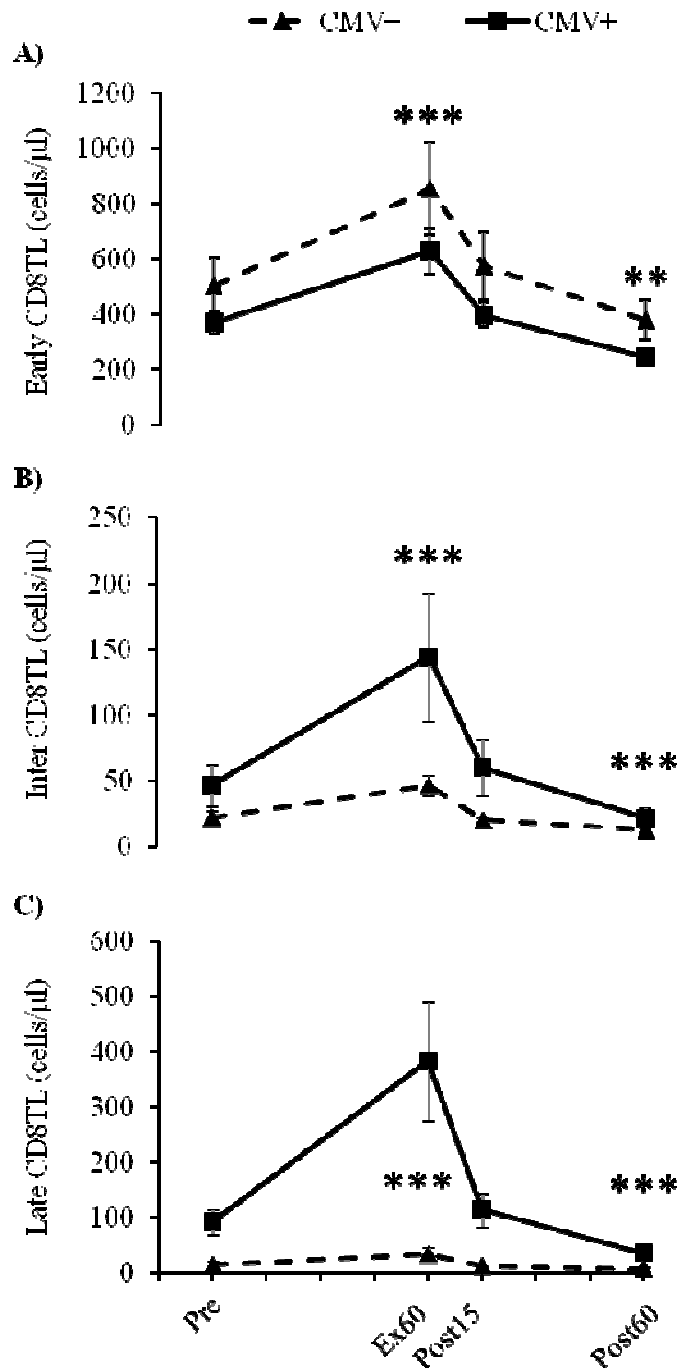
Changes in CD8TL sub-populations (cells/μl) identified by CD27 and CD28 expression. A) Early CD8TLs (CD27⁺CD28⁻). B) Intermediate CD8TLs (CD27⁺CD28⁻). C) Late CD8TLs (CD27⁻CD28⁻).

$*** p < .001$; $** p < .05$ comparison with baseline values. *See text for 'time by serostatus' interactions.* Data are Means ± SEM.

2.5. DISCUSSION

Recent studies have demonstrated a high specificity in the mobilisation of CD8TL subsets during exercise, whereby a subpopulation of memory cells, i.e., those with strong tissue-migrating capacity and effector potential, preferentially increase in the peripheral blood (Campbell et al., 2009; Simpson et al., 2008; Simpson et al., 2007a). In line with the assumption that this mobilisation is part of an immune surveillance response, we wondered if these memory cells might also show preferential egress from peripheral blood in the post-exercise period. Our results confirmed this expectation, showing a −60% decrease in late differentiated/effector-memory cells, compared to a −25% and −29% fall in total lymphocytes and naïve CD8TLs respectively. Thus, with exercise, cells that are relevant to immune surveillance, i.e., those with proven capacity to initiate immediate effector responses such as target killing, are preferentially redeployed into and out of peripheral blood. As their action is most likely to be required in tissue rather than peripheral blood, it seems probable that the egress, rather than peripheral release, is the most significant aspect of this evolved response.

While the mechanisms responsible for exercise-induced CD8TL mobilisation are well-researched, less is known about the post-exercise egress. Some researchers have contended that the fall in cell number is caused by exercise-induced apoptosis (Mars et al., 1998; Mooren et al., 2002; Mooren et al., 2004). However, both animal and human evidence provide only limited support for this idea (Dhabhar et al., 1995, 1996). For example, although following exercise lymphocyte numbers fall to less than 50% of baseline levels (Hansen et al., 1991; Shephard and Shek, 1999), only <10% of lymphocytes undergo post-exercise apoptosis (Mars et al., 1998;

Mooren et al., 2002; Mooren et al., 2004). Moreover, exercise-induced lymphocytopenia has been observed without evidence for apoptosis (Simpson et al., 2007b; Tanimura et al., 2008). More support exists for the idea that the post-exercise fall in cell numbers is due to lymphocyte homing into peripheral tissues. This migration is postulated to be part of an immune surveillance response (Dhabhar, 2000, 2002; Krüger et al., 2008; Krüger and Mooren, 2007). Cortisol has been proposed as a neuro-endocrine mediator of this lymphocytopenia (Fauci, 1976; McCarthy and Dale, 1988; Onsrud and Thorsby, 1981). However, recent evidence indicates that this hormone is unlikely to explain enhanced egress of late differentiated cells as seen in our study: Dimitrov and co-workers (2009) showed that although cortisol infusion caused lymphocytopenia, this response was restricted to naïve and lymphoid-homing (CD62L$^+$) CD8TLs, and that differentiated effector-memory cells were unaffected. It is possible that the post-exercise egress may reflect delayed effects of adrenergic stimulation (Ali et al., 2007), which would be consistent with the observation that this delayed decrease so closely parallels the pattern of catecholamine dependent mobilisation.

The CD8TL memory subsets that showed the strongest responses to exercise are known to be selectively increased by latent CMV infection (Appay et al., 2002; van Lier et al., 2003). Significantly, these differentiated memory CD8TL subsets are also known to express higher levels of the β2-adrenergic receptor, possibly explaining their higher sensitivity to exercise (Dimitrov et al., 2010; Holmes et al., 2005; Karaszewski et al., 1991). Based on these data, we anticipated that CMV serostatus would moderate CD8TL responses to exercise. The results indeed showed that both the mobilisation and post-exercise egress was nearly twice as large in CMV$^+$ individuals, as compared to CMV$^-$ individuals. Further analyses confirmed that this

augmentation was largely explained by the higher numbers of differentiated memory cells in CMV$^+$ individuals. For example, the number of CD27$^-$CD45RA$^+$CD8TLs was ~6-fold higher in CMV$^+$ participants, which is comparable with what has been reported in the literature (Chidrawar et al., 2009; Khan et al., 2002; Weinberger et al., 2007). Post-hoc analyses excluded that exercise intensity explained the response differences, as none of the performance indices were related to serostatus (i.e., $\dot{V}O_2$, energy expenditure, heart rate, and RPE). Further analyses also showed that CMV serostatus was unrelated to age, BMI, leisure time physical activity, fitness, and maximum heart rate. To our knowledge, this is the first study to demonstrate that infection history is a determinant of immunological responses to exercise.

CD4TL responses to exercise were not moderated by CMV serostatus. This was not unexpected as CD4TLs and their memory subsets are, compared to CD8TLs, generally less responsive to exercise (Campbell et al., 2009), and the effects of CMV infection on the composition of the CD4TL memory pool are modest (Fletcher et al., 2005; Khan et al., 2002; Pourgheysari et al., 2007; Wikby et al., 2002). Although no further lymphocyte subsets were examined, the null-finding for CD4TLs as well as for total lymphocytes suggests that the observed effects of serostatus are selective for CD8TLs.

What are possible implications of the current findings? Considering that the late differentiated CD8TLs which egress from peripheral blood have a strong tissue homing and inflammatory capacity, it could be argued that amplification of this response may promote protective immune surveillance, and thereby improve clearance of infection and vaccine responses (Edwards et al., 2007; Edwards et al., 2006). Conversely, it might be argued that enhanced mobilisation and tissue

migration of CD8TLs may have harmful effects by aggravating inflammatory processes (Dhabhar, 2000, 2002). This may, for example, be relevant in atherosclerosis whereby IFN-γ producing CD8TLs accumulate in atherosclerotic plaques (de Boer et al., 2003; de Boer et al., 1999; Gewaltig et al., 2008). Interestingly, CMV also facilitates transmigration of memory CD8TLs via infection of endothelial cells (Burns et al., 1999; Craigen et al., 1997), and the exaggerated CD8TL mobilisation in CMV$^+$ individuals might thus further promote cardiovascular disease risk (Strandberg et al., 2003). Moreover, as most cardiovascular diseases are associated with enhanced sympathetic activity (Fisher et al., 2009), it is conceivable that the cardio-protective effects of β-antagonists may be stronger in CMV$^+$ individuals via blunting of inflammatory CD8TL recruitment (Kuhlwein et al., 2001; Mills et al., 1999).

Another potentially significant implication is that the current observations identify CMV serostatus as a possible mediator, or confounder, in exercise and behavioural immunology. For example, increased infection rates among athletes following endurance exercise events (the so-called 'open window hypothesis') have in part, been attributed to decreased numbers and functional capacity of peripheral blood cells post-exercise (Nieman, 1995; Pedersen and Hoffman-Goetz, 2000). It now seems likely that individual differences in the magnitude of lymphocytopenia, and possibly alterations in *in vitro* cell function post-exercise, are at least in part related to infection history. The same would apply to research linking exercise- (and possibly stress-) induced lymphocyte redeployment to health status (e.g., hypertension) or demographics (e.g., age) (Ceddia et al., 1999; Mazzeo et al., 1998; Mills et al., 2003; Shinkai et al., 1998). This caveat seems to be strengthened by the association of positive CMV serostatus with ethnicity, increased age, lower socio-economic status (Dowd and Aiello, 2009; Lubeck et al., 2010; Staras

et al., 2006), as well as health outcomes such as atherosclerotic risk and susceptibility to infection (Dumortier et al., 2008; Hadrup et al., 2006; Olsson et al., 2000; Streblow et al., 2008; Strindhall et al., 2007). Taking a broader perspective, the fact that CMV infection expands a CD8TL population which expresses higher levels of the β2-adrenergic receptor (Dimitrov et al., 2009; Holmes et al., 2005; Karaszewski et al., 1991) has general relevance to neuro-endocrine immunology. For example, differentiated CD8TLs exhibit unique immunological features, such as increased IFN-γ production and cytotoxicity (Hamann et al., 1997; Sallusto et al., 1999). These functional characteristics may likewise become differentially affected by exercise, and possibly stress, in CMV$^+$ individuals.

Regarding the limitations of the present study, an important target for future research would be to determine if the enhanced CD8TL response to exercise is attributable to mobilisation of CMV-specific CD8TLs only, or that effector-memory CD8TLs with other antigen-specificities are similarly mobilised. Antigen-specific T lymphocytes can be identified using tetramers, which are synthetic HLA-molecule analogues tagged with an antigen-peptide, that are capable of binding virus-specific T cell receptors. To be able to answer this question conclusively, one would need to use tetramers for all immunogenic CMV epitopes and for all HLA types; we are not aware of a single laboratory that has access to such tetramer diversity. A second limitation of this study is that it is uncertain to what extent our observations would generalise to other latent micro-organisms. A number of viruses are known to affect composition of the lymphocyte pool, but these effects are different from those of CMV. For example, it seems unlikely EBV infection would have the same effect as CMV, because EBV predominantly increases the number of (exercise unresponsive) central-memory cells (Appay et al., 2008; van Lier et al., 2003). Similar

to CMV, HIV is known to increase the number of effector-memory cells (Appay et al., 2008; van Lier et al., 2003), which suggests that this infection may also alter CD8TL responses to exercise. A possible third limitation is that participants were all physically fit. Unfit individuals have been shown to exhibit larger CD8TL responses to exercise compared those who are fit (Hong et al., 2004; Hong et al., 2005; Mills et al., 2006), and thus it is conceivable that the effects of CMV serostatus may be moderated by fitness.

In conclusion, the results of the present study have shown that CD8TL mobilisation and egress in response to intensive exercise is driven by differentiated effector-memory cells. Additionally, we have shown, for the first time, that infection history can substantially modulate this bimodal CD8TL response to exercise by altering the composition of the memory pool.

2.6. REFERENCE LIST FOR CHAPTER TWO

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CHAPTER THREE

3.0. ASSESSMENT OF OXIDATIVE STRESS IN LYMPHOCYTES FOLLOWING EXERCISE

3.1. ABSTRACT

This study investigated whether changes in the cellular composition of blood during exercise, could partly account for observations of exercise-induced changes in lymphocyte oxidative stress markers. Markers of oxidative stress and antioxidant capacity were assessed before, after 60 minutes of treadmill running at 80% $\dot{V}O_2$ max, and 15 and 60 minutes later. Samples were collected from sixteen men (mean ± SD; age 32.6 ± 13.2 years; body mass index 23.8 ± 2.5 kg·m^{-2}, maximal-oxygen uptake 59.7 ± 5.2 ml·kg^{-1}·min^{-1}). Peripheral blood lymphocytes were assayed for protein carbonylation and plasma was assessed for lipid peroxidation and antioxidant capacity. In a separate study, intracellular thiol concentration was determined in lymphocyte sub-sets by flow cytometry, of which T cell memory populations were further identified on the basis of CD27, CD28, and CD45RA expression. The results show that total lymphocyte protein carbonylation was transiently increased with exercise and returned to baseline within 15 minutes ($p < .001$). This change was accompanied by increased plasma lipid peroxidation ($p < .05$) and total antioxidant capacity ($p < .001$). Correlation analyses showed that lymphocyte protein carbonylation was not related to changes in the cellular composition of peripheral blood during exercise. Natural killer cells (CD3$^-$CD56$^+$) and late-differentiated/effector memory cells (CD4$^+$/CD8$^+$CD27$^-$CD28$^-$/CD45RA$^+$) which mobilised most with exercise, showed high intracellular thiol content ($p < .001$). High thiol content suggests a lower 'oxidative load' carried by these lymphocytes. Thus, vigorous exercise resulted in a transient increase in lymphocyte oxidative stress, which was un-related to the alterations in the cellular composition of peripheral blood.

