

**THE IMPACT OF EXERCISE, ADIPOSITY
AND PERSISTENT VIRAL INFECTION ON
BLOOD T-CELL PHENOTYPE AND
FUNCTION**

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ABSTRACT

Human ageing is associated with a progressive decline in the function of the immune system, commonly referred as immunosenescence. This is characterized by the shrinkage of the naïve T-cell repertoire and a concomitant accumulation of highly differentiated effector-memory cells and dysfunctional senescent T-cells. These systemic immune alterations have clinical implications and have been associated with increased morbidity and mortality in the elderly. However chronological ageing may not be the only factor influencing immunosenescence and certain lifestyle factors may moderate or potentiate the rate at which immune alterations occur. The studies comprised within this thesis investigated the effects of lifestyle factors such as physical activity or obesity, along with latent viral infections on the proportions of highly differentiated and senescent T-cells.

Data were gathered from individuals of various age, physical activity, body composition and latent viral infection status to assess the effects of a wide range of lifestyle factors on T-cell proportions. The different levels of T-cell differentiation were assessed by four-colour flow cytometry using monoclonal antibodies specific to cell surface markers associated with T-cell phenotypes. The role played by leptin on T-cell activation was assessed by *in vitro* stimulation assays followed by cell surface phenotype and gene expression analysis. The effects of acute bouts of exercise on T-cells subsets were characterized using a submaximal cycling protocol.

Aerobic fitness was associated with lower proportions of senescent and higher proportions of naïve T-cells, particularly within the CD8⁺ T-cell compartment in healthy adult men. The beneficial impact of aerobic fitness, and consequently of regular physical activity, on the ageing immune system was independent of age, latent viral infection status and body composition. Furthermore, the moderating effect of higher estimated $\dot{V}O_{2\max}$ on the proportions of senescent T-cells suggested that a transition from low physical activity, characterized as having an estimated $\dot{V}O_{2\max}$ below average, to regular physical activity, characterized as having a $\dot{V}O_{2\max}$ above average, could prevent the age-associated accumulation of senescent T-cells during decades. Obesity and excess serum leptin in adolescents were shown to be associated with changes in T-cell subsets associated with immunosenescence, such as reduced proportions of naïve and early T-cell and increased proportions of effector-memory and senescent T-cells. In addition, high physiological concentrations of leptin enhanced the mitogen-induced T-cell activation *in vitro* suggesting a potential role in the accumulation of senescent T-cells observed in obese individuals. Latent CMV infection was also associated with similar reductions in naïve T-cell proportions and increased proportions of highly differentiated and senescent T-cells in young adults. Although CMV infection appeared to be associated with an amplified exercise-induced preferential mobilization of highly differentiated and senescent T-cells in blood, those cells may not have been specific for CMV.

It is concluded from this work, therefore, that chronological ageing is not the only factor associated with the accumulation of senescent T-cells in the elderly. By preventing obesity, and by potentially inducing senescent T-cells frequent mobilization and subsequent deletion via apoptosis, regular physical activity may prevent the

accumulation of highly differentiated and senescent T-cells in the elderly, and consequently reduce morbidity and mortality in later life.

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PUBLICATIONS

The following published papers and communications have been produced from the work contained within this thesis. Published papers are provided in Appendix 4.

Published Papers

- **Spielmann G.**, McFarlin. B.K., O'Connor D.P., Smith P.J.W., Pircher H., Simpson R.J. (2011). Aerobic fitness is associated with lower proportions of senescent blood T-cells in man. *Brain Behavior and Immunity*. **25(8)**: 1521-1529.

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- **Spielmann G.**, Campbell M.L., Guy K., Cosgrove C., McFarlin B.K., Smith P.J.W., Simpson R. (2008). A reduced frequency of senescent T-lymphocytes is associated with a greater maximal aerobic capacity in middle-aged men: a pilot study. *Journal of Sports Sciences*. **26(S1)**: 148-149.
- **Spielmann G.**, McFarlin. B.K., Cosgrove C., Guy K., Smith P.J.W., Simpson R.J. (2009). A greater $\dot{V}O_{2\max}$ is associated with a reduced frequency of senescent blood T-cells in middle-aged men but not younger men. *Medicine and Science in Sport and Exercise*. **41(5)**: 277.
- **Spielmann G.**, Johnston C.A., Simpson R.J., McFarlin B.K., Foreyt J.P. (2010) No impact of CMV or EBV seropositivity on the frequency of highly differentiated T-cells in Mexican-American adolescents. *International Journal of Exercise Science: Conference Abstract Submissions*. **2(2)**: 39.

- Bartlett, D.B., Cosgrove, C., **Spielmann, G.**, Guy, K. and Simpson, R.J. (2009). Exercise-induced T-cell activation in human subjects is affected by the mobilization of KLRG1+ T-lymphocytes into the peripheral blood. *Medicine & Science in Sports and Exercise*. **41**:5.

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ABBREVIATIONS AND SYMBOLS

AICD	Activation-Induced Cell Death
ANOVA	Analysis of Variance
APC	Allophycocyanin
APCs	Antigen Presenting Cells
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CKI	Cyclin-dependent Kinases Inhibitors
CMV	Cytomegalovirus
CVD	Cardiovascular Disease
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
FITC	Fluorescein isothiocyanate
Fsc	Forward Scatter
HDF	Human Diploid Fibroblast
HLA*A201	Human Leukocyte Antigen serotype “A201”
HR	Heart Rate
ICAM-	Intercellular Adhesion Molecule-
IL	Interleukin
IRP	Immune Risk Profile
KLRG1	Killer Lectin-Like Receptor G1
LFA-	Lymphocyte Function-associated Antigen-
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MHO	Metabolically Healthy Obese

NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NK	Natural Killer
OB-Rb	Long form of the Leptin Receptor
OB-Re	Soluble form of the Leptin Receptor
PA-R	Physical Activity Rating
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cells
PE	R-Phycoerythrin
PEDS-QL	Pediatric Quality of Life Inventory
PerCP Cy-5.5	Peridinin-chlorophyll proteins Cyanine 5.5
PMA	Phorbol Myristate Acetate
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-PCR'	Real Time Polymerase Chain Reaction
SD	Standard Deviation
SEM	Standard Error of the Mean
SIPS	Stress-Induced Premature Senescence
Ssc	Side Scatter
STASIS	Stress or Aberrant Signalling-Induced Senescence
STAT-	Signal Transducer and Activator of Transcription -
TCR	T-cell Receptor
TEMRA	Effector-Memory T-cell re-expressing CD45RA
TNF	Tumor Necrosis Factor
TRF	Telomere Restriction Fragment
$\dot{V}O_{2max}$	Maximal Oxygen Uptake
W	Watts
zBMI	Age- and Sex-adjusted Body Mass Index

CHAPTER 1
GENERAL INTRODUCTION

The immune system is a complex network of specialized cells, proteins, tissues and organs which primarily function to maintain host integrity and eliminate or neutralize any element recognized as non-self and potentially pathogenic. The immune system relies on two distinct but overlapping responses to fight pathogens efficiently, the innate and adaptive immune responses. Innate immunity is the primary response to injury and infectious agents, and consists of non-specific epithelial barriers and antimicrobial substances, effector cells such as macrophages, natural killer cells, eosinophils and neutrophils, and soluble proteins such as the complement system and cytokines secreted to co-ordinate the activities of both the innate and adaptive immune cells. Adaptive immunity is a slower, but more specific response against infective agents, relying on the creation of long-term immunological memory to create efficient and durable defenses against pathogens.

Along with B-cells, T-cells are one of the main cell types involved in adaptive immune responses, and their clonal expansion in response to an antigenic stimulus is a fundamental process of adaptive immunity. After recognizing an infectious agent, T-cells rapidly divide and initiate effector cell functions to combat the infection and prevent pathogenic dissemination in the host. Unfortunately, however, clonal expansion is not unending, and after repeated and excessive rounds of cell division, T-cells undergo replicative senescence. In this instance, T-cells fail to clonally expand in response to growth stimuli, compromising immune system integrity and increasing the opportunity for pathogens to spread and persist in the host. As most components of the immune system are subjected to senescence, the generic term immunosenescence has been used to describe the age-associated decline in the functionality of the immune system. Senescent T-cells are known to accumulate with increasing age, contributing to

poor vaccine efficacy, immunodepression and have also been linked with increased morbidity and mortality in the elderly.

Replicative senescence was initially observed *in vitro* in human diploid fibroblasts (HDF) cultures (Hayflick & Moorhead 1961) as a consequence of telomeric DNA attrition. These highly conserved non-coding hexameric repeats of the nucleotide sequence TTAGGG are located at the end of each chromosome (Moyzis *et al.* 1988), associated with the protein complex shelterin (de Lange 2005) that permit cells to make the distinction between DNA breaks from the natural ends of chromosomes and thus represses abnormal chromosomal fusions to be induced by DNA repair systems. Telomeres are progressively shortened with each round of cell division until they reach a critical size, known as the Hayflick limit, where the cell enters a state of replicative senescence. Senescent cells differ from quiescent cells by the permanent nature of the cell cycle arrest and by marked morphological, functional and phenotypical modifications (Bird *et al.* 2003). In comparison to cells retaining their proliferative capacities, senescent cells appears to be larger, flattened with a vacuolised, more granular cytosol and an increased nuclear volume (Bayreuther *et al.* 1988; Faragher *et al.* 1998). They also exhibit altered chromatin architecture and gene expression (Gire 2005) leading to molecular differences such as the expression of the altered senescence-associated β -galactosidase (van der Loo *et al.* 1998) or the decreased expression of heat shock proteins (Choi *et al.* 1990; Blake *et al.* 1991). Interestingly, while replicative senescence has been observed in a great variety of cell types, large scale microarray analysis has shown that the senescence-associated changes vary from one cell type to another (Shelton *et al.* 1999). Cellular senescence and senescence-associated changes have also been seen in immune cells, such as CD4⁺ and CD8⁺ T-cells (Grubeck-Loebenstein *et al.* 1994; Adibzadeh *et al.* 1995; Adibzadeh *et al.* 1996), suggesting

potential health-threatening consequences due to compromised adaptive immune responses. In addition to having impaired proliferative capacities, senescent T-cells appear to have a reduced sensitivity to apoptosis (Spaulding *et al.* 1999; Hsu *et al.* 2001) while exhibiting an increased pro-inflammatory cytokines production (Effros 2003; Effros *et al.* 2003). Consequently, in addition to the natural age-associated accumulation of dysfunctional senescent T-cells, their limited clearance from the so-called “immunological space”, a fixed homeostasis-regulated space occupied by the cellular components of the adaptive immunity, leads to a restricted T-cell repertoire, which in turn results in poor responsiveness to previously unseen or evolved pathogens and eventually to a higher rate of morbidity and mortality in the elderly (Koch *et al.* 2007). Certain lifestyle factors, such as smoking, physical inactivity or social-economical status (Valdes *et al.* 2005), and persistent viral challenges (Wikby *et al.* 2002) have been shown to influence the rate of immune ageing. Indeed, as a function of the normal ageing process, the accumulation rate of these dysfunctional cells can increase dramatically due to persistent viral challenges. Latent infections with the β -herpesvirus cytomegalovirus (CMV) and/or the γ -herpesvirus Epstein-Barr virus (EBV), although often clinically asymptomatic in the immunocompetent host, have been correlated to immunosenescence and the associated “Immune Risk Profile” (IRP), which has in turn been shown to predict mortality and morbidity in seniors (Wikby *et al.* 1998). The exact mechanisms underpinning immunosenescence are, however, yet to be discovered to allow the establishment of potential therapeutic interventions.

The beneficial effects of regular physical activity on immunity, particularly in older adults, are well-documented. Although many studies have shown increases in Natural Killer (NK) cell activity and T-cell functions in active compared to inactive elderly, the

mechanisms for enhanced immunity by regular exercise are not well understood. It has been shown that acute bouts of exercise preferentially mobilize senescent T-cells in the peripheral blood compartment (Simpson *et al.* 2007a) and also that acute exercise impacts aged individuals differently than younger individuals. If the exercise-induced leukocytosis observed in young adults is also seen in the elderly, fewer cells are entering the blood compartment after acute bouts of exercise in the elderly (Ceddia *et al.* 1999). Bruunsgaard *et al.* (1999) showed a greater mobilization of memory T-cells in younger subjects compared to older individuals following acute bouts of exercise. Interestingly, the same study showed that T-cells mobilized by acute bouts of exercise in the elderly population had a shorter telomere restriction fragment (TRF) mean length than the T-cells from their younger counterparts. As TRF mean length is a marker of terminal differentiation, or immunosenescence it can be advocated that acute bouts of exercise preferentially mobilize memory, or highly differentiated T-cells in young individuals, but mobilize senescent, or terminally differentiated T-cells in the elderly (Bruunsgaard *et al.* 1999).

Since latent herpesvirus infections, obesity and physical inactivity have been proposed to precipitate the onset of immune ageing in older individuals; it is legitimate to question the exact impact of age on immunosenescence. Indeed, although ageing is considered to be the major factor involved in immunosenescence, this could merely be an association, where the accumulation of senescent cells occurs due to infection history or/and lifestyle factors that are insidiously exacerbated with chronological ageing. Furthermore, if lifestyle factors do play a role in the accumulation of senescent T-cells and the concomitant shrinkage in naïve T-cell repertoire, engaging in regular physical activity and reducing obesity could have important clinical ramifications as a simple,

cheap and safe intervention with which to delay the onset of immunosenescence and considerably reduce the mortality rate in the elderly.

The studies comprised within this thesis aimed to examine some of the potential factors considered to influencing the onset of immunosenescence, such as latent viral infections, physical exercise, and obesity. These studies were completed in order to enhance understanding of the factors responsible for the increased proportions of senescent T-cells in peripheral blood, which is a major feature of immunosenescence and the associated IRP. This thesis is presented in seven subsequent chapters, detailed as follows:

- *Chapter 2:* This chapter provides a review of the current literature on immunosenescence and provides the necessary information relevant to the subsequent chapters of this thesis.
- *Chapter 3:* This chapter details the general materials and methods used to collect the data presented in the subsequent studies.
- *Chapter 4:* This chapter presents a study that investigated the effect of aerobic fitness on the proportions of senescent T-cells in men.
- *Chapter 5:* This chapter presents a study that investigated the effects of adiposity, latent viral infections, gender and quality of life on T-cell differentiation associated with immunosenescence in adolescent children.
- *Chapter 6:* This chapter presents a study examining the effects of physiological concentrations of leptin on T-cell activation *in vitro*, along with its effect on the constitutive and inducible expression of the leptin receptor in peripheral blood T-cells.

- *Chapter 7:* This chapter present a study aimed at examining the impact of latent CMV and EBV infections on the exercise-induced mobilization of senescent and viral-specific T-cells.
- *Chapter 8:* This chapter discusses the general findings of the studies comprised within this thesis. Conclusions from the work are drawn and research questions for future studies are proposed.