3.2. INTRODUCTION

Pro-oxidants or radical species are produced during nearly all strenuous forms of exercise (Finaud et al., 2006; Fisher-Wellman and Bloomer, 2009; Vollaard et al., 2005) and include free radicals (e.g., superoxide) and non-radical species (e.g., hydrogen peroxide). Radical species initiate cellular signalling which is crucial for cell function and might be key to the health benefits of exercise (Ji, 2008; Ji et al., 2006; Radak et al., 2005, 2008). However, if reactive species go unchecked they will rapidly oxidise proteins, lipids and nucleic acids (Sachdev and Davies, 2008). To prevent such damaging effects, pro-oxidants are balanced by intracellular molecules with antioxidant properties such as glutathione (GSH), which scavenge and buffer reactive species (Ji et al., 2006; Radak et al., 2005, 2008). However, in certain circumstances (e.g., vigorous exercise or some disease states), reactive species may overwhelm the antioxidant capacity of cells or tissues, resulting in 'oxidative stress' (Halliwell and Gutteridge, 1999). Oxidative stress can lead to impairment of cellular functions which may underlie ageing and pathologies such as cardiovascular disease (Beckman and Ames, 1998; Wu et al., 2004).

Oxidative stress can be assessed by measuring modifications, or adducts to plasma proteins and lipids (e.g., protein carbonylation, lipid peroxidation) or by quantifying the antioxidant capacity of plasma and whole blood (Bartosz, 2010; Finaud et al., 2006; Sachdev and Davies, 2008). When assessed in plasma, biomarkers such as protein carbonylation provide non-specific, whole body measures of oxidative stress (Berlett and Stadtman, 1997; Dalle-Donne et al., 2003; Finaud et al., 2006; Veskoukis et al., 2009). Recently protein oxidation has also been assessed in peripheral blood lymphocytes, and increased lymphocyte protein carbonylation has been

observed immediately after intensive swimming and endurance cycling exercise (Cases et al., 2006; Ferrer et al., 2009; Sureda et al., 2005; Tauler et al., 2006). There is however a largely unappreciated, but potentially important, caveat linked with these findings. Lymphocytes form a very heterogeneous group of cells, and exercise drastically alters the lymphocyte composition of peripheral blood (Anane et al., 2009; Campbell et al., 2009; Turner et al., 2010). It is not known if different lymphocyte subsets differ in the expression of oxidative stress markers, but if they do then it is possible that any exercise-induced changes in lymphocyte protein carbonylation, could reflect a change in blood composition, rather than effects of oxidative stress.

Lymphocytes can be sub-divided into cytotoxic $CD8^+$ and helper $CD4^+$ T cells, which together, account for 60%-80% of total lymphocytes. Natural killer cells and B cells constitute the remainder. Sub-populations of $CD8^+$ and $CD4^+$ T cells include antigen inexperienced 'naïve' cells (identified as $CD27^+CD45RA^+$), and three populations of antigen experienced cells; central memory (CM; $CD27^+CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$) and 'revertant' effector memory cells which re-express CD45RA (EMRA; $CD27^-CD45RA^+$) (Appay et al., 2008; Turner et al., 2010). Other researchers have used a different nomenclature to identify memory T cells, and distinguish between distinct stages of differentiation on the basis of CD27 and CD28 expression; early stage ($CD27^+CD28^+$), intermediate stage ($CD27^+CD28^-$) and late stage differentiated cells ($CD27^-CD28^-$) (Appay et al., 2002; van Lier et al., 2003). During exercise specifically the cytotoxic late-differentiated or effector memory populations, ($CD8^+CD27^-CD28^-/CD45RA^+$), as well as natural killer cells, are recruited into peripheral blood (Anane et al., 2009; Campbell et al., 2009; Turner et al., 2010). These subpopulations show evidence of multiple cell divisions, such as shorter telomeres, and display elevated DNA damage

(Effros et al., 2005; Monteiro et al., 1996; Scarpaci et al., 2003). These cells might therefore have an increased 'history' of oxidative stress, and it is possible that these exercise-sensitive cells also differ in protein carbonylation, or other markers of oxidative stress, such as intracellular GSH concentration.

The aim of this study was to examine whether the aforementioned changes in the cellular composition of blood in response to exercise may affect measurements, and therefore the interpretation, of exercise-induced oxidative stress in total lymphocytes.

3.3. METHODS

3.3.1. Participants

Sixteen healthy non-smoking men took part in this study, having abstained from taking vitamin supplements for at least six weeks (see Table 3.1). Participants provided informed consent, and the study was approved by the School of Sport and Exercise Sciences research ethics committee of the University of Birmingham.

3.3.2. Procedures

Participants undertook two graded exercise tests on a treadmill to accurately determine running speed for the exercise trial as previously described (Turner et al., 2010). Briefly, in the first test, participants ran at incremental speeds until exhaustion to measure $\dot{V}O_2$ max (Whaley et al., 2006). The second test consisted of sub-maximal running to assess the relationship between oxygen consumption ($\dot{V}O_2$) and treadmill speed. In addition, participants were asked to report leisure time physical activity using the international physical activity questionnaire (IPAQ) (Craig et al., 2003).

3.3.3. Exercise trial

One week after preliminary tests, participants returned to the laboratory between 06:00-07:00 following an overnight fast. Participants were instructed to refrain from exercising and drinking alcohol or caffeine during the day prior to the trial. Following a 15 minute rest, a baseline blood sample (Pre) was collected from forearm vein by an indwelling catheter, which was kept patent by flushing with saline. Participants then undertook an exercise trial, which consisted of treadmill

TABLE 3.1.

Characteristics of participants

	$n = 16$
Age (years)	32.6 ± 13.2
BMI (kg·m^{-2})	23.8 ± 2.5
Measured $\dot{V}O_2$ max (ml·kg^{-1}·min^{-1})	59.7 ± 5.2
Maximum heart rate at $\dot{V}O_2$ max (bpm)	187 ± 9
LTPA[a] (Median MET·min·week^{-1})[b]	3497 (720 - 7290)

Data are means ± SD.

[a] Leisure Time Physical Activity, as assessed by the international physical activity questionnaire (IPAQ).

[b] Data are medians (min-max).

running at a speed to elicit 80% of $\dot{V}O_2$ max for 60 minutes. Blood was collected at the end of exercise (Ex60), and again 15 minutes (Post15) and 60 minutes (Post60) post-exercise.

3.3.4. Blood sample processing

Blood was collected into ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Oxford, UK) and placed at 4°C until processing. Plasma was obtained by centrifugation at $1350 \times g$ for 15 min at 4°C, and stored at −80°C. Peripheral blood lymphocytes were isolated using density gradient centrifugation. Briefly, whole blood was diluted 1:1 with Phosphate Buffered Saline (PBS; Invitrogen, Paisley, UK) and carefully layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) before centrifuging at $300 \times g$ for 30 min. The lymphocyte layer was aspirated, and cells washed in PBS at $1000 \times g$ for 10 min. The pellet was re-suspended in either sodium carbonate buffer (50 mM, pH 7.4) and stored at −80°C for assessment of protein carbonylation, or re-suspended in PBS and processed for flow cytometric measurements.

3.3.5. General analytical procedures

Total lymphocyte protein concentration was determined using the bicinchoninic acid method as described by Smith *et al*. (1985). Biochemical parameters were expressed relative to protein concentration, or were corrected for changes in plasma volume according to Dill and Costill (1974) as appropriate. Haemoglobin and haematocrit was assessed using a Coulter ACT[diff] haematology analyser (Beckman-Coulter, High Wycombe, UK). Chemicals were obtained from Sigma-Aldrich, UK, unless otherwise stated.

3.3.6. Protein carbonylation

Total lymphocyte protein carbonylation was measured by enzyme-linked-immunosorbent-assay (ELISA) described by Buss *et al* (1997). Lymphocytes were lysed with RIPA buffer containing a protease inhibitor cocktail, and the supernatant used for analysis. Samples and standards (0.5 mg/ml in sodium carbonate buffer; 50 mM, pH 7.4, 50µl) were allowed to bind to 96-well multi-sorb plates for 60 min at 37°C in triplicate (Nunc, Fisher Thermo Scientific, UK). 2, 4-dintrophenylhydrazine (DNP; 1mM, 50µl) in 2 M hydrochloric acid was added, and plates incubated at room temperature for 60 min. Plates were blocked overnight at 4°C with 0.1% tris-buffered saline-TWEEN-20. Mouse anti-DNP antibody (1:1000, 50µl) was incubated with samples for 120 min at 37°C, followed by a peroxidise-labelled rat anti-mouse antibody (1:5000, 50µl), incubated for 60 min at 37°C. A citrate phosphate-based substrate (0.15 M, pH 5) was added, and plates left to develop in the dark for 30 min at 37°C. The reaction was stopped with 2 M sulphuric acid and plates read at 490-nm. Values were expressed as nmol/mg of protein.

3.3.7. Lipid peroxidation

Lipid peroxides were assayed in plasma using a modification of the method by el-Saadani *et al* (1989). Samples and positive and negative controls (1:1000 hydrogen peroxide and distilled water respectively) were added to 96-well plates in triplicate. Working reagent (0.2 M Potassium Phosphate, 0.12 M Potassium Iodide, 0.15 mM Sodium Azide, 2 g/l Triton-X, 0.1 g/l Alkylbenzyldimethylammonium, 10µM Ammonium Molybdate; 100 µl) was added, and plates incubated at room temperature for 30 min. Plates were read at 340-nm. Lipid peroxide concentration was calculated using the Beer-Lambert-Law with an extinction coefficient of 24600. Values were expressed as nmol/ml plasma.

3.3.8. Total Antioxidant Capacity

Total Antioxidant Capacity was assessed in plasma using the Ferric Reducing Ability of Plasma (FRAP) assay (Benzie and Strain, 1996). Briefly, standards (0-1000 µM ascorbic acid) and samples (10 µl per well) were added to 96-well flat-bottomed cell culture plates in triplicate. Working reagent (20 mM ferric chloride, 160 mM 2, 4, 6- tripyridyltriazine, 300 mM acetate buffer; 300 µl) was added to each well, and plates read at room temperature after eight minutes. Values were determined by linear regression from a 7-point standard curve (McAnulty et al., 2005), and expressed as µM of antioxidant power relative to ascorbic acid.

3.3.9. Flow cytometry

To examine the mobilisation of T lymphocyte sub-populations with exercise, fresh lysed whole blood was prepared as described previously (Turner et al., 2010). Fixed cell preparations were read within 36 h of exercise on a multi-parameter flow cytometer (BD FACS CANTO II, BD Biosciences). Lymphocytes were enumerated using a Coulter ACT[diff] haematology analyser (Beckman-Coulter, High Wycombe, UK).

In a separate investigation, intracellular glutathione was measured in lymphocyte sub-populations from eight healthy men (mean age ± SD; 31.0 years ± 10.4) at rest using a modification of the procedure described by Cossarizza *et al* (2009). Monobromobimane (MBB) is a non-fluorescent probe, which binds to intracellular thiols emitting light at 490-nm when excited with a 405-nm violet laser. GSH is the most abundant intracellular thiol therefore median fluorescence intensity

(MFI) of MBB can be considered a measure of intracellular GSH concentration (Cossarizza et al., 2009; Patsoukis and Georgiou, 2005).

Approximately 2×10^6 peripheral blood lymphocytes were incubated with monoclonal antibody panels for 20 minutes at room temperature. The first antibody panel included; anti CD19 FITC, anti CD56 PE (Pharmingen, San Diego, USA) anti CD3 PerCp (BD Biosciences, San Jose, USA). The second antibody panel included; anti CD45RA FITC, CD27 PE (Pharmingen, San Diego, USA), CD3 PerCP, CD28 PE-cy7, CD4 APC, CD8 APC-cy7 (BD Biosciences, San Jose, USA). Samples were washed in PBS by centrifugation at $300 \times g$. Cells were re-suspended in 1 ml of 37°C PBS, and a sample from each individual was incubated for 20 minutes at 37°C with 100 μM *N*-ethylmaleimide. Intracellular thiol content is depleted by *N*-ethylmaleimide therefore these preparations served as negative controls. Negative controls were washed and re-suspended in 1 ml of 37°C PBS. Samples and negative controls were incubated for 10 minutes at 37°C with 50 μM MBB (Invitrogen Molecular Probes, Paisley, UK). Cells were washed and re-suspended in PBS, and read within 30 min collecting 50,000 lymphocytes. Lymphocytes were gated on the forward *versus* side-scatter. T cells were identified by expression of CD3 in combination with CD4 or CD8. T cell sub-populations were identified on the basis of CD27 and CD45RA expression (Hamann et al., 1997) or CD27 and CD28 expression (Appay et al., 2002). B cells (CD3$^-$CD19$^+$) and Natural killer cells (CD3$^-$CD56$^+$) were also examined.

3.3.10. Statistical analysis

Data were inspected for normal distribution using the Kolmogrov Smirnov test. Non-normally distributed data were transformed logarithmically. Responses to exercise were examined with

repeated-measures Analyses of Variance (ANOVAs). Differences compared to baseline were examined using post-hoc paired samples *t*-tests. Statistical significance was accepted at the *p* < .05 level. Intracellular thiol content between lymphocyte sub-populations was examined using paired samples *t*-tests. Effect sizes are presented as η^2. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated. Data were analysed using SPSS statistical package version 18.0 for Windows (SPSS Inc, USA). Data presented on lymphocyte kinetics and correlations between lymphocyte number and protein carbonylation is from eleven subjects who also took part in the first empirical investigation in this thesis (see the Appendix for subject overlap between exercise studies).

3.4. RESULTS

3.4.1. Lymphocytes

A detailed analysis of lymphocyte responses to this bout of exercise has been undertaken by the authors and described previously (Turner et al., 2010; see Chapter two). Briefly, exercise mobilised CD8$^+$ T cells with a late-differentiated/effector memory phenotype (Turner et al., 2010). For illustrative purposes relevant to this investigation, alterations in the proportions of CD8$^+$ T lymphocytes at each extreme of the differentiation continuum are presented (i.e., naïve *versus* EMRA; see Figure 3.1A). Compared to rest, the most differentiated or 'older' EMRA cells constituted a larger proportion of total lymphocytes, whereas the least differentiated or 'younger' naïve cells were much smaller in proportion during exercise ($n = 11$; $F_{(3, 36)} > 9.6$, $p < .001$, $\eta^2 > .443$; see Figure 3.1A). CM and EM populations, and cells which did not express CD28 showed similar increases to EMRA with exercise, and an identical pattern was observed for CD4$^+$ T cell subsets (data not shown).

3.4.2. Protein carbonylation

Figure 3.1B shows total lymphocyte protein carbonylation in response to exercise. Protein carbonylation increased with exercise, and returned to baseline within 15 minutes ($F_{(3, 39)} = 16.7$, $p < .001$, $\eta^2 = .562$; see Figure 3.1B). Correlation analyses showed that lymphocyte protein carbonylation was un-related to the cell sub-populations that constitute total lymphocytes: No correlations ($r < .1$, $p > .5$) were observed between the numbers or proportions of any lymphocyte subset and the level of protein carbonylation measured in total lymphocytes (data not shown).