CHAPTER 2
LITERATURE REVIEW

2.1. The immune response

The term Immunity comes from the Latin *Immunis*, meaning relieved of the burden, where the word burden meant a tax or the subjection to a law and by extension a disease. During the 19th century the word immunity was first used in the context of human health, where it describes the properties of an organism to be resistant to some pathogens (Littre & Robin 1865). The primary function of the immune system is to maintain self integrity and to eliminate or neutralize any element recognized as non-self and potentially pathogenic. It is believed that the immune system development of higher vertebrates was induced for the defence against micro-organisms in their environment and maintenance of tissue integrity, i.e. surveillance against tumour, senescent or damaged cells (Manning 1979).

The immune system can be divided in two distinct but overlapping responses, the innate and the adaptive immune responses.

2.1.1. The Innate Immune System

The innate immune response is the first arm of the immune response and plays an essential role in the defence against pathogens. This innate response has been developed to be very aggressive and localised to avoid being detrimental for the host (Beutler *et al.* 2006). It allows the recognition of "non-self" intrusive agents using a system of soluble proteins and cell receptors. The recognition of microbial invasion by interactions among common pathogenic features, known as pathogen-associated molecular patterns (PAMPs), and toll-like receptors, acute-phase proteins or other cell-surface receptors, trigger a cascade of biological events that can lead to a rapid control of the infection.

Among these non-specific mechanisms, inflammation plays a predominant role by recruiting phagocytes, increasing vasodilation, capillary permeability and nociception. Immune effector cells such as NK cells, basophils, macrophages, eosinophils and neutrophils also play extensive roles in this non-specific response (Lehrer *et al.* 1988; Ouellette 1999; Krisanaprakornkit *et al.* 2000). The intra-tissue and intra-vascular coagulation is also activated during systemic infections. Soluble, constitutive and inducible proteins, such as the complement system and certain cytokines (common to both the innate and adaptive immune responses), are also critical factors in the primary response, enhancing antigen opsonisation and presentation of microbial antigenic determinants to the innate immune system. The engagement of the innate immune response is ultimately essential to help prevent the establishment of an infection and to induce an efficient specific adaptive and memory immune response.

2.1.2. The Adaptive Immune System

While innate immunity is principally orchestrated by non-specific effector cells, the adaptive immune response relies on two principal cell types, the T and B cells. The relative persistence of T and B cells, allow the establishment of immunological memory, increasing the host capacity to respond to specific antigens during future encounters. While B-cells and T-cells are morphologically similar, they mediate two different responses of the adaptive immune system, the humoral and cell-mediated immunity. The humoral response requires activated B-cells to differentiate into plasma cells and produce antigen-specific antibodies, leading to micro-organism phagocytosis, complement activation, pathogen neutralization, and destruction. Activated B-cells are also known to activate the cell-mediated immunity either by acting as antigen presenting

cells (APCs) or by secreting certain cytokines, such as IL-6 or TNF- α (Duddy *et al.* 2004). The cell-mediated branch of the adaptive immune response relies on the property of activated T-cells to kill virally infected, dysfunctional, abnormal or cancerous cells, and phagocytosed pathogens.

2.2. T-cells

There are two distinct sub-populations of T-cells: the T helper cells and the cytotoxic T-cells which express a common heterodimer called the T-cell receptor (TCR). This receptor consists of an alpha and beta chain in 95% of T-cells, whereas 5% of T-cells have TCRs formed by gamma and delta chains (Janeway 2011). After production in the bone marrow, pluripotent haemopoietic stem cells differentiate in lymphoid progenitor cells and migrate to a primary lymphoid organ, the thymus. In the thymus, the newly migrated thymocytes will undergo maturation and receptor gene rearrangement whereupon diverse surface glycoproteins will be expressed, such as CD2, CD25 and CD44, followed by antigen receptors (TCR). The ligation of CD2 to cell function-associated 3 (LFA-3) expressed by non-lymphoid thymic cells is essential for the extensive proliferation of the thymocytes (Denning *et al.* 1988). The T-cell receptor (TCR) is a heterodimer composed of either α/β chains or γ/δ chains divided in constant and variable regions. The constant region is attached in the cell membrane, whereas the variable region is available for interactions with a specific antigen presented by MHC molecules. Antigen recognition depends on the interaction between the TCR and a specific antigen-MHC complex. Thymocytes survival will depend on the functionality of their TCRs, and those expressing dysfunctional TCRs will be cleared by apoptosis. While the random nature of the TCRs gene rearrangement allows the highly specific

recognition of an enormous amount of antigens by the T-cells, it also increases the number of anergic and auto-reactive T-cells, requiring immature T-cells to be positively and negatively selected. During positive selection, only the immature T-cells with a high avidity with self-MHC molecules will survive, whereas those with a low-avidity with self-MHC molecules will die by apoptosis. The surviving immature T-cells that have strong self-antigen recognition will be cleared by apoptosis during the negative selection, to limit the number of circulating auto-reactive T-cells. Complete thymic maturation of the thymocytes is regulated by cytokines such as IL-15 or IL-7, which are known for their ability to induce immature and mature thymocyte proliferation (Suda & Zlotnik 1991; Herbelin *et al.* 1992). Once mature, T-cells emigrate from the thymus to join the T-cell repertoire, formed by the variety of T-cells with antigen-specific TCR. Two types of mature T-cells are found circulating in the periphery, and can be distinguished by the presence at the surface of the glycoprotein CD4 or CD8. The T helper cell generally expresses on its surface the CD4 co-receptor (CD4+ T-cell), whilst the cytotoxic T-cell expresses the CD8 co-receptor (CD8 + T-cell). Both types of mature naïve T-cells circulate in the bloodstream and through the secondary lymphoid organs until they are presented to their specific antigen coated in MHC molecules. The CD4 and CD8 co-receptors are essential for the development of an efficient immune response, by allowing the T helper cells and the T cytotoxic cells to recognize a MHC-peptide complex in the context of MHC class II or MHC class I respectively. While MHC class II molecules are found on the surface of APCs, MHC class I molecules are found on the surface of all nucleated cells. The CD4+ T-cell coordinates the immune response, and secretes cytokines that will play an important role in the activation of other immune system cells such as CD8+ T-cells, B cells and macrophages. The CD8+

T-cell has, once activated, a cytotoxic activity. Its function is to specifically eliminate altered self-cells, such as tumour cells and virus-infected cells (Goldsby *et al.* 2003).

2.2.1. T-cell activation

Naïve T-cells have never encountered the antigen for which they will be specific and cannot have effectors' activity until they have been activated by an APC or a virally infected or cancerous cell. These two cell types meet in secondary lymphoid organs such as the spleen and the lymph nodes. Naïve cells circulate steadily through the secondary lymphoid organs, passing from the peripheral blood compartment to the spleen or lymph nodes before returning into the bloodstream. The migration of T-cells through vascular endothelial cells, known as diapedesis, is initiated by the binding of the chemokine receptor CCR7 (CD197), present on T-cells, to its cognate ligand CCL21, present on vascular endothelial cells. This is rapidly followed by the ligation of the T-cell adhesion molecule L-selectin (CD62L) to two vascular addressins present on the vascular endothelial cells, GlyCAM-1 and CD34 (Janeway 2011). The relatively weak strength of these cellular bonds are however not sufficient to stop the T-cells from rolling on the surface of the endothelium and complete immobilisation of the naïve T-cells will require the action of integrins. All T-cells are characterised by the expression on their surface of the integrin $\alpha_L\beta_2$, also called lymphocyte function-associated antigen-1 (LFA-1) which bind to endothelial cells membrane glycoprotein belonging to the immunoglobulin superfamily, ICAM-1 (CD54). This family contains intercellular adhesion molecules (ICAM), ICAM-1, ICAM-2 and ICAM-3, which all bind the T-cell's LFA-1 integrin. ICAM-1 and ICAM-2 are found on endothelial cells and on APCs. Once bound their function is to prevent cells passing through the lining of blood vessels. ICAM-3 is expressed on all cells and can bind, in addition to LFA-1, the lectin

DC-SIGN expressed on dendritic cells. This feature makes ICAM-3 an important actor in cellular adhesion between T-cells and dendritic cells (Janeway 2011). Finally the immobilised naïve T-cell will pass through the endothelial intercellular junction by CD31 ligation. T-cells can travel this circuit once or twice a day. This constant cell movement increases the probabilities of meetings between an APC which presents a specific MHC-peptide complex and its specific T-cell. About one in 10^5 cell has a specific receptor complex for a specific MHC-peptide (Goldsby *et al.* 2003). In addition, this perpetual circulation of T-cells into the blood stream is believed to be necessary for their survival. Indeed, naïve T-cells need a regular signal provided by their contact to a MHC-self-peptide complex found on the surface of dendritic cells in order to survive in the peripheral blood (Janeway 2011).

T-cells encounter an APC, by the specific binding of the TCR molecule and its co-receptor (CD4 or CD8) to the MHC-peptide complex. The number of MHC-peptide complexes required for the T-cell activation varies with the MHC class involved and the T-cell maturity. For the MHC class I, this number can vary from less than a dozen to several thousand depending on the combination of MHC-peptide complex and clones of effector T-cells involved (Kageyama *et al.* 1995). A study however, has shown that a single MHC-peptide complex would be sufficient to activate an effector T-cell (Sykulev *et al.* 1996). Concerning the MHC class II, 200 to 400 complexes would be needed to induce cell stimulation. Nevertheless, for a full activation, 5000 MHC-peptide would be required (Reay *et al.* 2000), and a complete activation would result in the production of interleukin 2 (IL-2) and the proliferation of activated T-cells. The production of IL-2 begins to be detected when 1500 complexes are used to activate a naïve T-cell, while an effector cell only requires a hundred. Regarding the clonal expansion, a naïve T-cell

needs to be stimulated by 400 MHC-peptide to obtain a detectable proliferation, while an effector cell only needs 40 (Kimachi *et al.* 1997).

It was believed that the only function of integrins, such as LFA-1, was to allow the adhesion and the cessation of T-cells during their process of extravasation or migration to the tissues. However recent studies also highlighted the capacity of LFA-1 to deliver functional signals, including the activation of sphingomyelinase and c-jun amino-terminal kinase (JNK) when LFA-1 engagement by ICAM-1 synergise with TCR stimulation, reaching a level of activation comparable to the activation induced by the TCR/CD28 costimulation. This study has also described the induction of inositol-1,4,5-triphosphate (IP3) and Ca²⁺ flux when LFA-1 is costimulating the TCR (Gatta *et al.* 2002), suggesting that LFA-1 plays a crucial role in the T-cell activation process.

2.2.2. *T-cell differentiation*

The activation of T-cells in thymo-dependent areas lead to cytokines synthesis and clonal expansion of activated T-cells through three series of intracellular signals. The signal transduction cascade is initiated after ligation of the TCR-CD3 complex and an MHC-antigen complex, expressed on an APC. In order to mount adequate immune responses against every potential pathogens, both exogenous (such as bacteria) and endogenous (such as viruses) antigens are processed and bound to MHC class II or MHC class I molecules respectively. Following cellular uptake, peptides resulting from the enzymatic degradation of the exogenous antigen, are bound to MHC class II molecules and expressed on the cell surface. Endogenous antigens are processed through a different pathway due to their inherent presence in the infected cell. Ubiquinated endogenous antigens are degraded by the proteasome and bound to MHC

class I molecules before being expressed on the cell surface. Naïve CD4⁺ T-cells bind specifically to MHC class II molecules, specific to APCs, whereas Naïve CD8⁺ T-cells bind to MHC class I molecules, expressed on all nucleated cells. As a consequence of this difference in affinity, the two CD4⁺ and CD8⁺ T-cell subsets are designed to eliminate respectively extracellular pathogens, such as bacteria, or intracellular pathogens, such as viruses. In addition, activated CD4⁺ T-cell will secrete cytokines such as IL-2, IFN- γ and TNF- α , enhancing CD8⁺ T-cell activation and effector functions or cytokines such as IL-4 and IL-10, modulating the CD8⁺ T-cell response.

After ligation of the TCR complex to a specific MHC-antigen, Src family kinases such as Lck or Fyn are phosphorylating the immunoreceptor tyrosine-based activation motif (ITAM) of the CD3 complex (Kane *et al.* 2000) in order to allow the attachment of two other kinases, Syk and ZAP70. These two kinases have a specific role to phosphorylate adaptor proteins such as LAT, TRIM, SIT or PAG. If these molecules have been shown to lack enzymatic properties, they help to recruit cytosolic proteins (Leo *et al.* 2002), in particular the phospholipase C γ 1 which will hydrolyse the phosphoinositides membrane (PIP2) and release inositol triphosphate (IP3) and diacylglycerol (DAG) (Montixi & He 1999). The binding of IP3 on its receptor regulates the rate of intracellular calcium and mainly activates a calcium-dependent enzyme, the calcineurin. Once activated, the calcineurin will induce the dephosphorylation of a transcription factor: the NFAT (nuclear factor of activated T-cells). They will both then migrate from the cytoplasm to the nucleus to bind regulatory sequences of genes and induce cytokine synthesis such as IL-2. This calcineurin-dependent signal induces the G0 to G1 transition of mitotic cell cycle (Figure 2.1).

The second signal of costimulation is induced in the immunological synapse, by the binding of constitutive or inducible costimulatory receptors to their ligands, such as

CD40, CD80 and CD86, B7-H expressed on dendritic cells and CD40L, CD28, ICOS expressed on T-cells. The CD28 activation induces the phosphorylation of cytoplasmic SRC-homology 2 (SH2) tyrosine residues such as Vav1, leading to a protein kinase cascade and to the regulation of c-Fos and c-Jun expression. In addition, the DAG formed during the first TCR signal, activates the protein kinase C (PKC), which dissociates the cytoplasmic complex NF- κ B from its inhibitor I κ -B. The coordinated action of the TCR signal and the costimulatory signal, leads to the synthesis of the T-cells activation proteins IL-2 and α -chain of the IL-2 receptor (CD25).

The third signal of the cell cycle progression from phase G1 to phase S is influenced by the IL-2 binding on its high affinity receptor. This attachment causes the phosphorylation of the Janus kinase-1 (Jak-1) and Jak-3 and triggers a protein kinase cascade resulting in mTOR (mammalian target of rapamycin) production. Once released mTOR will initiate the mRNA translation and the synthesis of proteins regulating the cell cycle progression necessary for the phase M to take place. Because of its similarity to the IL-2, the macrophage's IL-15 can also provide this last signal. Consecutive T-cell differentiation will depend on the IL-2R signal strength and the amount of IL-12 as shown in Figure 2.1.

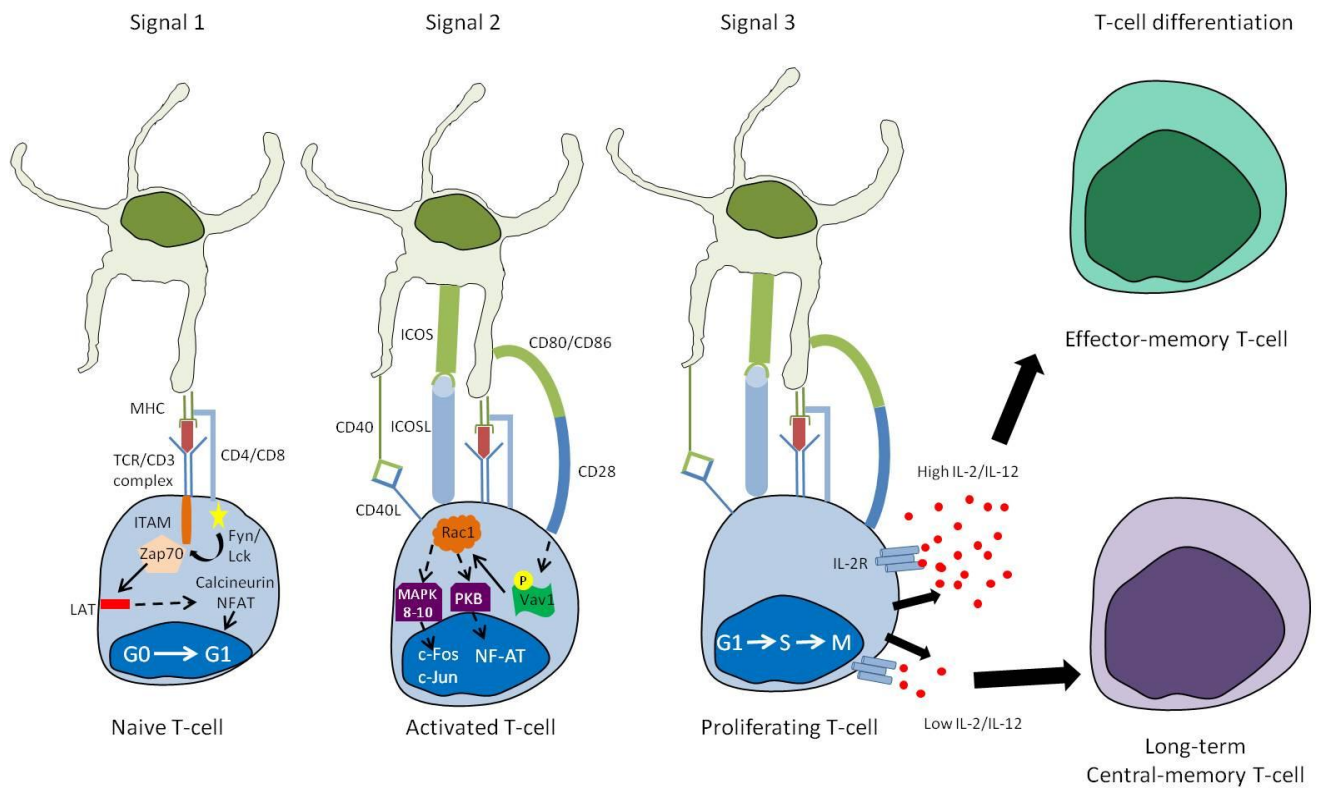


Figure 2.1. Activation signals required for complete T-cell activation and proliferation. The first activation signal occurs when the TCR/CD3 complex binds to the specific MHC-antigen complex, leading to an intracellular cascade and to the T-cell initial activation. The second signal of costimulation is generated by cognate interactions through costimulatory (or adhesion) molecules of the APC and the T-cell. The third signal is produced by IL-2 binding to its receptor, promoting the cell cycle progression to M phase, and starting cell proliferation. The cytokine-induced signal strength is believed to play a role on T-cell differentiation (Belz & Kallies 2010).

2.2.3. T-cell phenotype

Over the course of their maturation and differentiation, T-cells acquire and also lose the expression of different glycoproteins. As described above, all functional T-cells express a TCR-CD3 complex, allowing antigen recognition (Abbas *et al.* 2000) and a great majority of T-cells express either CD4 or CD8. Naïve T-cells also express CCR7 and CD62L, receptors allowing them to migrate to secondary lymph nodes and encounter antigens. When naïve T-cells encounter MHC-antigen peptide complex on an APC, they require the presence on their surface of the protein tyrosine phosphatase CD45, also known as the common leukocyte antigen. CD45, located on all nucleated haematopoietic cells, regulates the Src kinases during T-cell activation and is vital for T-cell functioning (Kung *et al.* 2000). This transmembrane receptor has the particularity to exist in three different forms on the T-cell surface, CD45RA, CD45RB and CD45RO (Okumura *et al.* 1996). The differential expression of CD45 molecules on T-cells have been widely used to identify their differentiation stage. Indeed naïve T-cells have been shown to constitutively express CD45RA while memory antigen-experienced T-cells were characterised by their acquired expression of CD45RO and the loss of CD45RA (Akbar *et al.* 1988; Sanders *et al.* 1988). However, recent studies have highlighted the potential of some CD8⁺ memory T-cells to re-express CD45RA, with or without CD45RO expression, but whilst keeping their effector and replicating capacities (Hamann *et al.* 1997; Sallusto *et al.* 1999; Dunne *et al.* 2002). Sallusto *et al.* (1999), proposed the analysis of CD45RA and CCR7 concomitant expression on T-cells as a more accurate representation of their true level of differentiation. Indeed they showed that naïve T-cells co-expressed CD45RA and CCR7 on their surface (CD45RA⁺/CCR7⁺) whereas central-memory T-cells only expressed CCR7 (CD45RA⁻/CCR7⁺) and effector-memory T-cells had lost the expression of both markers

(CD45RA-/CCR7-). Finally they showed that some effector-memory T-cells had re-acquired CD45RA while still lacking CCR7 (CD45RA+/CCR7-). CD45RA+/CCR7- T-cells, also called effector-memory T-cells re-expressing RA (TEMRA) are known to be very highly differentiated and to have undergone a great rounds of cell divisions. They have however been shown to keep replicative capacities and thus TEMRA T-cells can be considered as terminally differentiated T-cells but differ functionally from senescent T-cells (Dunne *et al.* 2002; Carrasco *et al.* 2006). A summary of the different T-cell phenotypes depending on their level of differentiation is shown in Figure 2.2.

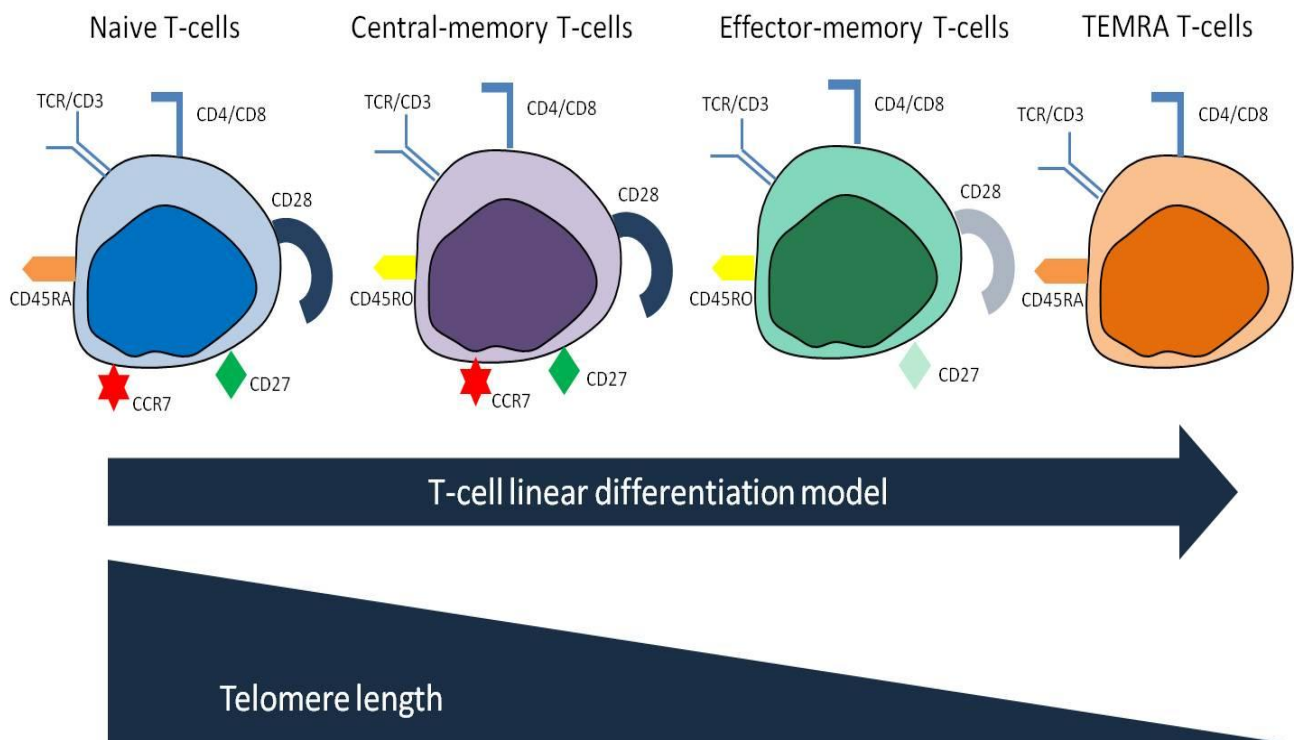


Figure 2.2. Phenotypic changes on T-cells during linear differentiation. Four distinct T-cell populations are defined according to the expression of CD27, CD28, CD45RA, CD45RO and CCR7. Relative telomere lengths indicating the cellular clonal expansion history are illustrated below each cell type according to current literature. Although the different T-cell subsets phenotypes are well defined, effector-memory T-cells are capable of expressing either CD27 or CD28 (Appay *et al.* 2008).

2.3. Immunosenescence

The term replicative senescence was first used by Hayflick and Moorhead (1961) to characterise the limited replication capacity of HDF *in vitro*. Indeed while trying to develop an immortal non-tumorigenic cell line in order to culture viruses and produce vaccines, they showed that after undergoing a number of replications, HDFs would enter into a state of permanent cell cycle arrest. The number of cell divisions necessary for a cell to enter a senescent state, varying between 40 to 50 generations, was described as a replication-threshold and coined the “Hayflick limit” (Hayflick & Moorhead 1961). In an attempt to link replicative senescence and its mechanisms to organism ageing, numerous researchers have studied the *in vitro* lifespan of multiple cell types (Cristofalo *et al.* 1994; Gardner & Mangel 1997; Pelicci 2004). Some of these studies have shown that immune cells, such as CD4+ and CD8+ T-cells, would also undergo cell cycle arrest after sustained and excessive rounds of replication when stimulated by specific mitogens or antigens *in vitro* (Grubeck-Loebenstein *et al.* 1994; Adibzadeh *et al.* 1995; Weng *et al.* 1995; Adibzadeh *et al.* 1996; Spaulding *et al.* 1999). Furthermore, Vaziri *et al.* (1998) have linked replicative senescence to telomeric DNA and telomerase activity, by showing that reconstitution of telomeric activity *in vivo* increased telomere length and more importantly cellular lifespan (Vaziri *et al.* 1997; Bodnar *et al.* 1998; Vaziri & Benchimol 1998).

2.3.1. Telomere-dependent senescence

2.3.1.1. Telomeres

Telomeres are non-coding nucleoprotein complexes composed of the highly conserved sequence TTAGGG and associated telomere binding proteins (Meyne *et al.* 1989). They are located at the chromosome ends and have an important secondary and tertiary structure which is believed to be as potent with regard to senescence as the telomere length itself (van Steensel *et al.* 1998). Their role is to protect the chromosome DNA from degradations potentially occurring at their extremities and facilitate chromosome replication by enhancing the chromosome binding to the nuclear envelope (Sharpless *et al.* 2004). They are shortened at each cell division and lose between 15 and 200 nucleotides, until they reach the Hayflick limit at an average length of 4 to 7 kb. A signal to the *p53* and/or *Rb* proto-oncogenes is then produced, which stops mitoses by expressing cyclin-dependent kinases inhibitors (CKIs) such as p21^{waf1}, p16^{INK4a} and p14/19^{ARF} (Collado & Serrano 2005). These CKIs are playing an anti-tumorigenic role by maintaining the retinoblastoma protein (pRb) hypophosphorylated, and thus inducing pRb binding to E2F to form the more stable complex pRb-E2F (Cao *et al.* 1992; Hagemeyer *et al.* 1993; Lees *et al.* 1993). The members of the E2F family are transcription factors targeting the principal genes involved in cell replication, such as cyclins, cyclin-dependent kinases, replication proteins etc. (He & Cress 2002; Bracken *et al.* 2004; Timmers *et al.* 2007), and their inactivation by pRb leads to a cell cycle arrest in G1 (Spaulding *et al.* 1999; Karlseder *et al.* 2002; Campisi 2005).

2.3.1.2. Oxidative stress

Telomeres are also shortened by direct physical telomeric single strand DNA damages caused by oxidative stress (Petersen *et al.* 1998). These alterations are independent to the telomeres size (von Zglinicki *et al.* 2000a; von Zglinicki 2002). Studies on telomeres have shown a possible structural cause of their premature shortening. Indeed, telomeric ends are composed of G-rich overhangs, the most oxidable nucleobase (Burney *et al.* 1999) produced by C-strand specific exonuclease which could contribute to telomere shortening (Makarov *et al.* 1997; McElligott & Wellinger 1997). Furthermore telomeric oxidative damages appears to be poorly repaired in comparison to similar chromosomal damages (von Zglinicki 2002) and researchers have managed to delay the onset of HDFs replicative senescence *in vitro* by reducing oxidative stress, using either oxygen depleted medium (Saito *et al.* 1995) or free radicals (von Zglinicki *et al.* 2000b). Interestingly increased levels of oxidative stress can also cause double strand breaks in chromosomal DNA, and thus triggering telomere-independent senescence (Chen *et al.* 2001; von Zglinicki *et al.* 2001).

2.3.1.3. Saturated fatty acid metabolism

When telomeres reach a critical size, a signal is transmitted to *p53* and *Rb*, leading to cell cycle arrest as described above. It is however important to note that *p53* also targets *fasn-1* and consequently inhibits the production of two crucial enzymes in the fatty acid metabolism, the fatty acid synthase and the stearyl-coenzyme A-desaturase 1 (D'Erchia *et al.* 2006). These two enzymes ensure the conversion of the saturated fatty acids palmitic and stearic acids to palmitoleic and oleic acid respectively (Nakamura & Nara

2004), limiting the accumulation of palmitic acid. High concentrations of palmitic acid, such as the one seen in obese patients, induce important mitochondrial and endoplasmic reticulum damages (Hickson-Bick *et al.* 2002) that can lead to cell cycle arrest and senescence (Chen *et al.* 2000). The important role played by stearoyl-coenzyme A-desaturase 1 in cell proliferation has been well documented, as reviewed by Igal (Igal 2010), and its inhibition could also help to trigger replicative senescence.

2.3.2. Telomere-independent senescence

Although T-cell senescence is believed to be primarily induced by telomere-dependent mechanisms, some studies suggest that some telomere independent-mechanisms may play an important role in immunosenescence. Indeed whereas telomere shortening occurs gradually with successive population doublings, some acute and chronic cell stressors may lead to premature senescence by up-regulating tumor-suppressor proteins with a 1-2 days kinetic, such as p16 and p21 constituting a rapid tumor-defence mechanism (Serrano 1997; Drayton *et al.* 2003).