FIGURE 3.1.
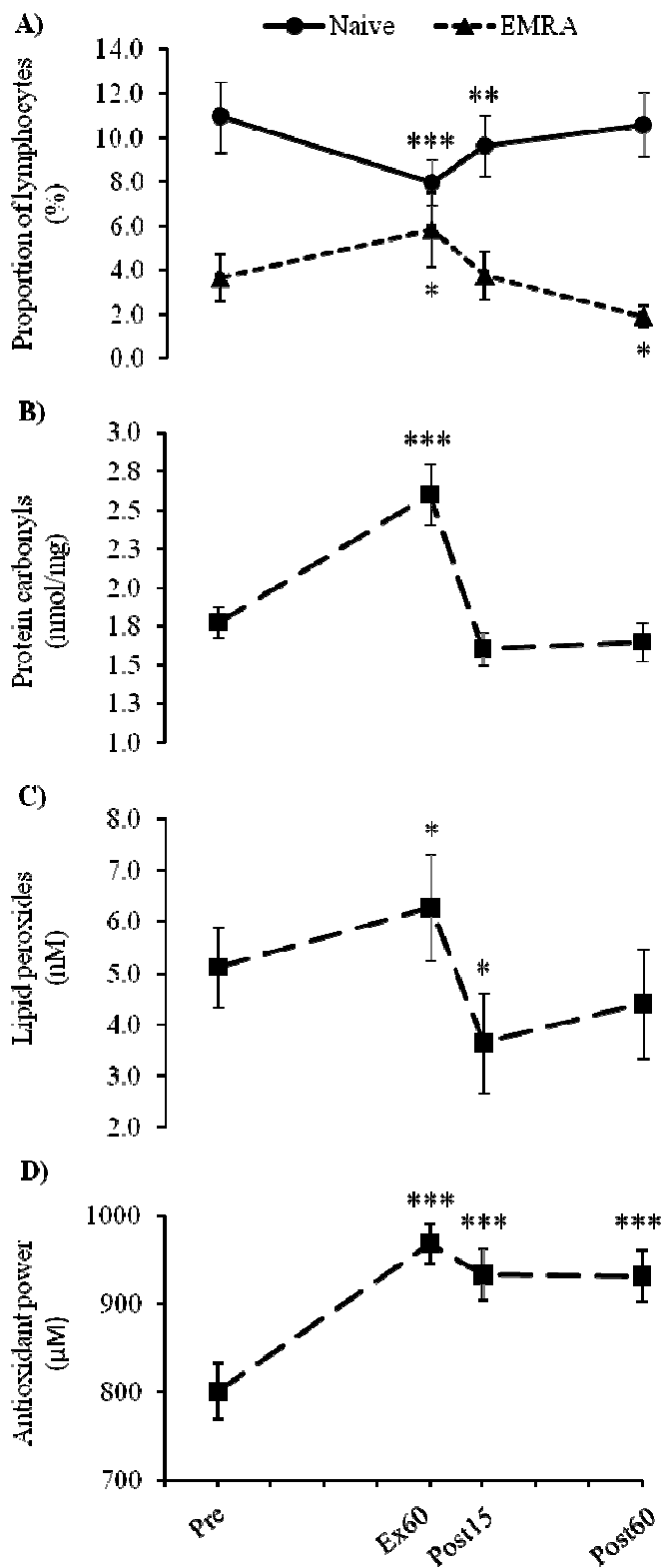
Immunological and oxidative responses to exercise. Data are means ± SEM. A) Effector memory CD8+ T cells which have re-expressed CD45RA (EMRA; CD27-CD45RA+) and naive CD8+ T cells (CD27+CD45RA+). Cells expressed as a proportion (%) of total lymphocytes. B) Lymphocyte protein carbonylation. C) Plasma lipid peroxidation. D) Plasma antioxidant capacity.

$*p < .05$, $**p < .01$, $***p < .001$ (paired samples t-tests to baseline).

3.4.3. Lipid peroxidation

Figure 3.1C shows plasma lipid peroxide concentrations in response to exercise. Lipid peroxidation increased with exercise, and following completion of the exercise bout, lipid peroxides fell below baseline levels within 15 minutes. Sixty minutes after exercise, lipid peroxide concentration had returned to pre-exercise levels ($F_{(3, 39)} = 3.6$, $p < .05$, $\eta^2 = .216$; see Figure 3.1C).

3.4.4. Total antioxidant capacity

Figure 3.1D shows the plasma antioxidant response to exercise. Total antioxidant capacity increased with exercise, and remained above baseline for 60 minutes ($F_{(3, 39)} = 18.2$, $p < .001$, $\eta^2 = .562$; see Figure 3.1D).

3.4.5. Lymphocyte intracellular glutathione concentration

In a separate investigation, eight healthy individuals provided a resting blood sample, and lymphocyte subsets were examined for intracellular thiol content. Natural killer cells exhibited higher thiol content than B cells, $CD4^+$ and $CD8^+$ T cells (see Figure 3.2A). Within the T cell populations, cytotoxic $CD8^+$ cells showed higher thiol concentrations than helper $CD4^+$ cells (see Figure 3.2A). The late-differentiated/effector memory $CD8^+$ sub-populations showed increased intracellular thiol concentrations compared to naïve/early stage differentiated cells (see Figures 3.2B and 3.2C). An identical pattern was observed between $CD4^+$ sub-populations, and these cells had lower intracellular thiol concentrations than the equivalent $CD8^+$ sub-populations (data not shown).

FIGURE 3.2.
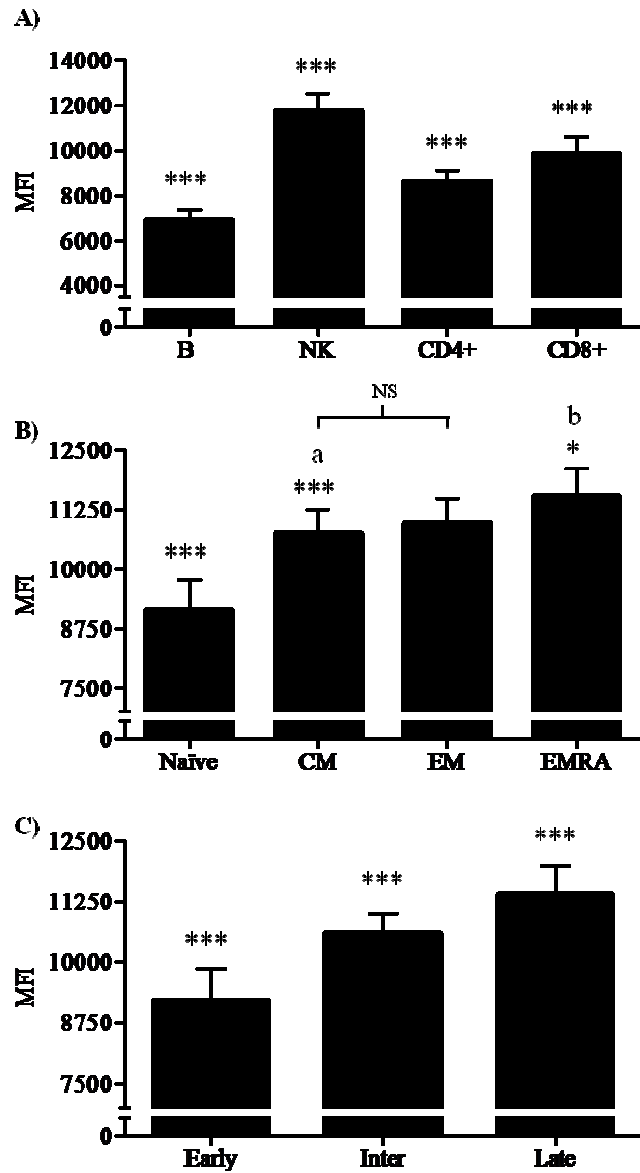
Lymphocyte sub-population intracellular thiol concentration. Data are means ± SEM. MFI = median fluorescence intensity. A) Major lymphocyte sub-sets. B) $CD8^+$ T cells identified on the basis of CD27 and CD45RA expression. Naïve ($CD27^+CD45RA^+$), central memory (CM; $CD27^+CD45RA^-$), effector memory (EM; $CD27^-CD45RA^-$), and revertant effector memory cells (EMRA; $CD27^-CD45RA^+$). C) CD8+ T cells identified on the basis of CD27 and CD28 expression. Early ($CD27^+CD28^+$), Inter (mediate) ($CD27^+CD28^-$), and late stage differentiated cells ($CD27^-CD28^-$). *** $p < .001$ compared to all subsets in figure sub-section. [a] *** $p < .001$ CM compared to EMRA. [b] * $p < .05$ EM compared to EMRA (paired samples t-tests to relevant sub-populations).

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3.5. DISCUSSION

In the present study, participants exercised at an intensity previously shown to cause oxidative stress (Lamprecht et al., 2009) and a transient increase in lymphocyte protein carbonylation was observed. This was accompanied by increases in plasma lipid peroxidation and elevated plasma antioxidant capacity. We hypothesised that 'aged' cells, which increase in peripheral blood with exercise, may carry a greater oxidative 'load' (i.e., increased markers of oxidative stress, when compared to naïve/early-differentiated cells). Thus, the present study investigated whether the observed lymphocyte protein carbonylation was likely a result of exercise-induced oxidative stress, or if this was partly attributable to changes in the cellular composition of peripheral blood.

Our measurements of protein carbonylation were un-related to the cell sub-populations that constitute total lymphocytes. No correlations were observed between the numbers or proportions of any lymphocyte subset and the level of protein carbonylation in total lymphocytes. For example, a higher number of 'aged' late-differentiated/effector memory cells within total lymphocytes, had no bearing on the degree of protein carbonylation in the total lymphocyte pool. In support, our separate investigations showed that the cells responsible for lymphocytosis (e.g., late-differentiated/effector memory cells and natural killer cells) had a higher intracellular thiol content compared to early-differentiated/naïve cells and B cells (the latter populations show minimal mobilisation with exercise). Cells with high thiol levels are usually associated with low levels of protein carbonylation and lipid peroxidation (Hernanz et al., 2000; Pandey et al., 2009). Although we did not measure protein carbonylation of individual lymphocyte subsets, on the basis of these results and previous literature, it seems unlikely that cell populations entering

peripheral blood due to exercise would carry increased protein carbonylation. We suggest, therefore, that the observed increase in oxidative stress to lymphocytes is likely to be a direct effect of exercise.

The results presented here show that cells selectively mobilised with exercise, carry high intracellular thiol content. The most abundant intracellular thiol is reduced GSH (Meister and Anderson, 1983; Townsend et al., 2003). GSH is a tripeptide synthesised from the amino acids cysteine, glycine and glutamic acid by the enzymes γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (Meister and Anderson, 1983; Townsend et al., 2003). The actions of γ-GCS and GSH synthetase are regulated through cellular signalling pathways and can be up-regulated to increase synthesis of GSH (Meister and Anderson, 1983; Townsend et al., 2003). Thus, it is possible that cells which have undergone previous oxidative insults might up-regulate GSH synthesis (Meister and Anderson, 1983), providing further protection against oxidative stress. In this investigation, the cells with the highest intracellular thiol content are highly proliferative and have been through repeated rounds of cell division (Nikolich-Zugich, 2008). These cells might therefore be described as 'aged'. Considering cellular ageing and exposure to pro-oxidants is sometimes associated with decreased antioxidant capacity, our finding that 'aged' cells have increased protection against oxidative stress might seem contradictory. However, these results are in line with the large body of evidence which show a protective and adaptive response in the face of oxidative challenge (Ji, 2001; Ji et al., 2006; McArdle et al., 2001; Radak et al., 2005, 2008).

Interestingly, and in addition, the role of intracellular thiols in lymphocytes is not limited to providing protection against pro-oxidants. Late-differentiated/effector memory cells and natural

killer cells are highly functional; they are able to divide rapidly upon exposure to antigen, home to inflamed tissue, and employ rapid cytotoxic effector functions without further need for differentiation (Appay et al., 2002; Appay et al., 2008; van Lier et al., 2003). The finding that these cells exhibit increased thiol levels is not surprising since intracellular thiol groups are essential for lymphocyte activation, proliferation and cytotoxicity (Droge et al., 1994; Liang et al., 1991; Smyth, 1991; Suthanthiran et al., 1990; Yamauchi and Bloom, 1993). Intracellular thiol content might therefore be up-regulated due to the memory/differentiation status of the lymphocyte, or as a result of oxidative stress, or as a result of both of these factors.

The present study showed that plasma antioxidant capacity was increased above pre-exercise levels for at least one hour following the exercise protocol. This 'antioxidant response' with exercise is well documented (Ji, 2008; Ji et al., 2006; Radak et al., 2008) and the present findings are in accordance with observations following similar exercise protocols (Aguiló et al., 2005; Michaildis et al., 2007). The most likely mechanism behind antioxidant responses to exercise is ascorbic acid efflux from the adrenal glands (Gleeson et al., 1987), however, it is also possible cells in contact with plasma (e.g., lymphocytes, erythrocytes, endothelial cells) directly influence the concentration of antioxidant molecules (Bartosz, 2010). This hypothesis is appealing considering the findings presented here, that cells which occupy peripheral blood with exercise show increased intracellular thiol content. In addition, the extracellular transport of lymphocyte derived GSH is proportional to intracellular thiol level (Meister and Anderson, 1983). Apoptosis also results in the release of GSH into the extracellular space (Ballatori et al., 2009) and up to 10% of lymphocytes undergo programmed cell death following exercise (Mars et al., 1998; Mooren et al., 2002; Mooren et al., 2004). It is therefore possible that the increased plasma

antioxidant capacity observed in the present study was partly mediated by an influx of cells into the blood which have an increased antioxidant capability. In addition to these factors influencing antioxidant capacity, rapid alterations may also be present at the gene transcription level. For example, redox-sensitive signalling leading to gene expression for enzymatic antioxidants is up-regulated within 15 minutes of exercise (Ji, 2008). It is argued that at least some of the benefits of regular physical activity are gained through redox-sensitive signalling (Ji et al., 2006; Radak et al., 2005, 2008).

It is widely accepted that engagement in regular exercise results in numerous health benefits (Kesaniemi et al., 2001). In the present study, intense exercise caused a transient increase in lipid peroxidation in plasma, which was followed by a fall in oxidised lipids to below baseline level 15 minutes after exercise cessation. Similar decreases in plasma lipid peroxides have been reported previously following exercise (Ginsburg et al., 1996; Kretzschmar et al., 1991), and it is argued that this might be one mechanism by which exercise exerts atheroprotective effects (Ginsburg et al., 1996; Kesaniemi et al., 2001).