2.3.2.1. Stress-induced senescence

Elegant *in vitro* studies have shown that diverse stressors such as intracellular oxygen species and oxidative stress (Chen & Ames 1994), exposure to high-doses of radiations (principally γ -ray) (Di Leonardo *et al.* 1994), inadequate cell culture conditions (Mathon *et al.* 2001; Shay & Wright 2001; Shay & Wright 2007) and dysregulated oncogenes (Herbig & Sedivy 2006) can lead to premature senescence before cells reach a critical telomere size. This state of premature senescence has been coined stress or

aberrant signalling-induced senescence (STASIS) or stress-induced premature senescence (SIPS) (Drayton & Peters 2002). Mechanistically, it has been shown that overexpression of the oncogenes *ras* (Hanahan & Weinberg 2000), with the concomitant reduction in *c-Myc* signalling (Guney *et al.* 2006), repeated exposure to H₂O₂ (Chen *et al.* 2001) and radiations (Suzuki *et al.* 2001) leads to the inhibition of the cyclin-dependent kinases 4 and 6 and the subsequent cell cycle arrest by the up-regulation of the CKI p16^{INK4a} (Serrano *et al.* 1997).

2.3.2.2 *Bim1*

T-cell mitogen and antigen-induced over-proliferation has also been proposed to be one cell stressor leading to premature senescence (Drayton *et al.* 2003). Heffner *et al.* (2007), has recently shown that the p53- and retinoblastoma-dependent pathway of cell cycle arrest may be activated by the telomere-independent down-regulation of Bmi1. Bmi1 is a transcription repressor, member of the Polycomb group, inhibiting the production of the p16^{INK4a} and p19^{ARF} tumour suppressor proteins, by repressing the transcription of the *Ink4a/Arf* locus. During T-cell activation, TCR ligation with the antigen induces Bim1 expression in naïve or non-senescent antigen-experienced cells; however Bim1 fails to be expressed in senescent T-cells. Heffner *et al.* (2007) advocate that this loss of the TCR-dependent expression of Bim1 could explain the elevated levels of p16^{INK4a} and p19^{ARF} observed in senescent cells.

2.3.3. Characteristics of senescent cells

Replicative senescence differs from other non-proliferative states like anergy or quiescence (Beverly *et al.* 1992) by irreversibly stopping their cell cycle between the non-division state (G0) and the phase preparing the DNA replication (G1) (Grubeck-Loebenstein *et al.* 1994; Weng *et al.* 1995; Adibzadeh *et al.* 1996; Levine *et al.* 1997). These senescent cells will stay in the G0/G1 phase disregarding stimulations with specific-antigens, pro-inflammatory cytokines or mitogens (Perillo *et al.* 1993). Weng *et al.* (1995) confirmed that the *in vitro* senescence model was an accurate representation of T-cells biological ageing, by measuring telomere length and proliferative capacities in an heterogeneous CD4+ T-cell population. This elegant study showed that naïve CD4+ T-cells had longer telomeres than the memory CD4+ T-cells. Furthermore the more differentiated T-cells had a reduced proliferative capacity compared to the naïve T-cells, when stimulated with antibodies anti-CD3 and anti-CD28 (Weng *et al.* 1995). Spaulding *et al.* (1999) suggested that senescent cells accumulate with age, after reporting senescent CD8+ T-cells with an increased resistance to apoptosis in comparison to cells with normal replicative capacities and a decreased activity of the enzyme caspase 3, an essential link in the extrinsic and intrinsic apoptosis pathways. Hsu *et al.* (2001) also reported a decreased expression on CD8+ T-cells of the upstream mediators Fas and Fas-ligand (FasL) of the extrinsic apoptotic pathway with ageing in mice. In elderly and nonagenarians, the same group reported an impaired apoptotic response in PHA-stimulated CD45RO+ memory T-cells and an increased proportion of CD28- CD95+ CD8+ T-cells, unable to up-regulate CD95L via CD28 stimulation, and consequently undergo Activation-Induced Cell Death (AICD) (Herndon *et al.* 1997).

Other mechanisms could also be responsible for the ability of senescent T-cell to escape apoptosis. Akbar *et al.* (1993) proposed that the senescent T-cell pool maintenance could be regulated by type-1 interferon (IFN), such as IFN- α , IFN- β or IFN- ω . Type-1 IFN have been shown to prevent apoptosis in activated T-cells while keeping them in a non-proliferative senescent state (Marrack *et al.* 1999), and although more research needs to be done to assess the impact of chronological and biological ageing on type-1 IFN concentrations, some encouraging results tend to support this hypothesis. Indeed cells from older mice have been shown to be more responsive to these interferons than cells isolated from younger mice (Bray 2001), and human dendritic cells isolated from the elderly appear to elicit an increased secretion of type-1 IFN in comparison to those isolated from younger donors (Agrawal *et al.* 2010). Furthermore IFN- β has been shown to down-regulate *c-myc* expression in fibroblasts, leading to growth inhibition and senescence (Jonak & Knight 1984; Dani *et al.* 1985; Jonak *et al.* 1987).

While this observation was made by several researchers (Spaulding *et al.* 1997; Posnett *et al.* 1999; Bryl *et al.* 2001a; Bryl *et al.* 2001b), some studies have also challenged it, by showing a higher propensity of senescent T-cells to undergo programmed cell death (Phelouzat *et al.* 1996; Phelouzat *et al.* 1997; Aggarwal *et al.* 1999; Dennett *et al.* 2002; Gupta 2002). When stimulated with TNF- α , CD4⁺ and CD8⁺ T-cells isolated from aged subjects appear to be more susceptible to undergo apoptosis than T-cells from younger donors (Aggarwal *et al.* 1999), and T-cells from aged subjects have a decreased expression of the anti-apoptotic gene Bcl-2 correlated with an increased expression of the pro-apoptotic gene Bax, compared to those from younger subjects (Aggarwal & Gupta 1998). Phelouzat *et al.* (1996) have made the same conclusion towards a higher sensitivity to apoptosis of senescent T-cells by demonstrating that high concentrations of PMA and ionomycin would lead to greater amounts of apoptotic cells in the elderly

than in young individuals. Consequently the disparities seen in senescent T-cells susceptibility to undergo apoptosis could be due to methodological differences, such as the use of different stimuli, experimental conditions or even phenotypes used to identify the different cell subsets.

Despite having an apparent increased resistance to apoptosis and an impaired capacity to divide, senescent T-cells maintain immediate effector-cell functions such as the recognition and killing of infected cells and the production of pro-inflammatory cytokines and other mediators. Indeed, Effros *et al.* (2003), by creating senescent T-cells *in vitro*, observed an increased production of the pro-inflammatory cytokines TNF- α , IFN- γ and IL-6. Moreover, the same features of senescent cells have been described *in vivo*, in older individuals (Effros 2003; Effros *et al.* 2003) and in centenarians (Vaziri *et al.* 1993). Recently, researchers have challenged the effector properties of some highly differentiated T-cells, classifying them as exhausted (Akbar & Henson 2011). They suggest that T-cell exhaustion occurs during high antigenic load and appears to primarily affect CD8⁺ T-cells. While exhausted T-cells share their phenotypic and proliferative characteristics with senescent T-cells, they lack T-cell effector functions and cytotoxic capacities (Akbar & Henson 2011).

Other interesting cellular characteristics of senescent T-cells include the accumulation of the protein p16^{INK4a} (Liu *et al.* 2009), the reduced production of stress proteins such as Hsp70 (Effros *et al.* 1994b), the presence of an unusual form of heterochromatin in discrete nuclear foci, called senescence-associated heterochromatic foci (SAHF) that appears to reinforce the development of immunosenescence (Narita *et al.* 2003) and the loss of telomerase (Plunkett *et al.* 2001; Valenzuela & Effros 2002). Activated T-cells

with intact proliferative capacities up-regulate telomerase to synthesise new telomere sequences (Broccoli *et al.* 1995; Hiyama *et al.* 1995) allowing them to clonally expand and establish an efficient immune response. Telomerase regulation has been shown to be linked to the co-stimulatory molecule CD28 (Shimizu *et al.* 1992; Weng *et al.* 1996; Valenzuela & Effros 2002; Akbar *et al.* 2004; Vallejo 2005), CD28 that has in turn been shown to be down-regulated on senescent T-cells following excessive rounds of replication both *in vitro* (Effros *et al.* 1994a) and *in vivo* (Ligthart *et al.* 1986; Azuma *et al.* 1993; Fiorentino *et al.* 1996; Weekes *et al.* 1999; Ouyang *et al.* 2003b; Klatt *et al.* 2005). Interestingly telomerase activity has been shown to differ between CD4⁺ and CD8⁺ T-cells. Indeed, CD8⁺ T-cells down-regulate telomerase much more rapidly than CD4⁺ T-cells, and when activated with identical stimuli CD4⁺ T-cells retain telomerase activity after 1.5 times more round of stimulations than CD8⁺ T-cells (Valenzuela & Effros 2002).

2.3.4. Senescent T-cell phenotype

The surface protein expression profile of senescent cells differs from phenotypic characteristics of normal cells. Effros *et al.* (1994; 1997) highlighted a significant decrease in the proportion of T-cells expressing the co-stimulatory glycoprotein CD28 in centenarians compared to younger donors. Thus, they have shown that, in senescent cultures, over 95% of T-cells lack CD28 expression (Effros *et al.* 1994a), due to the transcriptional silencing of the CD28 gene (Brzezinska *et al.* 2004). As a consequence of the important role in telomerase activity (Weng *et al.* 1996; Valenzuela & Effros 2002) and T-cell clonal expansion (June *et al.* 1990) played by CD28, CD28⁻ T-cells have impaired proliferative capacities (Azuma *et al.* 1993; Effros & Pawelec 1997;

Hamann *et al.* 1997) coupled with shortened telomeres (Effros *et al.* 1996; Monteiro *et al.* 1996). These characteristics have placed the lack of CD28 expression on T-cells as a useful senescent marker.

In correlation, Weekes *et al.* (1999) showed that another glycoprotein, CD57, which is not expressed on T-cells at birth, is up-regulated with repeated rounds of antigen-induced replications, while CD28 expression decreases (Wikby *et al.* 2002). The cell surface glycoprotein CD57, also known as Human Natural Killer-1 (HNK-1) or Leu-7 is expressed on NK cells and serves several functions such as cell recognition and adhesion by binding to laminin-1, -2 (Kizuka *et al.* 2008), L-selectin and P-selectin (Needham & Schnaar 1993). It has also been showed to be expressed on T-cells with shortened telomeres with an inability to proliferate (Brenchley *et al.* 2003). Furthermore CD57⁺ CD8⁺ T-cells have been shown to accumulate in the peripheral blood compartment with chronological ageing (Tarazona *et al.* 2000; Wikby *et al.* 2002; Brzezinska 2005). These observations made CD57 a marker of T-cell senescence, however a recent study from Chong *et al.* (2008), advocated the inaccuracy in senescent T-cell phenotyping when using CD57 alone as a marker, by showing that some CD8⁺/CD57⁺ T-cells were able to replicate and produce cytokines under the optimal conditions and stimulations. While some cases of T-cells expressing CD57 and lacking CD28 have been shown to proliferate in response to IL-2 and IL-15 stimulations (Yamada *et al.* 2007), they are generally believed to be irresponsive to cytokine stimulations (Brenchley *et al.* 2003).

Voehringer *et al.* (2001, 2002) demonstrated that a member of the Killer cell lectin-like receptors (KLRs) family, KLRG1, is expressed on CD8⁺ T-cells after repeated rounds

of stimulations and replications. Further research has also shown that KLRG1 is also expressed on mature and recently activated NK cells as well as CD4⁺ T-cells (Robbins *et al.* 2004). KLRG1 is a highly conserved trans-membrane C-type lectin inhibitory receptor with an extra-cellular part binding to conserved domains on N-, E- and R-cadherins in human and mice (Grundemann *et al.* 2006; Ito *et al.* 2006; Banh *et al.* 2009; Li *et al.* 2009c) and a cytoplasmic domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) responsible for the transmission of an inhibitory signal after ligation (Robbins *et al.* 2002). More specifically, KLRG1 ligation to the different ubiquitously expressed cadherins, leads to the recruitment of the phosphatase SHIP-1 and SHIP-2 that in turn will degrade the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in phosphatidylinositol (4,5)-bisphosphate (PIP2) and regulate phosphoinositide 3 kinase (PI3K) activity (Tessmer *et al.* 2007). PI3K plays an essential role in T-cell proliferation and survival, indeed by PIP3 phosphorylation, it regulates the recruitment of the phosphoinositide-dependent kinase 1 (PDK-1) and the mammalian target of rapamycin complex 2 (mTORC2) (or PDK-2) (Fresno Vara *et al.* 2004). These two enzyme will regulate the protein kinase b (Akt) activation by phosphorylating two specific sites, the Threonin 308 (Thr³⁰⁸) and Serine 473 (Ser⁴⁷³) respectively (Manning & Cantley 2007). Finally, the phosphorylated Akt will trigger cell division by inducing cyclin D1 expression (Ouyang *et al.* 2008) and p21/p27 phosphorylation (Liang *et al.* 2002; Zhou & Hung 2002).

As a consequence of KLRG1 specificity to the three forms of cadherins (Ito *et al.* 2006), it was hypothesised that KLRG1⁺ NK cells were playing a primordial role in immune surveillance and more specifically in the prevention of tumour development (Schwartzkopff *et al.* 2007). Indeed N-, E- and R-cadherins are respectively expressed on wild neurons, epithelial and retinal cells, but appears to be down-regulated or

mutated on tumour cells (Schwartzkopff *et al.* 2007). Consequently KLRG1 ligation to cadherins would inhibit NK cells cytotoxicity and protect healthy tissues while allowing clearance of cancer cells. Interestingly, KLRG1+ T-cells present alterations in their proliferative capacities, while keeping their ability to eliminate the antigen and produce cytokines such as IFN- γ (Voehringer *et al.* 2002). Repeated T-cells activation and clonal expansion induced by exposure to antigen or latent viruses (Thimme *et al.* 2005) lead to an accumulation of KLRG1+ dysfunctional T-cells in the elderly (Ouyang *et al.* 2003b). An elegant study conducted by Henson *et al.* (2009) on senescent CD8+ T-cells shed lights on the mechanistical role of KLRG1 in T-cell senescence, by showing that KLRG1 blocked Akt phosphorylation at the Ser⁴⁷³ level, inhibiting cell proliferation. Furthermore, Akt (ser⁴⁷³) phopshorylation being involved in hTERT phophorylation and the consequent telomerase up-regulation (Kang *et al.* 1999), KLRG1 prevents Akt (ser⁴⁷³) phopshorylation (Henson *et al.* 2009) may be a mechanism by which telomerase activity is greatly decreased in senescent T-cells (Plunkett *et al.* 2007). However some studies showing that KLRG1 blockade induced Akt (ser⁴⁷³) phosphorylation without inducing telomerase up-regulation (Henson *et al.* 2009), suggest that other pathways, such as the ERK pathway can play a role in hTERT phosphorylation and enhanced telomerase activity (Fauce *et al.* 2008).

Until recently, KLRG1 was thought to be the ideal senescent T-cell marker, however studies conducted by Beyersdorf *et al.* (2007) identified CD4+/KLRG1+ cells as regulatory T-cells able to proliferate. These primordial cells are insuring the immune system integrity by down-regulating the immune response, to avoid the development of autoimmune diseases. Furthermore some antigenic stimuli have been shown to up-regulate nuclear markers of proliferative ability, such as Ki67, in CD8+ T-cells expressing KLRG1 (Ibegbu *et al.* 2005), challenging their nature as senescent T-cells.

Consequently KLRG1 on its own cannot be considered as a marker of immune senescence on its own, but needs to be correlated with the down-regulation of CD28 and/or the up-regulation of CD57 (Ibegbu *et al.* 2005; Cosgrove C. 2007; Simpson *et al.* 2007a). It is also interesting to observe that CD57 appears to be up-regulated prior to CD28 down-regulation (Brenchley *et al.* 2003). Due to the lack of mitogenic-stimulation induced proliferation of some CD28⁺ T-cells, the combination of KLRG1 and CD57 coexpression is regarded as accurate phenotypic markers of senescent T-cells (Voehringer *et al.* 2002; Brenchley *et al.* 2003; Ibegbu *et al.* 2005).

Expressions of other cell surface glycoproteins have also been shown to be dysregulated in senescent T-cells. In addition to CD28 and CCR7 down-regulation, studies have highlighted the loss of the constitutive CD27 (Akbar *et al.* 1988; Hendriks *et al.* 2000) and the inducible T-cell co-stimulator ICOS (Larbi *et al.* 2008) in senescent T-cells. Furthermore other cell surface proteins such as the inhibitory killer immunoglobulin-like receptor KIR (Li *et al.* 2009a), the NK cell receptors KLRD1, KLRF1 and CD244 (Phillips *et al.* 1995), CDw60 (Larbi *et al.* 2008), CD85j (Czesnikiewicz-Guzik *et al.* 2008), NKG2D (Alonso-Arias *et al.* 2011), PD-1 (Appay *et al.* 2008) and the integrin alpha-subunit CD49d (Peres *et al.* 2003) are also up-regulated during T-cell senescence. Finally many researchers have identified an enzyme, the senescence-associated β -galactosidase (SA- β -galactosidase), to be found exclusively in human senescent cells (Reznikoff *et al.* 1996; Serrano 1997; van der Loo *et al.* 1998; Mishima *et al.* 1999) making its presence in the cells cytoplasm an indicator of replicative capacity. However a recent study from Kurz *et al.* (2000), have shown that an increase in SA- β -galactosidase in T-cells was due to an increase in lysosomal content, which although has been correlated to replicative senescence (Robbins *et al.* 1970), it cannot be considered as a good marker on its own.

2.4. The Immune Risk Profile

2.4.1. Immunosenescence and the IRP

Immunosenescence is the term used to describe the biological ageing of the immune system and has been linked with cardiovascular disease, myocardial infarction, obesity, various autoimmune disorders and cancer and is believed to directly or indirectly contribute to mortality in later life (Cawthon *et al.* 2003; Valdes *et al.* 2005; Starr *et al.* 2007). Although immunosenescence has an effect on almost all the components of the immune system, the adaptive immunity dysfunction (i.e. T-cells and B-cells) is thought to create the greatest health problems in the elderly (Pawelec *et al.* 2006). To predict morbidity and mortality in older adults, immunosenescence and several other immunological characteristics have been grouped under the IRP (Koch *et al.* 2006). The IRP emerged as a concept in the late 1990's. By following over 200 Swedish patients for more than a decade, the OCTO and NONA longitudinal studies identified the IRP as a collection of immune parameters which were strongly correlated to morbidity and mortality (Wikby *et al.* 1998; Wikby A. 2003). Strindhall *et al.* (2007) also showed that if the IRP is a good predictor of mortality, the rate of survival of centenarians is increased when in the normal immune profile (NIP).

The immune characteristics regarded as being part of the IRP are an inverted CD4+/CD8+ blood T-cell ratio, a reduced naïve T-cell repertoire and a poor blood T-cell response to mitogens and cognate antigens (Pawelec *et al.* 2006). These changes are linked to changes in T-cell surface protein expression such as the co-expression of CD57 and KLRG1 and the loss of the co-stimulatory molecules CD27 and CD28. The IRP also includes the presence of antibodies against the latent herpesvirus CMV and

EBV in the serum (Pawelec *et al.* 2006). A comprehensive overview of the IRP biomarkers is shown in the Table 2.1.

Table 2.1. Biomarkers associated with a Normal Immune Profile and with the IRP category in the elderly (Simpson 2011).

Biomarkers of a Normal Immune profile	Biomarkers of the IRP Category
<p>T-cell markers and Function: CD4:CD8 ratio > 1 ↑ T-cell proliferation <i>in vitro</i> ↑ CD27, CD28, CD45RA, CCR7 expression ↓ KLRG1, CD57, CD45RO expression ↑ Leukocyte Mean Telomere Length ↑ Telomerase activity</p>	<p>T-cell markers and Function: CD4:CD8 ratio < 1 ↓ T-cell proliferation <i>in vitro</i> ↓ CD27, CD28, CD45RA, CCR7 expression ↑ KLRG1, CD57, CD45RO expression ↓ Leukocyte Mean Telomere Length ↓ Telomerase activity</p>
<p>Latent virus Infection: CMV seronegativity EBV seronegativity</p>	<p>Latent virus Infection: CMV seropositivity EBV seropositivity ↑ CMV-specific T-cells ↑ EBV-specific T-cells</p>
<p>Cytokine Signalling: ↑ IL-2 secretion ↑ IL-2R expression</p>	<p>Cytokine Signalling: ↓ IL-2 secretion ↓ IL-2R expression</p>
<p>Low "Immune Space": ↑ Naive T-cell repertoire ↑ T-cell polyfunctionality → Apoptosis</p>	<p>Low "Immune Space": ↓ Naive T-cell repertoire ↑ T-cell oligoclonality ↓ Apoptosis</p>
Prolonged Health and Survival	Increased Morbidity and Mortality

2.4.2. Latent herpesvirus infection and IRP

Although the frequency of senescent T-cells increases with age, the accumulation rate of senescent cells is dramatically amplified due to viral challenges, such as frequent reactivations of latent CMV and EBV infection. Indeed persistent stimulation by the chronic viruses CMV and EBV have been shown to lead to enhanced expression of KLRG1 on the cell surface of virus specific CD8+ T-cells in mice and humans

(Thimme *et al.* 2005). CMV and EBV seropositivity is strongly correlated to immunosenescence and the associated "Immune Risk Profile" (Koch *et al.* 2006; Koch *et al.* 2007), which has, in turn, been shown to predict mortality in later life (Pawelec & Gouttefangeas 2006).

The IRP, amongst other immunological parameters, includes the presence of antibodies against the latent herpesvirus CMV and EBV in the serum (Pawelec *et al.* 2006). Although CMV infection is clinically asymptomatic in the immunocompetent host, the virus persists in cells of the myeloid lineage (Koch *et al.* 2006) and prevents them to undergo apoptosis by modifying the PI(3)K-Akt pathway (Johnson *et al.* 2001; Cooray 2004). As complete clearance of the virus is never achieved, CMV and EBV will be periodically reactivated in response to physical or psychological stress (Almanzar *et al.* 2005; Senchina & Kohut 2007) inducing the clonal expansion of virus-specific CD8+ T-cell clone and thus contributing to immune exhaustion (Koch *et al.* 2006). Numerous studies have supported this hypothesis by analyzing T-cells from older adults. It has been shown that CMV-specific CD8+ T-cells are mostly expressing KLRG1 while lacking CD28 expression (Ouyang *et al.* 2004; Thimme *et al.* 2005), whereas CMV-specific CD8+T-cells from younger individuals presented the opposite phenotype (Jackson *et al.* 1995). These results and the direct correlation between the proportion of CMV-specific CD8+ T-cells and the survival rate in elderly subjects (Koch *et al.* 2006) make CMV and EBV infections potential antecedents in early commencement of the IRP. While the IRP is an interesting collection of immune characteristics allowing an accurate assessment of the immune system in elderly, it appears even more interesting to understand all the mechanism responsible for entering this IRP. In addition to latent viral infections, markers of biological ageing (i.e. telomere length and p16^{INK4a} expression) have been linked to low socioeconomic status (Cherkas *et al.* 2006) and

increased proportions of effector-memory and senescent T-cells have been found in blood due to psychological stress, adverse working conditions (Bosch *et al.* 2009). Interestingly recent studies have linked certain lifestyle and occupational factors, such as cigarette smoking, positive energy balance, adverse working conditions, and low exercise levels with classic biomarkers of ageing such as blood leukocyte telomere shortening and p16^{INK4a} expression (Valdes *et al.* 2005; Cherkas *et al.* 2008; Song *et al.* 2010). These recent studies indicate that a sedentary lifestyle, in particular, promotes biological ageing and telomere shortening (Cherkas *et al.* 2008; Ludlow *et al.* 2008; LaRocca *et al.* 2010; Song *et al.* 2010), which is likely to result in early transition to the IRP category and premature mortality with advancing age.

2.5. Physical activity

2.5.1. Benefits of physical activity

Scientific studies on the relationship between physical activity and health conditions have been conducted for over fifty years. In the early 1950s, a study comparing 30,000 bus drivers (with a low level of physical activity) to 20,000 controllers (potentially tive) revealed that the controllers were less likely to develop cardiovascular diseases than their colleagues, drivers (Morris *et al.* 1953; Morris 1956). Since the late 1980s, researche has been conducted on the benefits of regular physical activity, reinforcing the relationship between physical activities and the reduction of premature mortality irrespective of cause (Siscovick *et al.* 1984a; Siscovick *et al.* 1984b; Barengo *et al.* 2004; Schnohr 2006; Schnohr *et al.* 2006).

Bouchard proposed in 1997 a mechanism highlighting the beneficial effects of moderate regular physical activity on cardiovascular disease (CVD) risks (Bouchard 1997). According to this study, regular sessions of moderate exercise induce a decrease in the total body fat mass and in the abdominal adipose tissue. This has a direct effect on the increase of insulin sensitivity and the expression of muscle lipoprotein lipase (LPL) decreasing the release of hepatic triglyceride lipase (TGL). This action on the enzymatic system will trigger lipoprotein catabolism and lipid oxidation and consequently decrease the plasma level of triglycerides and cholesterol.

Another mechanism by which physical activity reduces CVD risk is mediated by the reduction of blood pressure by weakening the adrenergic sympathetic activity and the peripheral resistance and inducing changes in the rennin-angiotensin-aldosterone system (Whelton *et al.* 2002).

By increasing insulin sensitivity, physical activity has also been shown to lower the risk of developing non-insulin-dependent diabetes mellitus (NIDDM) by 20-60% (Helmrich *et al.* 1991; Helmrich *et al.* 1994), with its maximum effect in populations presenting high risks of NIDDM (Manson *et al.* 1992). The need for insulin is being reduced by an augmentation in glucose-transportation protein (GLUT4), and an up-regulation of glycogen synthase leading to reinforcement of glucose storage (Hu *et al.* 1999; Booth *et al.* 2000). If these changes help to prevent the development of NIDDM in healthy subjects, they also reduce the symptoms of patients with NIDDM (Wallberg-Henriksson *et al.* 1998).

A strong correlation between physical activity and lower risk of colon cancer has been highlighted by numerous researchers (Thune & Lund 1996; Martinez *et al.* 1997; Slattery *et al.* 1997; Warburton *et al.* 2006). This can be explained by characteristics of exercise in altering prostaglandin synthesis and increasing intestinal mobility which reduces exposure time between the colon mucosa and potential carcinogens.

2.5.2. Role of regular exercise in weight regulation

Physical activity plays an essential role in weight regulation, as it consumes the excess of energy coming from food intake. Exercise on its own is not responsible for weight loss (Booth *et al.* 2000), however weight control is dependent to regular physical activity, and consequently, cannot be achieved without it (Byers 1995). Physical activity can regulate weight by creating and maintaining lean body mass, in particular in seniors, to avoid muscle loss or atrophy. It also regulates body weight by increasing total energy expenditure, eventually leading to the utilization of stored lipids contained in fat mass.

Lack of physical activity can create an unbalanced state of energy excess leading to obesity.

2.5.3. Obesity

Obesity can be defined as an excessive accumulation of adipose tissue with harmful consequences on health. The weight of an individual can be considered as a physiological regulated variable. An imbalance in this regulation with a reduction of the energetic expenditure, leads to an accumulation of energetic reserves and to obesity. The global measure of obesity which is the most commonly used is the body mass index (BMI) defined as: $BMI = \text{weight (kg)}/\text{height}^2 \text{ (m)}$ (Garrow 1985; World Health Organization 1995). This index, which is size-independent in human populations, is well correlated with fat mass.

The international definition of obesity recommended by the World Health organisation in 1997 and adopted in the United Kingdom is based on the relation between this index and mortality (Allison *et al.* 1997). In Caucasian populations, this relationship follows a U-shaped curve. The lowest risks are recorded for BMI between 18.5 and 25 kg/m². For high values, the risk starts to rise above 25 kg/m² and compared with the subjects between 18.5 and 25 kg/m², the relative risk exceeds 1.5 beyond 30 kg/m². The BMI, simple index which only needs the measurements of weight and size, is none-the-less an imperfect approximation of the body fat percentage in the body. While, when the index exceeds 30 kg/m², it generally indicates a significant increase in adipose tissue, in more normal values, this same index is dependent on lean mass. A simple illustration of this is the difference between men and women (Micozzi *et al.* 1986). Indeed Ley *et al.* (1992) showed that for a same BMI of 23 kg/m², the percentages of body fat through 20

year old women are doubled compared to 20 year old men, with respectively 26% and 13%. This tends to indicate that the more developed lean mass in men plays a great role in the BMI. However, the use of a common definition of obesity among men and women remains a requirement as the health-related consequences of excess body fat are less dramatic in women than men. Indeed, a second important parameter to take into consideration is the relationship between adiposity and mortality is body fat distribution. Since the first publications in the 1980s, many epidemiological studies have confirmed that various measures reflecting an overload abdominal fat (waist/hips ratio, waist, anterior-posterior diameter of the abdomen or sagittal diameter) were high risk factors of mortality from any cause and especially CVD (Ohlson *et al.* 1985). These indexes have a linear effect on mortality (Lapidus *et al.* 1984; Larsson *et al.* 1984; Welborn *et al.* 2003). In men, the excess of body fat is preferentially localised in the abdominal level, which probably explains why it is not well tolerated, on the metabolic and cardiovascular view, compared to the excess of adipose tissue in women. There is no consensual definition of the excess of abdominal adipose tissue, or agreement on its measure. However, from a Scottish adult population, thresholds of 94 waist/hips ratio (WHR1) and 102 (WHR2) cm for men and 80 (WHR1) and 88 (WHR2) cm for women were proposed to identify patients requiring the prevention of weight gain (WHR1) or weight loss (WHR2) (Lean *et al.* 1995). The waist/hips ratio which was first used tends to be replaced only by the waist measurement.