A limitation of the present investigation is that we did not assess protein carbonylation in individual lymphocyte sub-populations. In principle, this might be possible using fluorescence activated cell sorting (FACS); assaying the isolated lymphocyte sub-populations for protein carbonylation. However in reality, isolation of cells using FACS is very likely to cause oxidative stress and there are very few late-differentiated/effector memory cells per µl of blood – often in the region of just 10 or 20 cells. This would make it almost impossible to obtain enough cells for assessment of protein oxidation. Techniques for the assessment of oxidative stress on a per cell

basis in specific lymphocyte populations are lacking. We believe our chosen strategy is the most robust measure available for our particular application.

In conclusion, the results of the present study show that an acute bout of intense exercise caused a transient oxidative stress that could be detected by the measurement of protein adducts in lymphocytes. We suggest that the observed lymphocyte protein carbonylation following exercise is not caused by lymphocyte sub-populations carrying differing amounts of protein oxidation. We have shown that lymphocyte sub-populations show differential intracellular thiol concentrations. We propose that intracellular thiol content is related to the functional capacity and differentiation/memory stage of the lymphocyte subset.

3.6. REFERENCE LIST FOR CHAPTER THREE

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CHAPTER FOUR

4.0. PROLONGED DEPLETION OF ANTIOXIDANT CAPACITY

FOLLOWING ULTRA-ENDURANCE EXERCISE

4.1. ABSTRACT

The purpose of this study was to examine the short and long-term (up to 1-month) impact of an ultra-endurance running event on redox-homeostasis. Markers of oxidative stress and antioxidant capacity in peripheral blood were assessed following a single-stage 233 km (143 mile) running event. Samples were collected from nine men (mean age ± SD; 46.1 ± 5.3 years; body mass index 24.9 ± 2.3 kg·m^{-2}, maximal-oxygen uptake 56.3 ± 3.3 ml·kg^{-1}·min^{-1}). Peripheral blood lymphocytes were assayed for non-specific DNA damage (frank strand breaks) and damage to DNA caused specifically by oxidative stress (formamidopyrimidine DNA glycosylase (FPG)-dependent damage). Protein carbonyls and lipid peroxides were assessed in plasma. Reduced glutathione (GSH) was measured in whole blood. Lymphocyte frank strand breaks were elevated above baseline at 24 hours post-race ($p < .001$). FPG-dependent oxidative DNA damage was increased immediately post-race ($p < .05$). Protein carbonyls remained elevated for 7 days following the race ($p < .04$) whereas lipid peroxides were increased for 24 hours ($p < .05$) and fell below baseline 28 days later ($p < .05$). GSH, a measure of antioxidant capacity also showed a biphasic response, increasing by one third post-race ($p < .01$) and falling to one-third of baseline levels 24 hours later ($p < .001$). GSH remained depleted to approximately two thirds of pre-race values 28 days post-race ($p < .01$). Ultra-endurance exercise causes oxidative stress, which persists for at least 1 calendar month depending on the specific biomarker examined. These results suggest that ultra-endurance events are associated with a prolonged period of reduced protection against oxidative stress.

4.2. INTRODUCTION

Research consistently shows that ultra-endurance exercise results in the formation of reactive oxygen species (ROS) (Knez et al., 2006; Sachdev and Davies, 2008). The extent of this production has the potential to overwhelm antioxidant defences, causing oxidative stress (Halliwell and Gutteridge, 1999; Sachdev and Davies, 2008). Oxidative stress is associated with damage to proteins, lipids and DNA (Sachdev and Davies, 2008), which has been causally related to ageing, and the development of cancer and cardiovascular disease (Schwedhelm et al., 2004; Wu et al., 2004; Yeh et al., 2010). Consequently, there has been substantial debate as to whether the health benefits of physical activity extend to more extreme forms of endurance exercise (Kesaniemi et al., 2001; Knez et al., 2006). For example, epidemiological studies show that individuals taking part in an exceptionally large amount of physical activity have impaired cardiovascular health (Knez et al., 2006).

Exercise-induced oxidative stress is likely the result of superoxide ($O_2^{\bullet-}$) production by the electron 'leak' in mitochondria, xanthine oxidase in endothelial cells, or from the inflammatory response of activated leukocytes (Halliwell and Gutteridge, 1999; Sachdev and Davies, 2008). $O_2^{\bullet-}$ dismutates to hydrogen peroxide (H_2O_2) and, in the presence of transition metals, forms highly reactive hydroxyl radicals ($OH^{\bullet}$) which rapidly oxidise bodily molecules (Halliwell and Gutteridge, 1999). Arguably, the most robust and accurate methods for assessing oxidative stress are the measurement of ROS-induced modifications to plasma proteins or damage to DNA in peripheral blood lymphocytes (Halliwell and Gutteridge, 1999). These blood markers correlate

well with similar measurements in tissues, and therefore provide an indication of whole-body oxidative stress (Veskoukis et al., 2009).

ROS production during exercise can also result in alterations to antioxidant capacity. Measurement of blood antioxidant defences therefore provides another informative method to assess oxidative stress (Halliwell and Gutteridge, 1999). Conceivably, depletion of these defences could result in decreased protection against ROS. In contrast, an up-regulation of antioxidant defences is often observed following exercise (Halliwell and Gutteridge, 1999; Sachdev and Davies, 2008), and it is plausible that this process is one of the mechanisms behind the beneficial adaptations to exercise (Halliwell and Gutteridge, 1999; Sachdev and Davies, 2008). One of the most abundant antioxidant compounds in blood, as with most other body tissues, is reduced glutathione (GSH) (Townsend et al., 2003). GSH acts as an electron donor for the reductive detoxification of hydrogen peroxide (H_2O_2) into water (H_2O) by the enzyme GSH peroxidase (GPx). Decreased GSH is associated with cardiovascular disease and other non-communicable diseases, potentially due to impaired protection leading to oxidative stress (Shimizu et al., 2004). GSH also has other important functions, including regulation of immune function and acting as a co-factor for specific enzymatic reactions (Filomeni et al., 2002; Kinscherf et al., 1994; Suthanthiran et al., 1990). Thus, this molecule provides a useful measure of oxidative stress.

Many studies have investigated oxidative stress in the context of ultra-endurance exercise, but few have investigated a very prolonged and continuous bout of ultra-endurance exercise consisting of just one sporting activity (e.g., Skenderi et al., 2008). Several studies have examined ironman triathlons, which involve swimming, cycling and running (e.g., Neubauer et

al., 2008) Investigations of this kind therefore assume an equal contribution of effects from each sporting modality. Moreover, it is currently unknown whether oxidative stress persists for more than 19 days following such extreme exercise (Neubauer et al., 2008). In general, oxidative stress can last for up to 72 hours post-exercise, depending on the biomarker investigated (Machefer et al., 2004; Neubauer et al., 2008; Skenderi et al., 2008; Tauler et al., 2006). More prolonged effects are observed when antioxidant capacity is the primary measurement. For example, decreased levels of enzymatic antioxidants have been reported 19 days after an ironman competition (Neubauer et al., 2008). Although it appears the oxidative footprint left by ultra-endurance exercise is relatively acute, some data is clearly difficult to interpret due to complications in study design. Collecting samples before and after multiple days of exercise takes into consideration periods of rest and recovery (e.g., Machefer et al., 2004). Likewise, examining just one section of a multi-day event also includes effects caused by previous days of exercise (e.g., Tauler et al., 2006). Further studies are therefore required to answer the question of whether ultra-endurance exercise results in alterations to redox-homeostasis which are detectable one month later.

The present study examined the impact of a single-stage ultra-endurance running event on redox-homeostasis over a period of 28 days. On the basis of prior research, it was hypothesised that exercise would cause protein carbonylation, lipid peroxidation and damage to DNA, which would be accompanied by a reduction in whole blood GSH concentration. It was anticipated that all parameters would return to baseline levels within 28 days of the race.

4.3. METHODS

4.3.1. Participants

Nine healthy non-smoking men took part in this study (Table 4.1). Participants abstained from taking vitamin supplements for at least ten weeks prior to the ultra-endurance race. Participants provided informed consent, and the study was approved by the School of Sport and Exercise Sciences research ethics committee of the University of Birmingham.

4.3.2. Baseline measurements

Participants visited the laboratory prior to the ultra-endurance race to have height and body mass recorded, and skin-fold measurements taken for the calculation of percentage body fat (Durnin and Womersley, 1974). Maximal oxygen consumption ($\dot{V}O_2$ max) was directly measured with a graded exercise test on a treadmill adapted from the Bruce protocol (Whaley et al., 2006). Breath-by-breath measurements were recorded every 5 seconds throughout the test (Oxycon Pro, Jaegar, Germany). Heart rate and ratings of perceived exertion (RPE) were recorded during the final minute of each stage. In addition, participants had leisure time physical activity assessed using the international physical activity questionnaire (IPAQ) (Craig et al., 2003). IPAQ data was expressed as MET-minutes per week, where 1 MET (metabolic equivalent) is equal to resting metabolic rate.

TABLE 4.1.

Characteristics of participants and results from the ultra-endurance race (mean ± SD).

Characteristics of participants	$n = 9$
Age (years)	46.1 ± 5.3
BMI (kg·m^{-2})	24.9 ± 2.3
Body fat (%)	14.1 ± 2.4
LTPA[a] (Median MET·min·week^{-1})[b]	3393 (2040-4850)
Measured $\dot{V}O_2$ max (ml·kg^{-1}·min^{-1})	56.3 ± 3.3
Maximum heart rate at $\dot{V}O_2$ max (bpm)	175 ± 5
Ultra-endurance race results	
Running distance (km)	174.1 ± 60.0
Running duration (hh:mm)	30:02 ± 12:14

[a] Leisure Time Physical Activity, as assessed by the international physical activity questionnaire (IPAQ).

[b] Data are medians (min-max).

4.3.3. Ultra-endurance race

Participants took part in The Grand Union Canal Race; a single-stage 233 km (145 mile) race from Birmingham to London in the UK. The route comprised mainly gravel or paved tow-paths and bridleways adjacent to the canal. The race started at 06:00 on Saturday the 23rd of May 2009, and participants who completed the race, crossed the finish line between 21:15 on the next day, and 01:50 on Monday the 25th of May 2009. Participants undertook the race at their own pace. Diet was not controlled or recorded for logistical reasons and participants consumed food and fluids *ad libitum*. Ambient temperatures during the race were 6-9°C at night and 20-25°C during the day. Relative humidity was 57-64%.

4.3.4. Experimental design

Blood samples were collected from a forearm vein less than 60 minutes before the race and upon completion or drop out (mean 65 min ± 64 SD after). Additional blood draws were taken at 24 hours (mean 26 hours ± 4 SD after), 7 days, and 28 days post-race. Participants were seated for 5 min prior to all blood draws, and refrained from exercising for at least 7 days following the race.

4.3.5. Blood sample processing

Blood was collected into ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Oxford, UK) and immediately stored at 4°C. Peripheral blood lymphocytes and plasma were obtained within 60 min using a portable centrifuge (E8F Portafuge, LW scientific, Georgia, USA). Lymphocytes were isolated using density gradient centrifugation. Briefly, whole blood was diluted 1:1 with Phosphate Buffered Saline (PBS; Invitrogen, Paisley, UK) and carefully layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) before centrifuging

at $1350 \times g$ for 30 min. The lymphocyte layer was aspirated, and cells washed in PBS at $1350 \times g$ for 10 min. The pellet was re-suspended 1:1 in foetal bovine serum (FBS) 10% dimethyl sulfoxide (DMSO). Plasma was obtained by centrifuging whole blood at $1350 \times g$ for 15 min. Lymphocytes, plasma and whole blood were immediately frozen in multiple aliquots using dry ice, and later transferred to −80°C storage until analysis.

4.3.6. General analytical procedures

Total plasma protein concentration was determined using the bicinchoninic acid method as described by Smith *et al.* (1985). Biochemical parameters were expressed relative to protein concentration, cell number, or were corrected for changes in plasma volume according to Dill and Costill (1974) as appropriate. Haemoglobin and haematocrit was assessed using a Coulter ACT[diff] haematology analyser (Beckman-Coulter, High Wycombe, UK). Chemicals were obtained from Sigma-Aldrich, UK, unless otherwise stated.

4.3.7. Reduced glutathione

Reduced glutathione (GSH) was measured in whole blood frozen 1:1 with FBS (10% DMSO) using a commercially available fluorescence-based assay according to manufacturer's instructions (Promega GSH-Glo, Wisconsin, USA).

4.3.8. DNA damage

A non-specific measure of DNA damage, Frank strand breaks to lymphocyte DNA, was measured using the alkaline single-cell gel electrophoresis (comet) assay (Singh et al., 1988). DNA damage caused by oxidative stress was assessed using *Escherichia coli*

Formamidopyrimidine DNA glycosylase (FPG) digestion for the detection of oxidised purine lesions (FPG-dependent oxidative DNA damage) (Collins et al., 1993). Briefly, samples and controls were re-suspended in 1 ml of Dulbecco's modified Eagle medium (DMEM). Control samples (approximately 4×10^5 cells) were treated with 100 μM hydrogen peroxide or DMEM for 1 hour at 37°C. Samples and controls were centrifuged at $200 \times g$ for 5 min. Pellets were re-suspended and mixed in 25 μl DMEM and 300 μl low-melting point agar. 150 μl of this cell suspension was added to two separate glass slides (pre-coated with a thin layer of normal-melting point agar) and covered with a glass cover-slip. Slides were left for 30 min at 4°C to solidify. Cover slips were removed, and slides added to lysis buffer (2.5 M NaCl, 0.1 M Na$_2$EDTA, 10 mM Tris base, 1% Sodium *N*-lauryl sarcosinate, 10% DMSO, and 1% Triton-X 100, pH 10.0) for 1 hour at 4°C. Slides were washed (3 × 5 min) with FPG enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na$_2$EDTA and 0.2 mg/ml bovine serum albumin, pH 8.0). Parallel slides were treated with either 50 μl FPG enzyme buffer containing 1 unit of FPG enzyme (Trevigen, Maryland, USA) or 50 μl FPG enzyme buffer alone. Following treatment, slides were covered with cover-slips and incubated for 60 min at 37°C in a moist box. After incubation, cover-slips were removed, and slides were transferred to a horizontal electrophoresis tank containing electrophoresis buffer (300 mM NaOH and 1 mM Na$_2$EDTA, pH > 13.0) and left to stand for 20 min before electrophoresis at 32 V, 300 mA for 20 min (1.8 V·cm^{-1}). Slides were neutralised by flooding (3 × 5 min) in neutralisation buffer (0.4 M Tris, pH 7.5), and stained with 50 μl of 10 × Sybr Gold (Invitrogen Ltd, Paisley, UK). Slides were stored at 4°C in a moist box, and examined within 24 hours using a fluorescence microscope (20 × magnification, 515-560 nm excitation and 590 nm barrier filters respectively; Zeis Axiovert 10, Germany). Median percentage of DNA in

comet tails was analysed in 100 comets using computer-based image analysis software (Comet Assay IV, Perceptive Instruments, Suffolk, UK).

4.3.9. Protein carbonylation

Plasma protein carbonylation was measured by enzyme-linked-immunosorbent-assay (ELISA) described by Buss *et al* (1997). Samples and standards (0.5 mg/ml in sodium carbonate buffer; 50 mM, pH 7.4, 50µl) were allowed to bind to 96-well multi-sorb plates for 60min at 37°C in triplicate (Nunc, Fisher Thermo Scientific, UK). 2, 4-dintrophenylhydrazine (1mM, 50µl) in 2 M hydrochloric acid was added, and plates incubated at room temperature for 60 min. Plates were blocked overnight at 4°C with 0.1% tris-buffered saline-TWEEN-20. Mouse anti-DNP antibody (1:1000, 50µl) was incubated with samples for 120 min at 37°C, followed by a peroxidise-labelled rat anti-mouse antibody (1:5000, 50µl), incubated for 60 min at 37°C. A citrate phosphate-based substrate (0.15 M, pH 5) was added, and plates left to develop in the dark for 30 min at 37°C. The reaction was stopped with 2 M sulphuric acid and plates read at 490 nm. Values were expressed as nmol/mg of protein.

4.3.10. Lipid peroxidation

Lipid peroxides were assayed in plasma using a modification of the method by el-Saadani *et al*. (1989). Samples and positive and negative controls (1:1000 hydrogen peroxide and distilled water respectively) were added to 96-well plates in triplicate. Working reagent (0.2 M Potassium Phosphate, 0.12 M Potassium Iodide, 0.15 mM Sodium Azide, 2g/l Triton-X, 0.1 g/l Alkylbenzyldimethylammonium, 10µM Ammonium Molybdate; 100 µl) was added, and plates incubated at room temperature for 30 min. Plates were read at 340 nm. Lipid peroxide

concentration was calculated using the Beer-Lambert-Law with an extinction coefficient of 24600. Values were expressed as nmol/ml plasma,

4.3.11. Statistical analysis

Data were inspected for normal distribution using the Kolmogrov Smirnov test. Non-normally distributed data were transformed logarithmically. For the comet assay, measurements of percent (%) tail DNA were determined to assess the extent of DNA damage as recommended by Duez *et al* (Duez et al., 2003). Responses to exercise were examined with repeated-measures Analyses of Variance (ANOVAs). Differences compared to baseline were examined using post-hoc paired samples *t*-tests. Statistical significance was accepted at the $p < .05$ level. Effect sizes are presented as η^2. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated. Data were analysed using SPSS statistical package version 18.0 for Windows (SPSS Inc, USA).

4.4. RESULTS

Four men completed the ultra-endurance race (finishers) and five retired prematurely due to exhaustion (non-finishers). There were no significant physiological differences between finishers and non-finishers, nor were there differences in any of the biochemical or immunological parameters investigated between the two groups (data not shown). Running duration (hours), distance (km) or finishing status (i.e., finisher or non-finisher) did not confound any of the results reported below, as determined by analysis of covariance (Jennings and Stine, 2007). On this basis, and the fact that all non-finishers had undertaken exercise for more than 12 hours (hh:mm; mean 20:44 ± 07:15 SD), covering the distance of approximately three marathons (mean 126.7 km ± 29.7 SD), all participants were included in analyses.

4.4.1. DNA damage in lymphocytes

Figure 4.1 A shows frank strand breaks and FPG-dependent oxidative DNA damage before and after ultra-endurance exercise. Frank strand breaks increased with exercise (main effects of time; $F_{(4, 32)} = 11.7$, $p < .001$, $\eta^2 = .594$) and were elevated above baseline immediately and 24 hours after the race (paired samples t-tests; $t_{(8)} < -5.2$, $p < .01$, see Figure 4.1 A). An increase in FPG-dependent oxidative DNA damage was also observed (main effects of time; $F_{(4, 32)} = 2.8$, $p < .05$, $\eta^2 = .257$) and this was elevated above baseline immediately post-race (paired samples t-test; $t_{(8)} = -3.1$, $p < .05$, see Figure 4.1A).

4.4.2. Whole blood GSH

A biphasic antioxidant response was observed with ultra-endurance exercise (main effects of time; $F_{(4, 32)} = 26.0$, $p < .001$, $\eta^2 = .764$, see Figure 4.1B) which reflected the elevated markers of oxidative stress. GSH concentration increased immediately post-race (+32%; paired samples t-test; $t_{(8)} = -3.7$, $p < .01$) but was depleted to nearly one-third (−60%) of baseline levels 24 hours later (paired samples t-test; $t_{(8)} = 6.1$, $p < .001$). At 7 days and 28 days post-race, GSH remained at approximately half to two-thirds (−45 to −31%) of baseline levels respectively (paired samples t-tests; $t_{(8)} > 3.5$, $p < .01$, see Figure 4.1B).

4.4.3. Plasma protein carbonylation

Ultra-endurance exercise resulted in significant plasma protein carbonylation (main effects of time; $F_{(4, 32)} = 7.6$, $p < .001$, $\eta^2 = .370$) (see Figure 4.1C). Post-hoc comparisons to baseline showed that plasma protein carbonyl concentration was elevated for 7 days after the race (paired samples t-tests; $t_{(8)} > -2.5$, $p < .04$, see Figure 4.1C).

4.4.4. Plasma lipid peroxidation

Ultra-endurance exercise resulted in plasma lipid peroxidation (main effects of time; $F_{(4, 32)} = 14.0$, $p < .001$, $\eta^2 = .636$) (see Figure 4.1D). Plasma lipid peroxide concentration was elevated immediately post-race, and remained at this level for 24 hours, before falling below baseline 28 days later (paired samples t-tests; $t_{(8)} < 3.3$, $p < .05$, see Figure 4.1D).

FIGURE 4.1

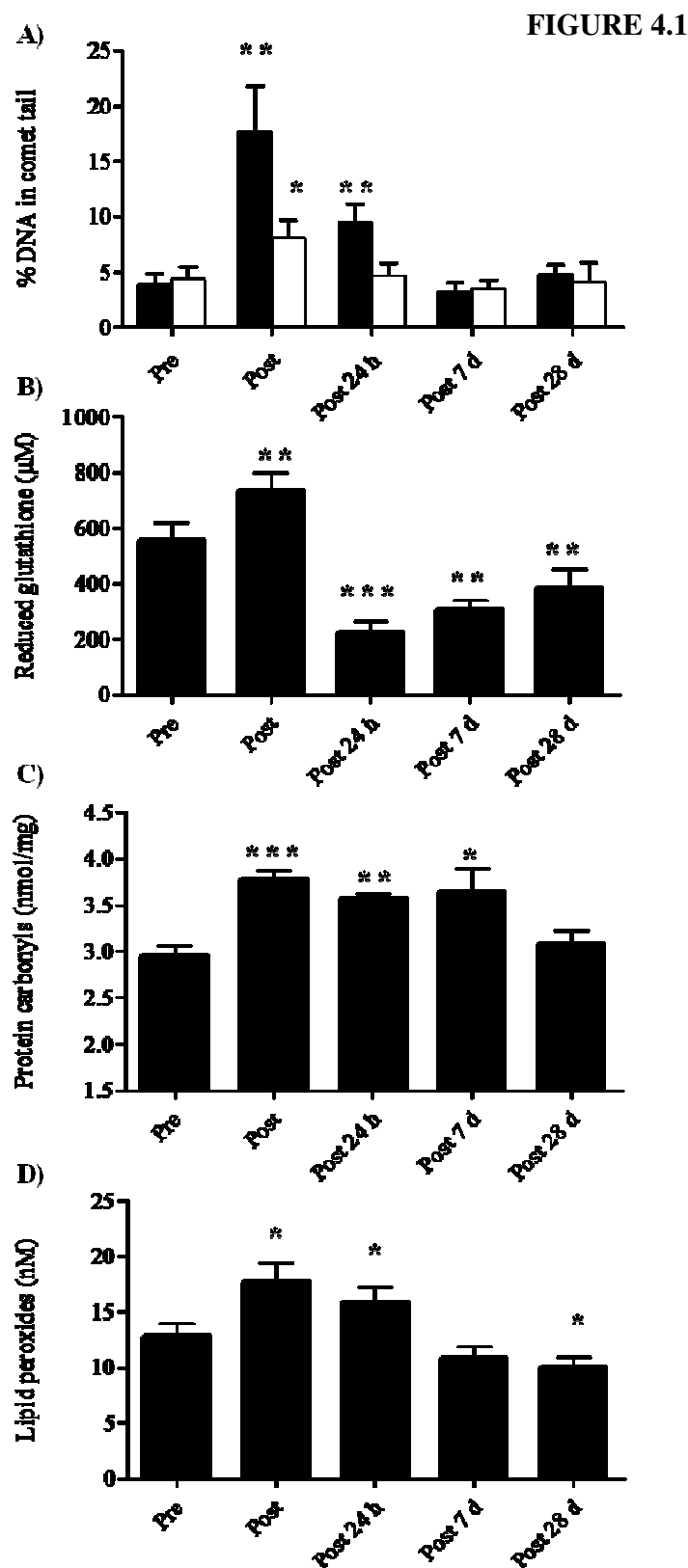Figure 4.1. A) Frank strand breaks to lymphocyte DNA (closed bars) and FPG-dependent oxidative DNA damage (open bars). Data expressed as percent (%) DNA in the comet tail. B) Whole blood reduced glutathione (GSH) concentration. C) Plasma protein carbonyl concentration. D) Plasma lipid peroxide concentration.

$*** p < .001$, $** p < .01$, $* p < .05$ compared to baseline (paired samples t-tests). Data are Means ± SEM.

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4.5. DISCUSSION

Studies have shown that ultra-endurance exercise results in oxidative stress, which is alleviated in a matter of hours or days depending on the biomarker assessed (Machefer et al., 2004; Neubauer et al., 2008; Skenderi et al., 2008; Tauler et al., 2006). The present investigation expands upon previous investigations by employing a more prolonged blood sampling regimen (up to 28 days post-race). This study measured several oxidative stress biomarkers, including a comparison between non-specific DNA damage (i.e., frank strand breaks) and oxidative-specific DNA damage (FPG-dependent oxidative DNA damage).

The results confirmed that ultra-endurance exercise is associated with increases in markers of oxidative stress. FPG-dependent oxidative DNA damage was increased immediately post-race, whereas frank strand breaks and lipid peroxidation were elevated for 24 hours. Augmented protein oxidation was detected 7 days after the race. In addition, we showed for the first time, that whole blood GSH exhibits a large biphasic response; increasing by one-third post-race, and falling to almost one-third of baseline levels 24 hours later. Strikingly, the antioxidant capacity of blood remained at two-thirds of initial values 28 days after the race - a similar level of GSH depletion as seen in Alzheimers disease patients (Bermejo et al., 2008).

The present study showed that lipid peroxides were elevated for 24 hours after ultra-endurance exercise, which is consistent with previous reports (Neubauer et al., 2008; Skenderi et al., 2008). This increase in lipid peroxidation was accompanied by a fall in lipid peroxide concentration 28 days post-race. Increased lipid peroxidation is associated with cardiovascular disease, and some

markers of oxidised lipids (e.g., malondialdehyde) are mutagenic to DNA increasing the risk of cancer (Niedernhofer et al., 2003; Schwedhelm et al., 2004). Conversely, decreased lipid peroxidation has previously been observed following exercise (Ginsburg et al., 1996; Kretzschmar et al., 1991) and it has been suggested that this may be one of the mechanisms behind the atheroprotective effect of physical activity (Ginsburg et al., 1996; Kesaniemi et al., 2001). Further, increased protein oxidation was detected for up to seven days post-race which could partly be attributed to the chemical stability of protein carbonyl groups (Dalle-Donne et al., 2003). Protein carbonylation has been associated with altered protein function and receptor interaction; for example carbonylation of the protein moiety of low density lipoprotein is known to increase uptake into blood monocytes (Aldred and Griffiths, 2004).

In the present study, ultra-endurance exercise caused an increase in frank strand breaks, which remained elevated for 24 hours. Damage to DNA is linked to increased cancer and cardiovascular disease risk (Wu et al., 2004), and elevated levels of DNA damage have been found in atherosclerotic plaques (Martinet et al., 2002). Following ultra-endurance exercise, some of this damage was attributable to exercise-induced ROS production, since FPG-dependent oxidative DNA damage was increased post-race, returning to baseline within 24 hours. The transient appearance of FPG-dependent oxidative DNA damage, and the subsequent return to baseline, likely reflects up-regulation of repair mechanisms in which oxidised purine bases (e.g., 8-oxoguanine) are repaired or removed by 8-oxoguanine DNA glycosylase (OGG1) (Radak et al., 2008). Indeed, OGG1 activity is increased approximately 16-18 hours after a marathon (Radak et al., 2003) which coincides with the return of FPG-dependent oxidative DNA damage to baseline in this study. It is possible that frank strand breaks did not follow the same pattern of repair,

because this includes oxidised pyrimidines which are not repaired by OGG1. Instead, pyrimidine lesions are repaired by endonuclease III homolog (NTH1), the activity of which, is not up-regulated following exercise (Radak et al., 2003). In addition, these pyrimidine lesions would not be detected (and therefore not included in our measure of oxidative DNA damage) by FPG in our assay. As well as the risks that DNA damage might confer, damage to lymphocytes might inhibit cell-mediated immunity post-exercise, which is in line with reports of increased infection risk following endurance exercise events (Nieman, 1995).

Perhaps the most striking finding of this study is that GSH was depleted to two thirds of baseline levels at 28 days post-race. The baseline GSH concentrations observed in this study were not elevated when compared to other reports in healthy individuals (e.g., Skenderi et al., 2008). GSH is a molecule that is key in cellular redox status regulation (Filomeni et al., 2002; Kinscherf et al., 1994; Suthanthiran et al., 1990; Townsend et al., 2003) and consequences of prolonged GSH depletion may include a compromise in immunity. For example, lower GSH is associated with decreased lymphocyte proliferation and increased viral reactivation. (Kinscherf et al., 1994; Staal et al., 1990; Suthanthiran et al., 1990)

A limitation of this investigation is that a control group was not included. However, prior studies employing a non-exercise control did not observe differences in oxidative stress during the resting control trial (e.g., Mastaloudis et al., 2001). A further potential limitation is that only 4 out 5 subjects completed the race. All subjects were included in analyses because even non-finishers engaged in more than 12 hours of continuous exercise, and covered the distance of more than three marathons. Importantly, there were no physiological, biochemical, or immunological

differences between finishers and non-finishers. Thus, as we expected, controlling statistically for finishing status (i.e., finisher *versus.* non-finisher), duration of exercise, or running distance had no effects on the results reported. Finally, markers of muscle damage were not measured in this study, and one might speculate that muscle damage-induced inflammatory responses could contribute to the observed oxidative stress. However, in the present study, the event route was relatively level, and therefore less damaging to muscle than races including sections of downhill running.