Obesity is now described as a worldwide epidemic by the World Health Organisation (World Health Organization 2000). In the USA, the number of adults with a BMI over 30 kg/m^2 rose from 14.25% in 1971 to over 31% in 2000 and in the UK, adult obesity rates rose from 6% of men and 8% of women in 1980, to 21% of men and 23.5% of women in 2000 (Mokdad *et al.* 2001; Deitel 2003). Furthermore, childhood obesity has

been a growing sanitary issue for the past 25 years, with the proportions of obese adolescents rising from an average of 5% of the 12-19 years old who were obese in 1980 to 18% in 2008 (Skelton *et al.* 2009). The frequency of 12-19 years old with an excess body mass reaches a third of the adolescent populations in the United States (Ogden *et al.* 2010). Developing countries are also affected by obesity as a result of changes in traditions, alimentation and physical activity. The increases in overweight and obesity have been linked to increases in chronic conditions including diabetes, hypertension, hypercholesterolemia, stroke, heart disease, certain cancers, and arthritis (Johnson *et al.* 1993; Burt *et al.* 1995; Allison *et al.* 1997; World Health Organization 1997; Harris *et al.* 1998). Although some of those conditions are only affecting the adult overweight and obese population, the emergence of some obese-related illness such as type 2 diabetes mellitus (American Diabetes Association 2000) and metabolic syndrome (Cook *et al.* 2003) in obese teenagers is considered as very alarming. Physical activity has been shown to reduce the risks of developing these chronic conditions in obese patients (Wei *et al.* 1999; Barengo *et al.* 2004). Consequently it is believed that obesity-related diseases may also be caused by a lack of physical activity.

2.5.3.1. Leptin: Linking feeding behaviour and the immune system

As a consequence of their pleiotropy, cytokines are known to affect more systems than the immune system alone, such as the reproductive system (Simon & Polan 1994) or even the digestive system. Indeed various cytokines are involved in the control of feeding behaviour by regulating the frequency, magnitude and duration of each food intake episode. IL-1 α , IL-1 β , IL-6 subfamily, IL-8, TNF- α , IFN- α and the adipokine leptin are believed to restrict food intake by directly acting on neuronal ion channels of various hypothalamic regions (Plata-Salaman 1991a; Plata-Salaman 1991b; Plata-

Salaman & Borkoski 1994; Plata-Salaman & French-Mullen 1994; Plata-Salaman 1996; Plata-Salaman 1998).

Hypotheses about weight and appetite regulation have been emitted for over 50 years. Ingalls *et al.* (1950) while looking for obesity treatment, created the first animal model allowing accurate studies of obesity by isolating a C57BL/6J obese mouse with an *ob/ob* phenotype. However, it is only in 1953 that Kennedy proposed the existence of a factor produced in the adipose tissue and targeting the brain to induce satiety (Kennedy 1953). This theory called lipostatic theory has been supported by a study where normal rats became hyperphagic and obese after electrically-inducing hypothalamic lesions (Hervey 1959). Another study, using genetically obese mice, highlighted the existence of a blood-borne factor in normal mice which induces a reduction of food intake (Hausberger 1959). It was only in 1994 that this blood-borne circulating factor was identified after isolation of the *ob* gene (Zhang *et al.* 1994). *Ob/ob* mice significantly lost weight after injection with the protein coded by the *ob* gene (Halaas *et al.* 1995; Pelleymounter *et al.* 1995; Rentsch *et al.* 1995).

The soluble protein, named Leptin, after the Greek *leptos* meaning lean, is a 16 kDa protein composed of 146 amino acids transcribed from the *ob* gene in 3.5 kb mRNA in the adipocytes. Leptin's three-dimensional structure is composed by four α -helix bundle motif, also found in the IL-6 superfamily (Zhang *et al.* 1997). Leptin is released in the bloodstream on a circadian rhythm, with a peak observed between 20 minutes and 4 hours, and reaches physiological concentrations ranging from 1 to 5 ng.mL⁻¹ in healthy lean subjects, but can attain 50 ng.mL⁻¹ in subjects with class IV obesity ("super-obesity") (Prolo *et al.* 1998), well above the blood-brain barrier threshold for the

adipokine ($15\text{ng}\cdot\text{mL}^{-1}$) (Wang *et al.* 1999). Various factors influence leptin secretion and serum concentration such as food intake and insulin concentration. An increase in those factors will induce an increase in leptin secretion, and thus in serum leptin concentration. This effect will be reversed as soon as there is a reduction in food intake or insulin concentration (Kolaczynski *et al.* 1996). Leptin concentration is also known to increase in response to pro-inflammatory molecules such as TNF- α (Gualillo *et al.* 2000). Interestingly sexual hormones have an opposite action on leptin secretion as testosterone inhibits leptin production while estrogens stimulate its release in the bloodstream (Blum *et al.* 1997; Castracane *et al.* 1998). Once in the bloodstream, leptin crosses the blood-brain barrier to bind to its receptor on the hypothalamic nuclei where it regulates food intake by inducing the expression of anorexigenic factors, inhibiting neuropeptide Y (NPY), orexin and agouti-related peptide (AGRP) neuronal activity (Stephens *et al.* 1995; Korner *et al.* 2001; Breen *et al.* 2005). It has also been shown that leptin will down-regulate level of NPY mRNA (Tartaglia 1997). When NPY is being released in the hypothalamus, it stimulates food intake, decrease thermogenesis, enhance insulin and cortisol production via the β_3 adrenergic receptors of the sympathetic nervous system (Morley *et al.* 1987). Consequently when leptin binds to its receptor on the hypothalamus, it induces a decrease in food intake, coupled with a reduction in insulin and cortisol production.

As described above, the leptin molecule presents structural similarities with the type I cytokine family (Matarese *et al.* 2002). Furthermore, leptin receptor (ObR) also belongs to the type I cytokines family receptors (Baumann *et al.* 1996; Tartaglia 1997) due to its similarities with the gp130 signal transduction arm of class I cytokine receptor family member, including interleukins (IL-6), granulocyte colony stimulating factor (G-CSF)

and leukaemia inhibitory factor (LIF) (Tartaglia 1997). These structural relative conformities, conjugated with studies showing that subjects with a leptin deficiency due to malnutrition or genetic disorders were more susceptible to infections and had an altered immune system (Chandra 1980; Ozata *et al.* 1999), suggested an important role for leptin in immunity. This altered immune response during long term fasting could be explained by the need for the body to store energy for more important functions, such as cardiovascular and cerebral functions, but it could also be explained by the potential role played by leptin in lymphopoiesis (Bennett *et al.* 1996).

Many studies have highlighted the impact of leptin on both the innate and adaptive arm of the immune response. Indeed serum leptin activates and induce macrophage and monocyte proliferation by triggering leukotriene B₄, phospholipase A₂ (Mancuso *et al.* 2004) and pro-inflammatory cytokines production (Gainsford *et al.* 1996; Loffreda *et al.* 1998) which will enhance phagocytic function (Mancuso *et al.* 2002). Leptin also up-regulates CD38, the transferrin receptor (CD71), the α -chain of the IL-2 receptor (CD25) and adhesion molecules, such as the integrins CD18, CD11b and CD11c on monocytes and macrophages (Santos-Alvarez *et al.* 1999; Lord 2006). Finally, leptin prevents monocyte undergoing apoptosis, via MAPK activations (Lin *et al.* 2002) and enhances the differentiation, activation, proliferation and cytotoxicity of NK cells (Zhao *et al.* 2003). Contrasting with these results, many studies have shown leptin to have anti-inflammatory properties by reducing pro-inflammatory cytokines during sepsis in primates (Xiao *et al.* 2003) and increasing anti-inflammatory cytokines production, such as IL-4 (Jaworek *et al.* 2002) and IL-1 receptor antagonist (IL1RA) (Gabay *et al.* 2001). Consequently leptin should be regarded as an immunomodulatory adipokine.

As stated above, leptin also effects the adaptive immune system. Similarly to its effect on monocytes and macrophages, leptin up-regulates the expression of CD71, CD25, CD69 on CD4⁺ and CD8⁺ T-cells (Martin-Romero *et al.* 2000) and adhesion molecules such as ICAM-1, and very late antigen 2 (VLA-2) on CD4⁺ T-cells (Park *et al.* 2001). It also appears to induce CD45RA⁺ ('naïve') T-cell proliferation via STAT3 activation (Akaishi *et al.* 1998; De Rosa *et al.* 2007) when cultured in presence of a mitogen, but it tends to inhibit CD45RO⁺ ('memory') T-cell proliferation (Lord *et al.* 2002) while eliciting them to shift to a more T_{h1} cytokine-profile response (Lord *et al.* 1998; Mito *et al.* 2004; Goldberg *et al.* 2005; Lord 2006). This shift to a T_{h1} profile has multiple consequences, such as a reduction of anti-inflammatory cytokine secretion, such as IL-4, while increasing TNF- α and IFN- γ production, but it also promotes IgG2a production by B-cells while inhibiting IgG1 formation (Faggioni *et al.* 2001). Furthermore, observations on thymic atrophy in *ob/ob* mice and on diminished thymic function during acute caloric restriction are suggesting an action of leptin in thymic output and naïve T-cells formation (Lord *et al.* 1998). This is supported by studies showing that thymic output can be restored in cases of malnutrition by leptin administration (Howard *et al.* 1999; Lord *et al.* 2001). Finally leptin increases anti-apoptotic proteins such as Bcl-2, Bcl-X_L (Fujita *et al.* 2002) and T-bet (Lord *et al.* 2002), protecting thymocytes and mature T-cells from undergoing apoptosis.

Interestingly Valdes *et al.* (2005) has shown an inverse correlation between telomere length, BMI and serum leptin concentration. This could be explained by an increased proliferation rate of naïve T-cells in the presence of leptin, coupled with a Th-1 cytokine-profile response and a reduced clearance of redundant memory cells via apoptosis, potentially highlighting leptin as the central actor of premature senescent in

obesity. This however remains hypothetical and as very few *in vivo* studies have been conducted and most of the *in vitro* studies founding an effect of leptin on immune cells have used supra-physiological doses of leptin (Martin-Romero *et al.* 2000), chronic exposure of this adipokine in cases of obesity and severe obesity remains to be tested. High serum leptin concentrations alone cannot however explain the state of immune depression seen in obese patients.

2.5.3.2. *Impact of obesity on the immune system*

The highly deleterious effect of excess weight and obesity on the cardiovascular system has been of interest for many years. However the potential link between obesity and immunosuppression has only been proposed recently. Obesity increases the risk for cardiovascular disease (Larsson *et al.* 1984), that are in turn correlated with shortened leukocyte mean telomere length. Indeed Brouillette *et al.* have repeatedly demonstrated that healthy subjects retain longer leukocytes mean telomere length than subjects with premature myocardial infarction (Brouillette *et al.* 2003; Brouillette *et al.* 2007; Brouillette *et al.* 2008). This observation was supported by a study conducted on the Scottish elderly, where participants who suffered chronic heart diseases had shorter leukocyte mean telomere length than healthy age-matched subjects (Starr *et al.* 2007; Starr *et al.* 2008). The hypothesis that obesity has a direct or indirect link with immunosuppression was further advocated by researchers when an increased BMI was shown to be combined with a reduced leukocyte mean telomere length in women (Valdes *et al.* 2005), with reduced response to tetanus vaccination in children (Eliakim *et al.* 2006) and with an increased risk of bacteremia infections following surgery (Munoz *et al.* 2004).

The mechanisms linking obesity and immunosuppression are still unclear and require to be investigated thoroughly. It is reasonable to believe that the high levels of oxidative stress (Furukawa *et al.* 2004) and the elevated pro-inflammatory cytokines secreted by the excess of adipocytes (Dandona *et al.* 2004) observed in obese individuals increases the risk of single strand breaks in telomeric DNA and thus accelerate telomere shortening and the onset of replicative senescence (von Zglinicki *et al.* 2000a; von Zglinicki 2002). Finally as described previously, fatty acid metabolism can also play a role in the onset of replicative senescence and calorie restriction is known for restoring a robust cellular proliferative response that was reduced during obesity (Apte *et al.* 2002). More research on the impact of fatty acid metabolism on immunosenescence are required before proposing that a diet-related elevated serum concentration of fatty acid coupled with chronological ageing could accelerate immunosenescence.

2.5.4. Exercise immunology

Regular physical activity has been shown to prevent numerous health complications in the elderly, such as cardiovascular events (Goldstein 2010), certain cancers (Shepard 1990), bone loss (Cousins *et al.* 2010), muscle atrophy (Kuh *et al.* 2005) and depression (Ruuskanen & Ruoppila 1995; Hassmen *et al.* 2000). Furthermore, the beneficial impact of regular physical activity on body weight is widely accepted. Indeed, regular physical activity and a higher fitness level are associated with both reduced obesity and obesity-related diseases (Martinez-Gonzalez *et al.* 1999; Mortensen *et al.* 2006; Chaput & Tremblay 2009). Although relatively few studies have examined the link between regular exercise and the immune system as a function of age (Nieman *et al.* 1994; Nieman *et al.* 1995; Nieman 1997; Bruunsgaard *et al.* 1999; Buyukyazi 2004;

Buyukyazi *et al.* 2004; Drela *et al.* 2004; Simpson & Guy 2010), regular physical activity helps to maintain a healthy lifestyle in the elderly by reducing risks of depression (Strawbridge *et al.* 2002) and by reducing both infection risks and infection severity among older adults (Leveille *et al.* 2000).

2.5.4.1 T-cell Responses to Moderate Exercise

As mentioned above, the beneficial effect of regular moderate physical activity, such as bi-weekly recreational and non-exhaustive exercise, has been shown to be especially marked in older adults (Bruunsgaard *et al.* 1999; Buyukyazi 2004). Kohut *et al.* (2002) recently highlighted the favorable effect of vigorous exercise on the immune response of seniors to the influenza vaccine. Moderate physical activity will have a different effect on the immune system compared to vigorous exercise. Indeed, in healthy young adults, moderate physical activity will direct the immune response into a T_{h1}-type cytokine response (IL-2, IL-12) and an intense physical activity will lead to an indirect T_{h2}-type cytokine response, by down-regulating the T_{h1}-type cytokine response. (Ostrowski *et al.* 1999; Suzuki *et al.* 2002; Suzuki *et al.* 2003). However, animal and human studies have shown that the elderly will respond differently to exercise than younger individuals. Older adults preferentially elicit a T_{h2}-type cytokine response when subjected to moderate exercise (Shearer 1997; Kohut *et al.* 2001) and should consequently have a more efficient humoral immune response than their younger counterparts. The beneficial effects of moderate physical activity on health was further suggested by Nieman *et al.* (1993), who reported a negative correlation between high levels of physical activity and self-reported scores on symptoms of upper-respiratory tract infections. More recently, Ludlow *et al.* (2008) proposed another mechanism

explaining the positive effect of moderate regular exercise on infection in older adults, by finding that leukocyte telomere lengths were longer in physically active seniors compared to inactive seniors, indicating a lower frequency of senescent T-cells in physically active seniors compared to their inactive counterparts. These findings were supported by a study conducted in 2008 by Cherkas *et al.* where a strong correlation has been observed between shortened telomeres in mixed blood leukocytes and a sedentary lifestyle.

2.5.4.2. *T-cell Responses to Acute Exercise*

Regular or chronic physical activity is often opposed to acute exercise as a consequence of the apparent dissimilarities between them. Indeed while regular physical activity appears to have beneficial effect on the immune system, a growing number of studies have highlighted the potential detrimental effects of frequent bouts of acute high-intensity exercise on the immune system (Nieman D.C. 1990; Nieman 1997). Although it was hypothesised that the beneficial impact of regular physical activity on the immune system could be due to the periodic repetition of acute bouts of exercise performed over an extended period of time (Simpson 2011), it remains to be tested.

Individuals frequently exercising at high intensity such as elite athletes, present a high prevalence of symptoms of upper respiratory tract infections (URTIs) before and after competition potentially linked to the increase in training volume and intensity (Nieman D.C. 1990; Nieman 1997). Similar effects have been observed in animal models, where mice appear to be at a greater risk of infection to viruses after acute exercise, compared to non-exercising control (Murphy *et al.* 2008). The mechanisms underlying this apparent state of immune dysfunction after acute bouts of exercise have been widely

investigated and changes in T-cell numbers and functions have been highlighted. Buyukyazi (2004) observed a reduction in the proportion of CD4⁺ T-cells in athletes with an increasing estimated $\dot{V}O_{2max}$. This is consistent with studies showing a decrease in the CD4/CD8 T-cells ratio during athlete's training season (Rebelo *et al.* 1998). Furthermore, T-cell function is altered with acute bouts of exercise and significant reductions in activation and proliferative responses have been documented post training and competitive events (Nieman *et al.* 1994; Bury *et al.* 1996; Vider *et al.* 2001a). T-cell activation can be modulated by hormonal changes such as those observed during exercise. The release of the catecholamines epinephrine, norepinephrine and dopamine (Bahr *et al.* 1991) occurs with physical exercise. Bergquist *et al.* (1997) showed that dopamine might enhance peripheral blood mononuclear cell apoptosis by up-regulating Fas/FasL and pro-apoptotic molecules such as Bax. However, while high concentrations of dopamine inhibit T-cell proliferations (Cook-Mills *et al.* 1995), limited doses of dopamine seem to enhance T-cell division (Basu *et al.* 1993; Tsao *et al.* 1997). Furthermore, the circadian release of epinephrine and norepinephrine have been shown to regulate the preferential mobilization of highly differentiated effector and senescent T-cells (Dimitrov *et al.* 2009). As training routines include repeated and daily bouts of exercise, highly differentiated and senescent athlete's T-cells would be constantly mobilized in the peripheral blood compartment and exposed to high concentrations of dopamine during training periods (Sutoo & Akiyama 2003).

Acute exercise has also been shown to decrease T-cell responsiveness to mitogenic stimuli (Tharp & Preuss 1991; Nieman *et al.* 1994; Nieman *et al.* 1995; Ceddia *et al.* 1999). Finally, acute bouts of exercise appear to have a great effect on cell shifts from and to the peripheral blood compartment. Larrabee (1902) was the first to investigate this effect by showing that exhaustive bouts of exercise such as marathons, induced

leukocytosis suggesting the development of an inflammatory response. It has been later shown that during and immediately after acute bouts of exercise, the observed lymphopenia is preceded by the entry of T-cells in the peripheral blood compartment (Simpson *et al.* 2007a; Simpson *et al.* 2008). Interestingly, studies have shown a preferential mobilization of senescent CD4⁺ and CD8⁺ T-cells. Indeed, in both young and old individuals there is a large influx of KLRG1⁺, CD57⁺ and CD28⁻ T-cells to the peripheral blood compartment (Bruunsgaard *et al.* 1999; Simpson *et al.* 2007a; Simpson *et al.* 2008). While the preferential mobilization of terminally differentiated T-cells following acute bouts of exercise have been well documented (Simpson *et al.* 2007a; Simpson *et al.* 2008), other studies have shown the same effect of exercise on highly differentiated, non-senescent T-cells. Indeed Campbell *et al.* (2009) showed that acute exercise induced a greater mobilization of effector-memory and TEMRA CD8⁺ T-cells in the peripheral blood compartment, than naïve and central-memory T-cells. Following these encouraging results, these researchers recently investigated the impact of exercise on T-cell mobilization in CMV seropositive individuals (Turner *et al.* 2010). In addition to confirming the results previously obtained by Campbell *et al.* (2009), they observed a greater mobilization of TEMRA and EM CD8⁺ T-cells in the blood compartment of CMV seropositive subjects after acute exercise compared to the mobilization of the same cell populations seen in CMV seronegative subjects. They advocated that infection history may have an effect on T-cell mobilization, which could be a protective mechanism of exercise against ageing immunity. While these studies are providing valuable information on the impact of exercise on memory T-cells in CMV seropositive subjects, they have failed to assess the impact of acute exercise on dysfunctional, senescent T-cells in these individuals. It is plausible to propose that repeated acute bouts of exercise would mobilize senescent T-cells in the peripheral blood compartment and

free some “immunological space” by promoting their elimination via mechanisms such as apoptosis or AICD (Simpson 2011). Figure 2.3 is presenting the hypothetical impact of acute bouts of exercise on the immune system.

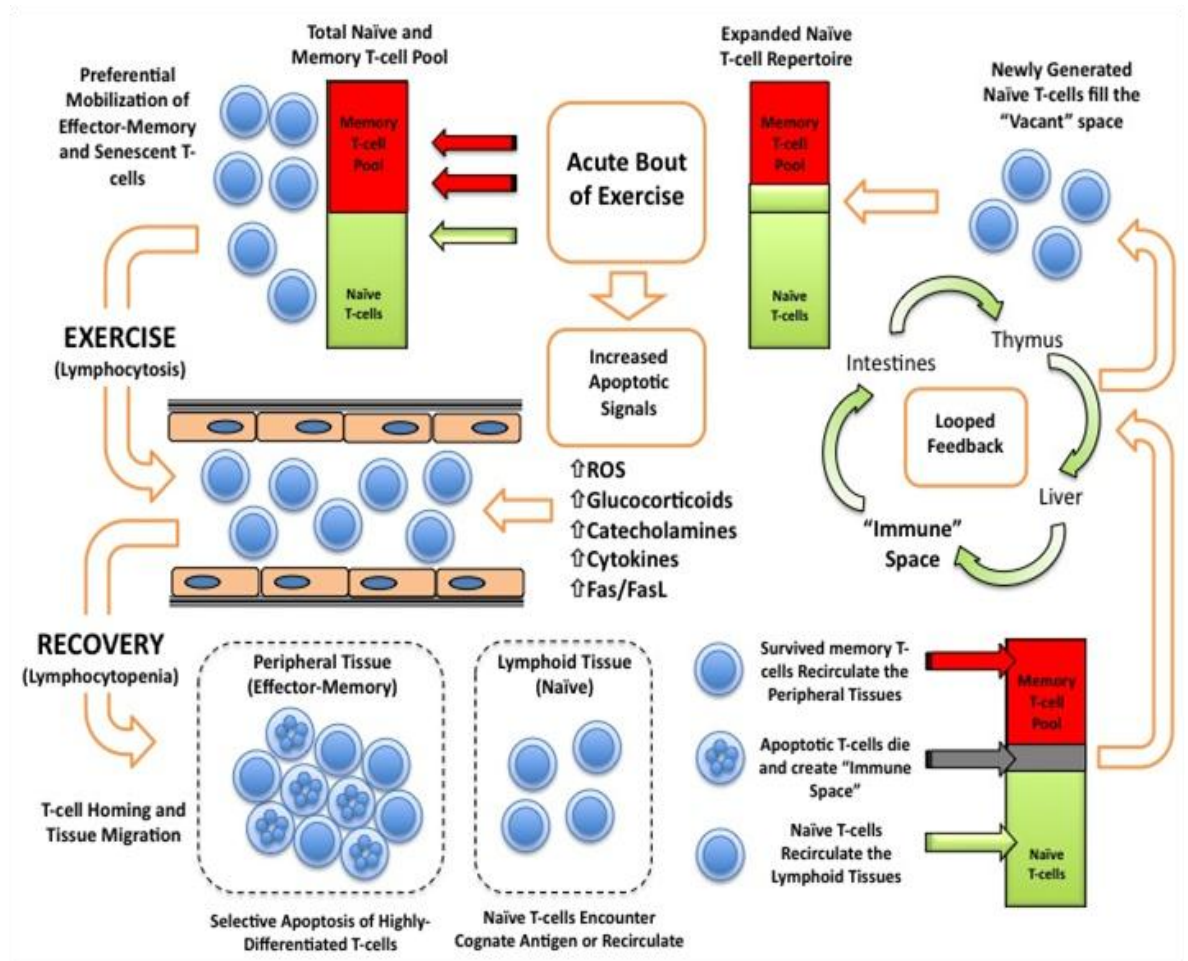


Figure 2.3. Proposed model by Simpson (2011) highlighting the potential impact of repeated bouts of acute exercise on the immune space. Highly differentiated and senescent T-cells are mobilized in the peripheral blood compartment during each bout of exercise. Mobilized T-cells are exposed to multiple apoptotic stimuli, eventually leading to the programmed cell death of apoptosis-susceptible cells during the recovery phase and creating more “immune space”. Naïve T-cell output will increase to maintain immune homeostasis and repeating this process will help maintaining a healthy the naïve T-cell repertoire (Simpson 2011).

2.6. Summary

The effect of chronological ageing on the immune system has been well documented, and it is clear that the accumulation of senescent T-cells and the concomitant shrinkage of naïve T-cell repertoire lead to increased risks of morbidity and mortality in the elderly. However, immunosenescence is not affecting older adults equally, and although some elderly are in a state of immunodepression and at risk of infection, others are not immunocompromised and have mastered the art of healthy longevity.

Lifestyle factors such as reduced physical activity and positive energy balance along with a lifetime of recurrent viral challenges could be the main culprits of the inter-individual differences observed in the current literature. Indeed elevated levels of oxidative stress, as seen in obese individuals, can trigger premature senescence by accelerating telomere attrition or inducing excessive DNA damage. Furthermore, the correlation between obesity and increased levels of serum adipokines, such as leptin suggests a potential link between obesity and immune senescence. Indeed, leptin is known to have a stimulating effect on peripheral blood T-cells which could ultimately lead to the accumulation of senescent T-cells. In addition to the potential activation effect of high leptin concentrations in obese individuals, recurrent viral activation by latent herpesviruses over a prolonged period of time could explain the disparity in proportions of senescent T-cells seen among the elderly. Indeed CMV and EBV infections have been associated with a state of immunodepression in the elderly termed Immune Risk Profile (IRP), which in turn is associated with increased morbidity and mortality in older adults.

The mechanisms underpinning the beneficial effects of regular physical exercise on ageing immunity are not currently known, but could be explained by a combined

beneficial effect on excess body weight and by its influence on CMV/EBV-induced immunosenescence. Frequent bouts of acute exercise might serve as a mechanism to remove virus-specific T-cells from the “immunological space”, thus triggering a release of naïve and newly functional T-cells from the thymus and subsequent expansion of the naïve T-cell repertoire. The different studies presented in this thesis were conducted to provide new insights in the factors leading to the onset of premature immunosenescence and to assess the effect of regular physical activity on the immune system. The data discussed in the subsequent chapters could introduce physical activity as a cost-effective and safe clinical intervention to reduce or delay the onset of immunosenescence.

2.7. Specific aims of the Studies described within this Thesis

The overarching aim of the studies presented in this thesis was to examine the effects of different factors potentially influencing the onset of immunosenescence independently of chronological ageing. The specific aims of the studies conducted and described within the data chapters of this thesis were:

Chapter 4:

- (1) The aim of this study was to examine the association of physical activity and aerobic fitness, indicated by estimated $\dot{V}O_{2max}$, and the age-related increase of effector-memory and senescent T-cells in a population of healthy adult men.

Chapter 5:

- (2) The main aim of this study was to examine the relationship between adiposity and excess body mass and the proportions of blood T-cell subsets in an age-controlled population of Mexican-American adolescents.
- (3) The second aim of this study was to examine the association between serum leptin concentrations and the proportions of effector-memory and senescent T-cells in an age-controlled population of Mexican-American adolescents.
- (4) The last aim of this study was to examine the effect of potential confounding factors such as latent CMV and EBV infections, quality of life and gender on the associations between excess body mass and serum leptin concentrations and the proportions of blood T-cell subsets.

Chapter 6:

- (5) The aim of the study presented in this chapter was to examine the effect of physiological concentrations of plasma leptin on T-cell activation to assess the potential role of high serum concentrations of this adipokine on the T-cell repertoire of obese subjects.
- (6) The second aim of this study was to assess both the constitutional and inducible expression of the functional form of the cell surface leptin receptor and mRNA in T-cells.

Chapter 7:

- (7) The main aim of this study was to examine the impact of latent CMV and EBV infections on blood T-cell phenotypes in healthy young adult men.

- (8) The second aim of this study was to examine the impact of the latent CMV and EBV infections on the exercise-induced changes in T-cell concentration in peripheral blood.
- (9) The third aim of this study was to determine the contribution of viral-specific T-cells to the mobilization of memory and senescent T-cells with exercise.

CHAPTER 3
GENERAL MATERIALS AND METHODS

3.1. Subjects

3.1.1. Subject Selection

All peripheral blood samples were taken by a qualified phlebotomist from healthy non-smoking male subjects. Subjects with excessive alcohol intake (>14 units of alcohol per week), taking medication affecting the immune system, routinely using ibuprofen and/or aspirin, anti-depressants, medications designed to alter blood pressure or cardiovascular function and hormone replacement therapy were excluded from the subject pool. Subjects reporting major affective disorders, HIV infection, hepatitis, chronic/debilitating arthritis, central or peripheral nervous disorders, previous stroke or cardiac events, were bedridden in the past three months, suffer from known cardiovascular disease or autoimmune diseases were all excluded from the study. All volunteers gave written informed consent (Appendix 1) and ethical approval was obtained from the School of Life Sciences, Edinburgh Napier University and the Committee for Protection of Human Services at the University of Houston.

3.1.2. Anthropometric Measurements

All donors completed an assessment of percentage body fat determined by a bioelectrical impedance method (Bodystat, Douglas, UK), unless stated otherwise. Electrodes were placed on the dominant ipsilateral wrist and ankle as designated by the subject and measurements were duplicated to obtain greater accuracy. Resistance and reactance were measured in the normally hydrated volunteers in the supine position. All subjects were weighed, measured and waist and hips circumference were obtained by the same researcher to limit inter-tester inaccuracies. Waist circumference was

measured midway between the lateral lower rib margin and the iliac crest. Hip circumference was measured at the level of the major trochanters, through the pubic symphysis.

3.2. Exercise protocol and measurement of physiological variables

3.2.1. Physical activity assessment

Each participant from the studies described in *Chapter 4* and in *Chapter 7* provided his physical activity score using a non-exercise 7-level questionnaire (Jackson et al. 1990) (Appendix 2). The physical activity rating questionnaire (PA-R) was divided in 3-categories: minimal physical activity defined by infrequent light physical activity such as walking or infrequent (0-1) moderate physical activity defined by 10 minutes to an hour of weekly regular moderate physical activity such as weight lifting or yard work (2-3) and vigorous physical activity such as running from 1 mile to 10 miles per week (4-7). Measures of $\dot{V}O_{2\max}$ were estimated from both the questionnaire and the submaximal cycling exercise protocol described below. The relationship between $\dot{V}O_{2\max}$ estimated from the non-exercise questionnaire and the $\dot{V}O_{2\max}$ estimated from the submaximal cycling test was $r = 0.88$ ($p < 0.001$).