This study confirms that ultra-endurance exercise causes oxidative stress, which persists for one calendar month post-exercise, depending on the specific biomarker examined. For the first time, we show that blood antioxidant capacity, as measured by GSH, is significantly depleted below baseline for 28 days post-race. These results suggest that ultra-endurance events are associated with a period of reduced protection against oxidative stress.

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CHAPTER FIVE

5.0. A MILD IMMUNE RISK PROFILE IN CMV SEROPOSITIVE

HEALTHY YOUNG ADULTS

5.1. ABSTRACT

In the elderly, a set of immunological markers, collectively termed the 'Immune Risk Profile' (IRP), predict susceptibility to infection, accelerated cognitive decline, frailty, and mortality. The IRP appears, at least in part, driven by latent cytomegalovirus (CMV) infection. The purpose of this study was to investigate whether an IRP signature is also present in CMV infected healthy young adults. 158 university student volunteers (mean ± SD; age 20.9 ± 2.7 years, body mass index 22.7 ± 2.7 kg·m^2) took part. Volunteers were assessed for CMV serostatus, the numbers and proportions of CD4$^+$ and CD8$^+$ differentiated/effector-memory cells (i.e., CD27$^-$CD45RA$^{-/+}$/CD28$^-$), plasma interleukin-6 (IL-6), and antibody responses to a half-dose influenza vaccine. Approximately 30% (n = 48) of volunteers were CMV$^+$. A higher lymphocyte and CD8$^+$ T cell count (both $p < .01$), and a lower CD4:CD8 ratio ($p < .03$) was observed in CMV$^+$ individuals. Notably, four CMV$^+$ individuals (8%) exhibited a CD4:CD8 ratio < 1.0, whereas none of the CMV$^-$ participants showed this inverted ratio. The numbers of CD4$^+$ and CD8$^+$ differentiated/effector memory cells were approximately 4-fold higher with CMV$^+$ serostatus ($p < .001$). Plasma IL-6 was also increased in CMV$^+$ individuals ($p < .05$) and was independently associated with the numbers of CD8$^+$CD27$^+$CD28$^-$ T cells ($p < .03$). Finally, there was a significant negative correlation between A/Brisbane antibody responses and anti-CMV IgG titres, as well as with total lymphocytes, CD8$^+$ T cells, and late-differentiated/effector memory T cells ($p < .05$). Although mild, this study shows that an IRP-like phenotype can be observed in young healthy adults infected with CMV, suggesting that this latent virus may be associated with rudimentary aspects of immunosenescence early in life.

5.2. INTRODUCTION

Ageing is associated with a gradual decline in immune competence, termed immunosenescence, which is associated with increased susceptibility to infection, accelerated cognitive decline, frailty, and increased mortality (Akbar et al., 2004; Larbi et al., 2008; Wikby et al., 2005). Longitudinal studies have identified a set of immunological markers, collectively known as the 'Immune Risk Profile' (IRP), which appear to be predictive of these outcomes in elderly adults. The IRP consists of an inverted CD4:CD8 ratio (< 1.0) which appears to be largely driven by an accumulation of differentiated $CD8^+$ T cell subsets (i.e., $CD27^-CD28^-/CD45RA^+$), and evidence of increased inflammatory activity dubbed 'inflammageing'(Franceschi et al., 2000). Latent infection with Cytomegalovirus (CMV) is also subsumed under the IRP (Karrer et al., 2009; Olsson et al., 2000; Wikby et al., 2002; Wikby et al., 2006).

It has recently become clear that several features of the IRP are to a significant extent determined by CMV (Derhovanessian et al., 2009; Pawelec et al., 2004). CMV is a beta-herpes virus that establishes lifelong latency in the body after primary infection. Seroprevalence, as determined by CMV-specific antibodies in serum or plasma (anti-CMV IgG) is high, ranging between 35% among children to 90% among elderly individuals (Lubeck et al., 2010; Staras et al., 2006). The most robust effects of latent CMV infection is a dramatic accumulation of memory T cells that have a differentiated or 'effector' phenotype (i.e., $CD27^-CD28^-/CD45RA^+$) (Appay et al., 2002; van Lier et al., 2003). Recent data has shown that CMV infection drives this expansion, considered a hallmark of immunological ageing, independently of calendar age (Chidrawar et al., 2009; Khan et al., 2002; Weinberger et al., 2007). In older individuals, increased anti-CMV IgG

levels as well as the number of differentiated CD8^{+} T cells have been associated with lower antibody responses to influenza vaccination, and higher circulating levels of inflammatory markers (Almanzar et al., 2004; Goronzy et al., 2001; Saurwein-Teissl et al., 2002; Trzonkowski et al., 2003; Wikby et al., 2006).

It remains largely unexplored if, and to what extent, CMV infection also drives immunity towards the IRP in young individuals. If markers of weakened immunological health initiated by infection can be observed prior to the significant reduction in thymic output with ageing, then this will be relevant for the type and timing of interventions that may ameliorate or prevent immunosenescence (Adler, 2008; Campbell et al., 2010; Griffiths, 2009; Pawelec et al., 2010; Tang et al., 2009). Hence, we studied the associations between CMV and features of the IRP in healthy young adults, including the CD4:CD8 ratio, numbers of differentiated/effector memory T cells, plasma IL-6 levels, and antibody responses to a novel antigen (a half-dose influenza vaccine).

5.3. METHODS

5.3.1. Participants

One hundred and fifty eight healthy university students were recruited by local campus advertisement (Edwards et al., 2010). Mean ± SD age and body mass index (BMI) were 21 years ± 3, and 22.7 kg·m^2 ± 2.7, respectively. An equal number of males and females were recruited. Exclusion criteria were smoking and self reported history of immune or cardiovascular disease, self-reported pregnancy or suspected pregnancy, and use of prescription-medication in the past month (excluding the contraceptive pill). Participants self reported no influenza-like illness in the past year, and no symptoms of acute infection at the time of vaccination and follow up measurements. All participants provided written informed consent and the study protocol was approved by the Black Country Local Research Ethics Committee.

5.3.2. Procedures

Participants visited the laboratory between 16:00 and 17:00 to provide a resting blood sample and receive an influenza vaccination. Before arrival, participants were instructed to abstain from vigorous exercise and over-the-counter medication for 24 hours, alcohol for 12 hours, and food or caffeine for 2 hours prior to their visit. A sub-group of participants undertook a 20 minute exercise intervention before vaccination involving a series of weight lifting exercises. The results of this manipulation are described elsewhere (Edwards et al., 2010).

5.3.3. Blood sample processing

Blood samples for serum and plasma were collected before vaccination (baseline), 24 hours post-vaccination, and 28 days later. Serum was obtained by centrifuging blood in plain Vacutainer tubes (Becton-Dickinson, UK) at 3400 g for 5 min at 21 °C, and stored at −20 °C. Plasma was obtained by centrifuging blood in potassium ethylene-diamine-tetra-acetic acid (K_3EDTA) Vacutainer tubes at 3400 g for 10 min at 1 °C, and stored at −80 °C.

5.3.4. Vaccination

Influenza vaccination was used as a marker of *in vivo* immune function (Burns and Gallagher, 2010). To increase sensitivity of this marker, a half-dose (0.25ml; 50% adult recommended dose) of the 2008/2009 northern hemisphere influenza vaccine (Fluarix, GlasxoSmithKline, Inactivated Split Virion, Lot No. AFLUA384AB) was administered. A nurse administered the vaccine via intra-muscular injection into the deltoid muscle.

5.3.5. Assays

Influenza antibody titre was determined in serum at baseline (pre-vaccination) and 28 days post-vaccination using a haemagglutination inhibition test as previously described (Edwards et al., 2010). An antibody titre represents the highest serum dilution to inhibit the agglutination of test erythrocytes which bind together into a lattice-like structure upon exposure to influenza virus particles (Burns and Gallagher, 2010). At baseline, CMV serostatus and IL-6 concentration were assessed in plasma. CMV serostatus was determined on the basis of anti-CMV IgG > the 3 IU/ml cut-off control by ELISA, according to manufacturer instructions (Genesis Diagnostics, UK). IL-6 was measured using a high sensitivity ELISA according to manufacturer instructions

(Quantikine HS Human IL-6 ELISA, R&D Systems, UK). Assay sensitivity was 0.1 IU/ml and 0.039 pg/ml for the CMV and IL-6 ELISAs respectively. Only one individual fell below the sensitivity threshold for IL-6 (0.02 pg/ml). Intra- and inter-assay precision (CV %) were <10% for both assays. CV values were based upon duplicate measures of eight samples, and for inter-assay comparisons, four plates were compared.

5.3.6. Flow cytometry

Whole blood cell counts were conducted on K_3EDTA blood 24 hours post-vaccination and repeated 28 days later (Coulter ACT^{diff} haematology analyser; Beckman-Coulter, High Wycombe, UK). Flow cytometry was performed on fresh lysed whole blood as described previously (Turner et al., 2010). Fixed cell preparations were read within 36 hours on a multi-parameter flow cytometer (BD FACS CANTO II, BD Biosciences). Lymphocytes were gated on the forward *versus* side-scatter. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were examined for CD27 expression in combination with CD45RA or CD28 to identify naïve ($CD27^+CD45RA^+$), central memory ($CD27^+CD45RA^-$; CM), effector memory ($CD27^-CD45RA^-$; EM) 'revertant' effector memory cells which re-express CD45RA ($CD27^-CD45RA^+$; EMRA), early- ($CD27^+CD28^+$), intermediate- ($CD27^+CD28^-$) and late-differentiated ($CD27^-CD28^-$) sub-populations (Appay et al., 2002; Hamann et al., 1997; Romero et al., 2007; Turner et al., 2010).

5.3.7. Statistical analyses

Data were inspected for normal distribution using the Kolmogrov-Smirnov test. Non-normally distributed data were transformed logarithmically. Differences between CMV^+ and CMV^- participants were assessed with univariate ANOVA (continuous variables) or Chi-squared tests

(categorical variables). Anti-influenza antibody titres were assessed between baseline and 28 days post-vaccination with repeated measures analyses of variance (ANOVA). To identify factors moderating the vaccination response, key variables (e.g., anti-CMV IgG) were entered into ANOVAs as individual covariates and examined for significance (Analysis of co-variance; ANCOVA) (Jennings and Stine, 2007). Significant covariates were further examined adjusting for sex, CMV serostatus, baseline influenza antibody titre, and the pre-vaccine exercise-intervention (Jennings and Stine, 2007). Pearson's correlations established the direction of relationships between key variables (e.g., anti-CMV IgG) and fold change of influenza antibody titre. Fold change was calculated by dividing the geometric mean antibody titre by the baseline titre. Effect sizes are represented by η^2. Data are presented as means ± standard error of the mean (SEM) unless otherwise stated. Data were analysed using SPSS statistical package version 18.0 for Windows (SPSS Inc., USA).

5.4. RESULTS

5.4.1. CMV serostatus

Approximately 30% of individuals ($n = 48$) were CMV$^+$ as determined by anti-CMV IgG titres > 3 IU/ml. CMV$^+$ serostatus was not associated with age, sex or BMI ($p > .1$).

5.4.2. CMV serostatus is associated with a higher lymphocyte count and lower CD4:CD8 ratio

Lymphocyte and sub-population data was averaged for the two collection days as analyses showed that the proportions of cells were similar ($p > .38$), with the exception of the most differentiated subsets ($p < .01$), and correlated between days (average correlation $r = .72$, $p < .001$). The results reported below remained the same when collection days were analysed separately.

CMV infection was associated with a higher lymphocyte count and a greater number of CD8$^+$ T cells ($F_{(1, 152)} > 8.1$; $p < .01$, $\eta^2 > .050$; data not shown). CMV infection was associated with a significantly lower CD4:CD8 ratio ($F_{(1, 152)} = 5.1$, $p < .03$, $\eta^2 = .032$; see Figure 5.1C). A CD4:CD8 ratio < 1.0 was rare in the study population, but it is notable that this was found in 4 CMV$^+$ individuals (8%) and none of the CMV$^-$ individuals.

5.4.3. CMV infection is associated with a robust expansion of differentiated CD4$^+$ and CD8$^+$T cells

The numbers and proportions of CD4$^+$ and CD8$^+$ EM, EMRA, and late-differentiated cells were significantly increased in CMV$^+$ individuals ($F_{(1, 152)} > 30.6$; $p < .001$, $\eta^2 > .167$, see Figure 5.1A,

and 5.1B and Table 5.1). In CMV$^+$ participants, weak positive correlations were observed between anti-CMV IgG titres and the numbers of total lymphocytes, CD8$^+$ EMRA, CD4$^+$/CD8$^+$ and late-differentiated cells (correlations between $r = .24$, $p < .02$ and $r = .39$, $p < .008$, respectively).

5.4.4. Plasma IL-6 is increased with CMV infection and associated with the number of CD8$^+$ CD28$^-$ cells

CMV$^+$ individuals exhibited higher plasma IL-6 compared to CMV$^-$ ($F_{(1, 152)} = 4.3$, $p < .05$, $\eta^2 = .027$; see Figure 5.1E). IL-6 was positively correlated with both intermediate and late-stage differentiated CD8$^+$ cells ($r > .18$, $p < .03$). Post-hoc regression analyses showed that intermediate stage differentiated cells predicted IL-6 concentration independently of CMV serostatus ($F_{(2, 144)} = 3.8$, $p < .025$, adjusted $r^2 = .037$). The relationship with late-differentiated cells was lost when adjusting for CMV serostatus ($p > .1$).

5.4.5. Higher anti-CMV IgG titres confer weaker responses to A/Brisbane antigen

There were no differences in vaccination response (defined as a > 4 fold change in antibody titre 28 days post-vaccination) to any of the influenza strains with CMV serostatus ($p > .1$). Repeated measures ANCOVA showed that a higher concentration of anti-CMV IgG was associated with a smaller increase in A/Brisbane antibody titres ($F_{(1, 152)} = 4.1$, $p < .05$, $\eta^2 = .026$; $r -.16$ $p < .05$; see Figure 5.1D). This effect remained significant after adjusting for sex, A/Brisbane baseline antibody titre, and the pre-vaccine exercise intervention (Adjusted analyses; $F_{(1, 155)} > 3.9$, $p < .05$, $\eta^2 > .025$).

TABLE 5.1.

Proportion of lymphocyte subpopulations in CMV^- and CMV^+ participants (mean ± SD)

Cells % [†]	CMV −	CMV +
CD4+ [#]	39.44 ± 6.18	38.53 ± 5.55
CD4+ naïve	46.26 ± 10.27	44.81 ± 10.96
CD4+ CM	48.61 ± 9.23	46.16 ± 9.54
CD4+ EM	4.90 ± 2.31	7.50 ± 4.22***
CD4+ EMRA	0.22 ± 0.49	1.53 ± 3.83***
CD4+ early	93.60 ± 2.60	89.68 ± 7.32*
CD4+ inter	1.27 ± 0.81	1.28 ± 1.03
CD4+ late	0.19 ± 0.43	3.29 ± 6.07***
CD8+ [#]	20.77 ± 4.12	23.42 ± 5.36**
CD8+ naïve	57.77 ± 10.52	48.69 ± 13.71***
CD8+ CM	34.38 ± 9.35	30.10 ± 11.97**
CD8+ EM	3.88 ± 2.68	7.23 ± 5.48***
CD8+ EMRA	3.96 ± 5.32	13.98 ± 11.12***
CD8+ early	79.38 ± 9.14	65.98 ± 14.88***
CD8+ inter	12.78 ± 6.44	12.81 ± 8.18
CD8+ late	5.39 ± 5.54	15.90 ± 12.87***

[†]Cells as a proportion of $CD4^+$ or $CD8^+$ T lymphocytes

[#]Cells as a proportion of Lymphocytes

*** $p< .001$, ** $p< .01$, * $p< .05$, Univariate ANOVA

5.4.6. Lymphocytes and differentiated T cells are associated with smaller responses to A/Brisbane antigen

Repeated-measures ANCOVA showed that a weaker A/Brisbane antibody response was also associated with a higher total lymphocyte and CD8$^+$ T cell count, and increased numbers of late-differentiated CD4$^+$ and CD8$^+$ T cells, and CD8$^+$ EM and EMRA cells ($F_{(1, 156)} > 4.4$, $p < .05$, $\eta^2 > .041$; $r > -.16$, $p < .05$). These effects remained significant after adjustment for sex, A/Brisbane baseline antibody titre, and the pre-vaccine exercise intervention (Adjusted analyses all; $F_{(1, 155)} > 4.2$, $p < .05$, $\eta^2 > .019$). After adjustment for anti-CMV IgG or CMV serostatus, most of these associations were lost, except for total lymphocytes and total CD8$^+$ T cells ($F_{(1, 155)} > 4.2$, $p < .05$, $\eta^2 > .026$).

FIGURE 5.1.

A) T cell phenotypes CD27/CD45RA

B) T cell phenotypes CD27/CD28

C) CD4:CD8

D) A/Brisbane fold change

E) Plasma IL-6
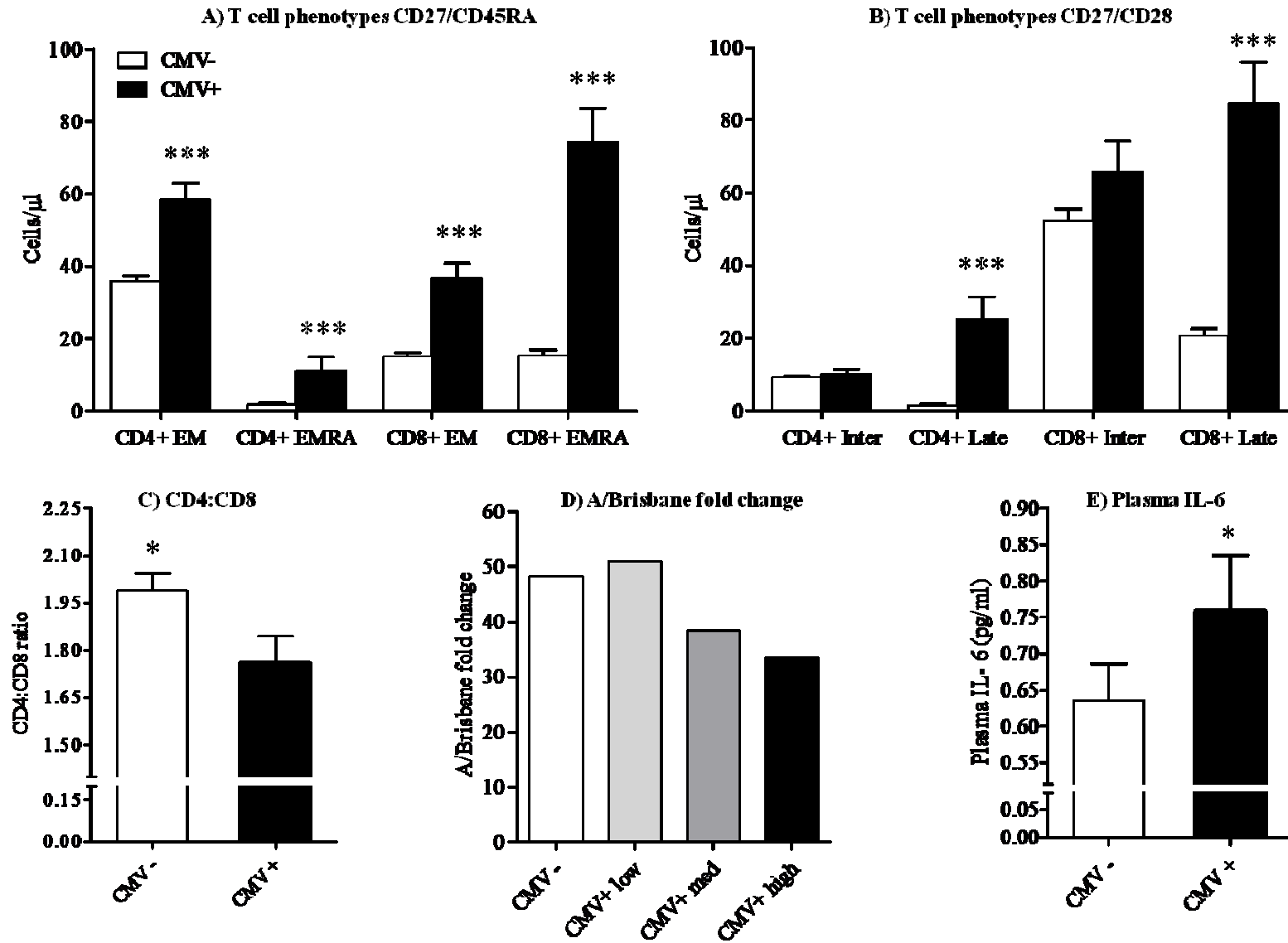
LEGEND FOR FIGURE 5.1.

A mild Immune Risk Profile in CMV seropositive healthy young adults. **A)** CD4$^+$ and CD8$^+$ effector memory (EM; CD27$^-$CD45RA$^-$) and 'revertant' effector memory (EM; CD27$^-$CD45RA$^+$) T lymphocytes. **B)** CD4$^+$ and CD8$^+$ T intermediate-stage (CD27$^+$CD28$^-$) and late-differentiated (CD27$^-$CD28$^-$) T lymphocytes. **C)** Ratio of CD4$^+$ to CD8$^+$ T lymphocytes. Four CMV$^+$ individuals (8%) exhibited a ratio < 1.0. **D)** Data depicts the significant correlation between anti-CMV IgG titre and A/Brisbane fold change with influenza vaccination ($r - .16$, $p < .05$). Figure based upon quartiles of anti-CMV IgG; CMV$^-$ < 3.0 IU/ml, CMV$^+$ low 3.0-5.1 IU/ml, CMV$^+$ med 5.1-9.1 IU/ml, CMV$^+$ high > 9.1 IU/ml. **E)** Plasma IL-6 concentration. Data are means ± SEM. *** $p < .001$, * $p < .05$ CMV$^+$ compared to CMV$^-$.

5.5. DISCUSSION

The results of this study show that young adults infected with CMV exhibit mild signs of the Immune Risk Profile including elevated inflammatory activity. Moreover, anti-CMV IgG titres, total lymphocytes, $CD8^+$ T cells, and the numbers of late-differentiated/effector memory cells were associated with smaller antibody responses to a half dose influenza vaccination.

To the best of our knowledge, we have shown for the first time that healthy young adults latently infected with CMV exhibit increased levels of IL-6. In addition, we also show that elevated IL-6 is independently associated with the numbers of $CD8^+CD27^+CD28^-$ T cells. It is unclear however, if the association between IL-6 and $CD8^+$ T cell phenotypes was causal or what the direction of causality would be. $CD8^+CD28^-$ cells are efficient producers of inflammatory cytokines (e.g., tumour necrosis factor-α; TNF-α and IFN-γ,) and hereby could underlie an elevated systemic IL-6 level that appears to be associated with CD28 down-regulation (Bryl et al., 2001; Clerici et al., 2001; Lorre et al., 1994; Sansoni et al., 2008; Zanni et al., 2003). Indeed, associations between systemic inflammation and an accumulation $CD8^+CD28^-$ cells are present in older adults (Wikby et al., 2006; Zanni et al., 2003), as well as in a variety of auto-immune diseases, including rheumatoid arthritis, multiple-sclerosis and Graves' disease (Markovic-Plese et al., 2001; Schmidt et al., 1996; Sun et al., 2008). Together, the present results and previous literature implicate a role for 'inflammageing' and T cell senescence even in young adults.

In the present study, CMV infection was associated with a ~17% increase in plasma IL-6. This is comparable to the ~20% increase in IL-6 with men and women who develop coronary heart

disease later in life, compared to those who remain healthy (Pai et al., 2004). Further, CMV has been implicated in the development of cardiovascular disease through its effects on endothelial cells (Burns et al., 1999; Craigen et al., 1997; Murayama et al., 1997; Roberts et al., 2010; Scholz et al., 1999; Soderberg-Naucler, 2006; Strandberg et al., 2009). Individuals with a Guanine > Cytosine single-nucleotide-polymorphism (SNP) at position -174 in the promoter region for the IL-6 gene show ~60% increase in IL-6 levels, and are at greater risk of cardiovascular disease (Bennermo et al., 2004; Fishman et al., 1998; Rafiq et al., 2007). It would be interesting to examine whether being CMV seropositive with this SNP carries a further increased risk of disease (Bonafe et al., 2001; Pai et al., 2004; Riikola et al., 2009; Soderberg-Naucler, 2006).

We found that an increased anti-CMV IgG titre was weakly associated with smaller antibody responses to vaccination as well as with increased numbers of late-differentiated/effector memory T cells. In turn, a high number of these cells also associated with lower vaccine responses, but this was an artefact of CMV infection. These findings provide further support that infection with CMV is the major driving force behind the IRP and immunosenescence (Derhovanessian et al., 2009; Moss, 2010; Pawelec et al., 2004; Pawelec and Derhovanessian, 2010; Pawelec et al., 2009).

It is currently unknown exactly how infection with CMV promotes lower antibody responses to vaccination. Several mechanisms are plausible, and it is unclear whether these processes are direct or indirect effects of CMV. Immunosenescence and chronological ageing is associated with impaired function of antigen presenting cells (Gomez et al., 2008; Panda et al., 2009). Rather than a direct effect of CMV, research shows that dendritic cell function is affected by 'senescent'

CD8$^+$ T cells. For example, 'suppressor' CD8$^+$CD28$^-$ cells promote expression of negative regulatory receptors on dendritic cells (e.g., immunoglobulin-like transcripts 3 and 4; ILT3 and ILT4) (Chang et al., 2002; Manavalan et al., 2003). In addition, CD8$^+$CD28$^-$ cells cause a down-regulation of the co-stimulatory molecule CD154 (CD40-ligand) on CD4$^+$ T helper cells (Ciubotariu et al., 1998; Cortesini et al., 2001; Weng et al., 2009). The ensuing impaired responsiveness of CD4$^+$ T helper cells may in turn inhibit antibody production, which is a hallmark of B cell immunosenescence (Cancro et al., 2009; Siegrist and Aspinall, 2009; Weng et al., 2009). Thus, despite the complex, and as yet undetermined mechanisms which might underpin vaccine unresponsiveness, the results of this study show that infection with CMV may have implications for the immune response to novel antigens.

Thus far only one study by Trzonkowski *et al* (2003) has examined CMV as a factor influencing vaccine responses in pre-senescent individuals (mean age ± SD; 32 ± 12 years) (personal communication). As with the present results, individuals with high anti-CMV IgG titres, and a greater number of CD8$^+$CD28$^-$ T lymphocytes, exhibited impaired responses to influenza vaccination (measured as < 4 fold increase in antibody titre one month post-vaccination). The present study confirms and extends these observations, showing that a CMV associated IRP-like phenotype may be central to these observations. One implication is that current findings suggest a different theoretical approach to T cell immunosenescence. Traditional thinking is that senescence is primarily driven by thymic involution, whereby CMV infection could act as a secondary or amplifying factor. The current observations instead suggest a more primary role of CMV in establishing rudimentary features of immunosensencence very early in life, whereby subsequent thymic involution may potentially act as a secondary feature.

5.6. REFERENCE LIST FOR CHAPTER FIVE

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CHAPTER SIX

6.0. DISCUSSION

6.1. SUMMARY AND OVERVIEW

The research in this thesis investigated markers of immunosenescence and oxidative stress in healthy adults. The aim of the first study (chapter two) was to assess whether lymphocyte sub-populations that characterise a senescent immune system, which are known to mobilise with exercise, are also the cells which extravasate from peripheral blood during post-exercise lymphocytopenia. Secondly, because infection with CMV dramatically expands these differentiated 'senescent cells', it was investigated whether CMV serostatus influenced the magnitude of CD8$^+$ T cell mobilisation and egress with exercise. The findings confirm that these differentiated CD8$^+$ T cells, which have a strong tissue-homing and inflammatory potential, show the largest decrease post-exercise, paralleling the pattern of mobilisation during the exercise bout. It was also shown that the mobilisation and subsequent egress of CD8$^+$ T cells and subsets post-exercise were larger in CMV seropositive individuals.

The second study in this thesis (chapter three) investigated whether exercise-induced alterations in the cellular composition of blood influenced measures of oxidative stress in total lymphocytes. This chapter confirmed that vigorous exercise caused oxidative stress in lymphocytes. Correlation analyses showed that a higher number of 'aged' late-differentiated/effector memory cells within the total lymphocyte pool, was unlikely to have bearing on the degree of protein carbonylation in total lymphocytes. Further analyses showed that cells responsible for lymphocytosis, such as CD8$^+$CD27$^-$CD45RA$^+$ (EMRA), had a higher intracellular thiol content compared to cells which show minimal mobilisation with exercise (e.g., CD8$^+$CD27$^+$CD45RA$^+$; Naïve). As high intracellular thiol level is usually associated with lower oxidative stress

(Hernanz et al., 2000; Pandey et al., 2009), this further confirms that cell populations entering peripheral blood with exercise would be unlikely to show increased protein carbonylation to start with. Thus the well documented changes in the cellular composition of blood do not appear to confound measures of oxidative stress markers in total lymphocytes with exercise.

The aim of the third study in this thesis (chapter four) was to examine oxidative stress with ultra-endurance exercise; an activity which is increasing in popularity, but the health benefits of such extreme exercise have been subject to debate (Knez et al., 2006). The results of this chapter show that running for between 12-40 hours, covering a distance of between 3-5 standard marathons, causes a significant oxidative stress. Depending on the specific biomarker examined, oxidative-modifications to bodily molecules were detectable for up to one week after the race. Strikingly, the antioxidant capacity of blood was significantly reduced for one month post-race, to levels which could be compared to those seen in Alzheimer's disease (Bermejo et al., 2008). Thus, these results suggest that ultra-endurance exercise can be associated with a relatively prolonged period of reduced protection against oxidative stress.

The final study in this thesis (chapter five) examined whether rudimentary features of the IRP, a pattern of immune alterations that characterise a decline in immunity with ageing, are observed in a seemingly healthy and young population. This research showed that infection with CMV in young adults was associated with several immunological changes consistent with an IRP-like phenotype. CMV seropositive individuals showed lower CD4:CD8 ratios, marked accumulations of $CD27^-CD28^-/CD45RA^+$ cells, increased IL-6, and smaller antibody responses to vaccination. Thus it was concluded that the earliest appearances of the IRP are observed in CMV seropositive

but otherwise healthy young adults. Adoption of this phenotype in CMV seropositive individuals suggests a more primary role of CMV infection in immunosenescence.

6.2. IMMUNOSURVEILLANCE AND EXERCISE

Lymphocytosis and lymphocytopenia are well established immunological responses to acute exercise (Gleeson and Bishop, 2005). These processes are thought to 'prepare' the immune system for potential challenges such as wounding or infection (Dhabhar, 2002). The results of chapter two suggest that differentiated memory T cells that have a marked presence in immune ageing, are highly sensitive to exercise. These cells, characterised by high cytotoxicity, ability for homing to inflamed tissue, and increased production of inflammatory cytokines (e.g., IFN-γ), extravasate from peripheral blood post-exercise. This is likely to reflect an immunosurveillance response whereby cells are redeployed into the peripheral blood so that they can migrate into the tissues. This interpretation is in line with investigations in animals (Dhabhar, 2000, 2002; Dhabhar and McEwen, 1996; Dhabhar et al., 1995; Krüger et al., 2008; Krüger and Mooren, 2007). For example, in mice, exercise is associated with a redistribution of lymphocytes to the lungs (Krüger et al., 2008; Krüger and Mooren, 2007). This might be an adaptation to the increased chance of pathogen encounter with elevated ventilation during exercise. This literature, together with the findings of chapter two, suggests that exercise brings about changes in the cellular composition of peripheral blood which are adaptive processes of immunosurveillance.

6.3. LYMPHOCYTE OXIDATIVE STRESS WITH EXERCISE

The results of chapters three and four collectively suggest that exercise-induced oxidative stress can be measured in peripheral blood lymphocytes. Measurement of protein oxidation in these cells might offer an additional measure of oxidative stress, which could be employed with more established markers, such as DNA damage. It appears that the influx of 'aged' cells into the periphery with exercise (as shown in chapter two), does not explain the observations of protein carbonylation in these cells with exercise. Thus, previous observations of protein carbonylation in lymphocytes in the literature are likely to be true effects of exercise, and not a caveat of cell population changes (Cases et al., 2006; Ferrer et al., 2009; Sureda et al., 2005; Tauler et al., 2006).

Based on the report that CD45RO^{+} memory cells show increased DNA damage compared to CD45RA^{+} naïve cells (Scarpaci et al., 2003), it might be suggested that increased damage to lymphocyte DNA with ultra-endurance exercise (as shown in chapter four) is caused by an influx of memory cells into peripheral blood with exercise. This reasoning is unlikely to explain our observation of increased lymphocyte DNA damage in chapter four, because we did not observe changes in the numbers of lymphocytes and lymphocyte subsets following the race (data not presented in this thesis). Thus, the results from chapters three and four suggest that lymphocytes are susceptible to oxidative stress during exercise, and that measurements in these cells are not confounded by alterations in the composition of peripheral blood.

The fact that lymphocytes are susceptible to oxidative stress during exercise might suggest that there is a role for oxidative stress in the observed impairment of lymphocyte function post-exercise (Gleeson, 2007; Romeo et al., 2010). Lymphocyte responses to mitogens, proliferation, and cytotoxicity are transiently suppressed with exercise (Niess et al., 1999; Shephard and Shek, 1999). These same lymphocyte functions are also suppressed upon exposure to pro-oxidants *in vitro* (Gleeson, 2007; Niess et al., 1999). Thus, exercise-induced oxidative stress is a possible mechanism for post-exercise immune suppression. Impaired immune function following exercise is transient, and generally returns to normal levels within several hours (Gleeson, 2007; Romeo et al., 2010). Conceivably, repeated bouts of extreme exercise might cause an accumulation of oxidative damage, which could result in long term alterations to cellular functioning (Beckman and Ames, 1998; Sohal and Weindruch, 1996).

6.4. IMPLICATIONS OF ULTRA-ENDURANCE EXERCISE

The results of chapter four add to a growing literature base suggesting that ultra-endurance exercise is associated with a significant oxidative stress (Knez et al., 2006). Compared to the transient increase in lymphocyte oxidative stress with one hour of running in chapter three, chapter four shows that more extreme exercise causes an oxidative stress which can be detected for up to 28 days depending on the specific biomarker examined. Indeed, chapter four shows that individuals taking part in ultra-endurance exercise, experience a prolonged depletion of blood antioxidant defences (GSH). This suggests a prolonged period (up to one month) of reduced protection from oxidative stress. The consequences of this observation are unknown however it might be recommended, based on these results, that subsequent ultra-endurance exercise, which

causes oxidative stress, is not undertaken during this period. It is reasonable to speculate that further oxidative insult during GSH depletion might cause deleterious alterations to cell function, as it has been shown that GSH is essential for lymphocyte activation, proliferation and cytotoxicity (Droge et al., 1994; Liang et al., 1991; Smyth, 1991; Suthanthiran et al., 1990; Yamauchi and Bloom, 1993).

Extreme exercise is associated with psychological stress, adrenergic activity, immune activation, inflammation, and oxidative stress (Cooper et al., 2007; Knez et al., 2006). All of these factors have been implicated in viral reactivation (Mehta et al., 2000; Prösch et al., 2000; Soderberg-Naucler, 2006; Stowe et al., 2007; Zanghellini et al., 1999). Although speculative, ultra-endurance exercise might therefore result in reactivation of latent herpes viruses, which could exacerbate immunosenescence. The findings of chapter four partly support this hypothesis as it has been shown that viral reactivation occurs with cellular depletion of GSH (Baruchel and Wainberg, 1992; Mihm et al., 1995; Staal et al., 1990). In addition, loss of viral control following ultra-endurance exercise may occur due to impaired lymphocyte function with GSH depletion (Liang et al., 1991; Smyth, 1991; Suthanthiran et al., 1990; Yamauchi and Bloom, 1993). Further research in this area seems warranted.

6.5. INFLUENCE OF CYTOMEGALOVIRUS ON IMMUNITY

Chapters two and five examine the way in which infection with CMV shapes our immunobiology. Together, the findings of these two chapters show that CMV is capable of altering our basal immune profile; infection causes a large accumulation of late-

differentiated/effector memory CD8^{+} T lymphocytes. Although formerly considered a hallmark of immune aging, these effects can be observed even in young adults. As these 'senescent' differentiated memory cells are highly responsive to exercise, their expansion through CMV infection appears to govern the magnitude of our immunological responses to exercise. The results of chapter two showed that individuals latently infected with CMV exhibited a mobilisation and subsequent egress of cells which was nearly twice as large as with CMV seronegative individuals. This data provides the first evidence that infection history governs the magnitude and kinetics of immunological responses to exercise. These findings might have important implications for previous research, showing reduced lymphocyte proliferation in the elderly following exercise (Ceddia et al., 1999; Mazzeo et al., 1998) and increased CD8^{+} T cell mobilisation with psychological stress in hypertensive patients (Mills et al., 2003). It would be interesting to examine if CMV serostatus is driving these differences. In addition, the amplification of immunological responses by CMV might enhance immunosurveillance, thereby facilitating detection and clearance of infection (Dhabhar, 2000, 2002). However, it is also possible that enhanced migration of IFN-γ producing cells could aggravate inflammatory processes by infiltrating atherosclerotic plaques, and promoting cardiovascular risk (de Boer et al., 2003; de Boer et al., 1999; Strandberg et al., 2009).

The results of chapter five suggest that even in young adults CMV infection exerts alterations to immune functioning that are typically associated with ageing. The study showed that mild IRP-like phenotypes are observed in young adults infected with CMV, which may impact public health, for example when considering the most appropriate age to employ interventions to boost vaccination efficacy (Campbell et al., 2010; Tang et al., 2009). In addition, one could speculate

that the onset of immunosenescence might begin during young adulthood as a result of CMV infection, potentially amplified by subsequent thymic involution. Thus, methods to ameliorate immunosenescence, such as prevention of CMV infection with vaccination, would need to be implemented early in life (Adler, 2008; Griffiths, 2009; Pawelec et al., 2010).

6.6. REMAINING ISSUES AND FUTURE PERSPECTIVES

A key issue identified in this thesis is that infection history is an important determinant of immunological responses to exercise. As discussed in chapter two, an important direction for future research would be to ascertain if infection with other micro-organisms similarly governs CD8^{+} T cell responses to exercise. The results of chapter five also permit further investigation into how other latent viruses and infections shape our biology. Prime candidates might be the two other beta-herpes viruses which share similar characteristics to CMV: human herpes virus (HHV) 6 and 7 (Campadelli-Fiume et al., 1999; Ward, 2005). Approximately 90% of adults are infected with these viruses, and as with CMV, viral reactivation in healthy individuals is asymptomatic but causes life threatening complications the immune suppressed (Campadelli-Fiume et al., 1999; Ward, 2005). Importantly, as with CMV, these viruses appear to have significant effects on the immune system. For example, HHV-6 causes immune suppression by inhibiting production of interleukin-2 and IFN-γ in T cells, and inducing apoptosis in naïve and CM cells (Flamand et al., 1995; Gupta et al., 2009). At the same time, this virus also increases proliferation and production of inflammatory cytokines by EM and EMRA cells (Gupta et al., 2009). It is currently unknown what effects, if any, HHV-6 and 7 have on the composition of the T cell pool, and whether infection also contributes to immunosenescence. Thus, understanding

whether HHV-6 and 7 exert similar effects to CMV on immunity, and whether seropositivity for all three of these viruses further exacerbates immunosenescence, is a possible target for future work.

A novel finding presented in chapter five, is that young individuals infected with CMV exhibit low grade inflammation, as measured by a ~17% increase in plasma IL-6. This is comparable to the ~20% IL-6 elevation with men and women who develop coronary heart disease later in life, compared to those who remain healthy (Pai et al., 2004). Individuals with a Guanine > Cytosine single-nucleotide-polymorphism (SNP) at position −174 in the promoter region for the IL-6 gene, show ~60% increase in IL-6 levels (Bennermo et al., 2004; Fishman et al., 1998; Rafiq et al., 2007). These individuals are at greater risk of cardiovascular disease, and considering CMV has been directly implicated in atherosclerotic risk, being CMV seropositive with this SNP might carry a further increased risk for disease (Bonafe et al., 2001; Pai et al., 2004; Riikola et al., 2009; Soderberg-Naucler, 2006). IL-6 SNPs have also been associated with increased susceptibility to upper respiratory tract infections (Cox et al., 2010; Revai et al., 2009). Considering the weakened immunological health observed with CMV infection, it would be interesting to see if IL-6 SNPs in CMV seropositive individuals confer a greater infection risk.

The final issues for future research relate to exercise-induced oxidative stress. A novel and striking finding shown in chapter four, was that whole blood GSH level (a measure of antioxidant capacity) was depleted below baseline for one month after ultra-endurance exercise. As well as establishing how long this period of possible reduced protection against oxidative stress persists, it would be important, but perhaps unethical, to examine whether subsequent

vigorous exercise causes more severe damage. It should be acknowledged however, that this is only one measure of antioxidant capacity, and does not necessarily reflect plasma antioxidant capabilities. An additional issue is whether lymphocytes exhibiting increased oxidative stress markers, as shown in chapters three and four, exhibit impaired cellular function independently of exercise. Finally, it would be of great interest to understand if lymphocyte sub-populations show increased or decreased resistance to oxidative stress, considering the findings of a differential intracellular thiol content between lymphocyte sub-populations shown in chapter three. However, with the exception of probes to detect radical species by flow cytometry (Chen et al., 2010; Cossarizza et al., 2009; Eruslanov and Kusmartsev, 2010), tools for the assessment of oxidative biomarkers in lymphocyte sub-populations are lacking. Clearly, development of sensitive techniques which can be used to investigate different cell populations is a target for future work.

6.7. CONCLUSIONS AND SUMMARY

The research in this thesis presents several novel findings:

- Exercise induces a strong egress of late-differentiated CD8$^+$ T cells post-exercise.
- Infection with CMV amplifies the CD8$^+$ T lymphocyte responses to exercise by expanding the numbers and proportions of the highly exercise-sensitive 'senescent' effector-memory CD8+ T cells.
- Exercise causes protein oxidation in peripheral blood lymphocytes, and this is unlikely to be confounded by alterations in the cellular composition of peripheral blood.

- Lymphocyte sub-populations exhibit differential intracellular thiol concentrations which might be related to oxidative stress, to the differentiation/memory/functional characteristics of these cells, or all of these factors.

- Ultra-endurance exercise is associated with whole body and lymphocyte oxidative stress, which is accompanied by depletion of some antioxidant defences for at least one month.

- Early appearances of the Immune Risk Profile are detectable in CMV-infected 'healthy' young adults, suggesting an early onset of immunosenescence can occur independently of thymic involution as a result of CMV infection.

In conclusion, the current findings support the notion that oxidative stress and infection with micro-organisms shape our biology and can accelerate aspects of ageing.

6.8. REFERENCE LIST FOR CHAPTER SIX

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APPENDIX

Volunteer participation and overlap in the exercise-based studies presented in this thesis.

Participant	Chapter two	Chapter three	Chapter three sub-investigation	Chapter four
101		×		
102		×		
103		×		
104		×		
105		×		
106	×	×	×	
107	×	×		
108	×	×		
109	×	×		
110	×	×		
111	×		×	
112	×			
201	×	×		×
202	×	×		×
203	×	×		×
204	×	×		×
205	×	×		×
206	×			×
207	×	×		×
208				×
209				×
301			×	
302			×	
303			×	
304			×	
305			×	
306			×	