3.2.2. Indoor cycling trainer calibration

To ensure accuracy and repeatability of the exercise protocol for each individual subject, the computrainer was calibrated for rolling resistance prior to the exercise. The load generator and bike tire were mechanically warmed to an optimal temperature by

pedaling at a low-comfortable speed and 150 W for 10 minutes. This was done by a researcher while the subject remained seated, to prevent a premature start of the exercise protocol. Subjects were then asked to pedal and reach a speed of 25 mph before coasting to a stop without applying pressure on the pedals. Rolling resistance was adjusted and set to 2.00 lbs for each individual subject during the computrainer calibration to ensure homogeneity in the exercise protocol throughout the studies.

3.2.3. Submaximal exercise test to estimate maximal oxygen uptake and maximum cycling power

After providing the resting blood sample, all participants from the studies described in *Chapter 4* and *Chapter 7* had their $\dot{V}O_{2\max}$ estimated using a submaximal cycling exercise protocol (Astrand 1960). Subjects completed four 3-minute heart-rate adjusted incremental stages on a Trek road bike mounted on a calibrated indoor cycling trainer (Computrainer Lab, Racermate, WA, USA). The subjects' heart rate (HR) was measured continuously during the exercise protocols by short-range telemetry (Suunto T6, Vantaa, Finland) and the average HR during the last minute of each stage was used to determine the age-adjusted wattage to apply to the following increment. Maximal oxygen uptake and maximum power was estimated from the test using the equations provided by Adams *et al.* (1998).

3.2.4. Cycling Exercise Protocol

All participants from the study described in *Chapter 7* completed the cycling exercise protocol within 1-3 weeks after the initial $\dot{V}O_{2\max}$ estimation test. Subjects reported to

the laboratory after an overnight fast between 07:30 and 09:00 hours and provided a resting blood sample. Subjects were then fitted with a heart rate monitor and completed a 30-min cycling protocol at a fixed intensity corresponding to 85% of the pre-determined estimated maximum wattage. Further blood samples were collected immediately on completion of the exercise test then again 1h after exercise cessation. Serum samples were collected and tested for CMV and EBV as described below.

3.3. Peripheral blood collection and mononuclear cell isolation

Blood sampling was consistent throughout the different studies comprised within this thesis. Intravenous blood samples were collected from an antecubital vein in 10mL vacutainers® tubes (BD vacutainer™, Franklin Lakes, NJ USA) spray-coated with lithium heparin, using 3/4" BD Vacutainer Safety-Lok™ (BD Biosciences, Oxford, UK). Serum samples were collected in 10mL serum-separating tubes SST™ (BD vacutainer™, Franklin Lakes, NJ USA) coated with clotting agents and stored frozen at -80 °C for further analysis, including serum viral antibody titers. Peripheral Blood Mononuclear Cells (PBMC) were isolated from whole blood using density gradient centrifugation (Lymphoprep®; Axis-Shield, Oslo, Norway or Histopaque®; Sigma-Aldrich, Irvine, Scotland). Whole blood was diluted with an equal volume of 0.9% NaCl (Baxter™, Deerfield, IL USA), and 6mL of the diluted blood was layered over 3 ml of Lymphoprep®. Samples were then centrifuged for 25 minutes at 800g. Following centrifugation, the distinct band formed by the PBMC was carefully removed using a Pasteur pipette (Figure 3.1) and washed twice in 0.9% NaCl for 10 minutes at 250g, then again in either cell culture medium RPMI 1640 (Sigma-Aldrich, Irvine, Scotland) when cells were cultured or in Phosphate Buffer Saline + 2% bovine serum albumin +

0.02% Sodium Azide (Sigma-Aldrich, Irvine, Scotland) when cells were stained for flow cytometry analysis immediately following isolation. Finally, the cell pellet was re-suspended in RPMI 1640 or PBS-BSA- NaN_3 and quantified.

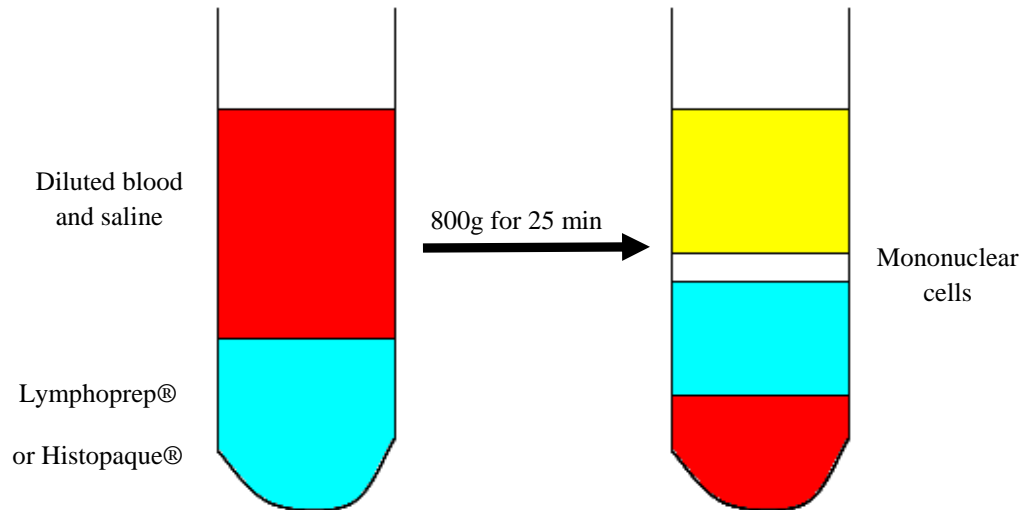


Figure 3.1. Mononuclear cell separation by centrifugation

3.3.1. PBMC Quantification

The cells were counted using the Nucleocounter® automated analyzer (Sartorius, Edison, NJ, USA) and using optical microscopy by coupling a Malassez haemocytometer with the use of the cell dye trypan blue. When using the Nucleocounter® automated analyser, the cells' nuclei were stained with a fluorescent dye, propidium iodide (PI), after lysis of the cell membrane by specific reagents. An equal amount of cells was directly stained with PI without lysing the cells, in order to quantify the non-viable or dead cells already present in the suspension. The fluorescence was read by a fluorescent microscope in the Nucleocounter. The fluorescence intensity is directly correlated to the number of cells. A percentage of viability was also obtained using this technique.

3.3.2. Mitogen titration and PBMC stimulation

The experimental procedures used for the data presented in *Chapter 6* required PBMC *in vitro* stimulation. Isolated PBMC were cultured to assess the effect of leptin exposure on their activation state. According to the Tunable Activation Threshold (TAT) Theory (Grossman & Paul 1992) and to previous studies on T-cell activation (Martin-Romero *et al.* 2000), we mildly stimulated the isolated PBMC with optimal concentrations of the mitogen Phorbol 12-Myristate 13-Acetate (PMA). PMA is a polyfunctional diterpene phorbol ester mimicking diacylglycerol (DAG), a natural activator and ligand for the C1 domain of the Protein Kinase C, thus activating T-cell independently of the TCR-signaling pathway. As a consequence of its lack of specificity, the concentration of PMA required to obtain the activation of between 40-50% of a T-cell population will vary from batch to batch.

Two different batches of commercially available PMA (Sigma-Aldrich, Irvine, Scotland) were re-suspended in 1 mL of DMSO to obtain a stock solution at 1 mg.mL⁻¹. Freshly isolated PBMCs (1x10⁶) were cultured in 1 mL of RPMI 1640 culture medium with various concentrations of PMA during 4 hours at 37 °C with 5% of CO₂. The cells were then taken out of culture, washed with an excess of PBS-BSA-NaN₃ for 10 minutes at 250g and labeled with anti-CD3 FITC-conjugated and anti-CD69 PE-conjugated antibodies. The optimal concentrations of PMA from the batch 1 and batch 2 to activate an optimal proportion of 40 to 50% of T-cells was found to be respectively 0.25 ng.mL⁻¹ and 0.125 ng.mL⁻¹ (Figure 3.2).

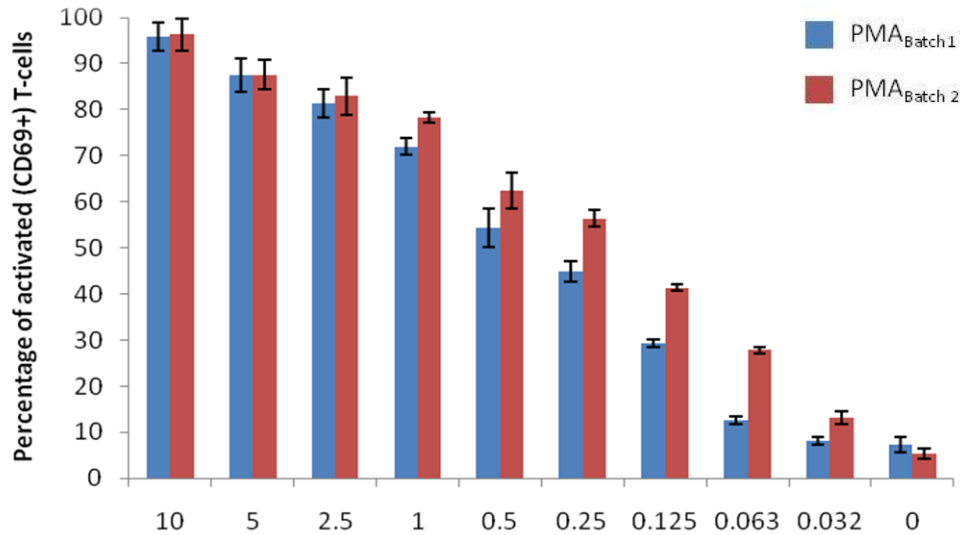


Figure 3.2. The proportions of activated CD3⁺ T-cells after 4 hours of incubation in RPMI 1640 and various concentrations of PMA (ng.mL⁻¹) from two different batches. All values are Mean \pm SD.

3.4. Serological Testing for CMV and EBV IgG Antibodies

Subject serostatus was required to analyse the data presented in the *Chapter 4*, *Chapter 5* and *Chapter 7*. Serum samples were analysed within 8 weeks of storage, in duplicate for CMV and EBV IgG antibodies using separate commercially available ELISA kits in accordance with the manufacturer's instructions (GenWay Biotech, CA, USA). Diluted serum (1/100) was added to CMV or EBV-antigen pre-coated wells (Genway Biotech, CA, USA). The anti-virus IgG specific antibody, if present, bound to the antigen. Unbound materials were washed away from the wells with the provided washing buffer before allowing the horseradish peroxidase (HRP) enzyme conjugate to bind to the antibody-antigen complex. The substrate, 3,3',5,5' tetramethylbenzidine (TMB), was added to the wells and the plate was incubated for 10 minutes at room temperature (22 °C) to allow complete TMB hydrolysis. The intensity of the colour generated being proportional to the amount of anti-CMV or anti-EBV IgG specific antibody present in the serum sample, spectrophotometric analysis was performed on each well with a 96-

wells plate reader (SpectromaxM2, Molecular Devices, CA, USA). The Antibody index of each sample was calculated relative to the provided calibrator. As instructed by the manufacturer, subjects were considered to be seronegative for CMV or EBV if the antibody index was below 0.9, and CMV or EBV seropositive if the antibody index was greater than 1.1. Subjects with an antibody index of 0.9 to 1.1 (borderline) were excluded from the studies. The antibody index of two resting samples were analysed per subject in order to exclude subjects with a recent reactivation of the latent virus (defined as a % increase in serum IgG).

3.5. Cellular staining for flow cytometric analysis

3.5.1. Direct Immunofluorescence Assays

PBMCs (1×10^6) were labelled with 100 μ L of pre-diluted directly conjugated monoclonal antibodies (mAbs) in a three or four colour procedure. After labelling, the PBMCs were incubated for 45 minutes at room temperature. Commercial stock solutions of the mAbs were diluted in PBS + 0.5% BSA + 0.02% Sodium Azide (PBS-BSA-NaN₃) at concentrations previously identified as being optimal for analysis by flow cytometry. Details on the different mAbs used in the different experiments conducted in this thesis are presented in the Table 3.1. The excess unbound mAbs was washed away in PBS-BSA-NaN₃ for 10 minutes at 250g and the cell pellet was resuspended in 400 μ L of PBS-BSA-NaN₃ before flow cytometric analysis.

Table 3.1. Monoclonal antibodies used in the detection of cell surface protein and the optimal dilutions determined after titrations.

Specificity	Isotype	Clone	Conjugate	Optimal dilution	Species reactivity	Manufacturer
CD3ε	IgG2a, κ	MEM-57	FITC	1/100	Human	Immunotools, Friesoythe, Germany
CD3ε	IgG2a, κ	HIT3a	APC	1/100	Human	BD Bioscience, San Jose, CA, USA
CD3ε	IgG2a, κ	MEM-57	APC	1/100	Human	Immunotools, Friesoythe, Germany
CD4	IgG1, κ	MEM-241	PE-Cy5	1/50	Human	Immunotools, Friesoythe, Germany
CD4	IgG2b, κ	OKT-4	PerCP-Cy5.5	1/50	Human	BD Bioscience, San Jose, CA, USA
CD8α	IgG2a, κ	MEM-31	FITC	1/100	Human	Immunotools, Friesoythe, Germany
CD8α	IgG2a, κ	MEM-31	PE-Cy5	1/50	Human	Immunotools, Friesoythe, Germany
CD8α	IgG1, κ	RPA-T8	PerCP-Cy5.5	1/50	Human	BD Bioscience, San Jose, CA, USA
CD25	IgG1, κ	MEM-181	PE	1/50	Human	Immunotools, Friesoythe, Germany
CD27	IgG1, κ	LG.7F9	PE	1/50	Human	BD Bioscience, San Jose, CA, USA
CD28	IgG1, κ	KOLT-2	FITC	1/100	Human	Immunotools, Friesoythe, Germany
CD28	IgG1, κ	CD28.2	PE	1/100	Human	BD Bioscience, San Jose, CA, USA
CD45RA	IgG2b, κ	MEM-56	FITC	1/100	Human	Immunotools, Friesoythe, Germany
C45RA	IgG2b, κ	HI100	FITC	1/100	Human	BD Bioscience, San Jose, CA, USA
CD45RO	IgG2a, κ	UCHL1	PE	1/50	Human	Immunotools, Friesoythe, Germany
CD45RO	IgG2a, κ	UCHL1	PE	1/100	Human	BD Bioscience, San Jose, CA, USA
CD57	IgM	NK-1	PE	1/300	Human	Abcam, Cambridge, UK
CD69	IgG1, κ	FN50	PE	1/50	Human	Immunotools, Friesoythe, Germany
CD71	IgG1, κ	MEM-75	PE	1/50	Human	Immunotools, Friesoythe, Germany
CCR7	IgG2a, κ	4B12	PE	1/100	Human	BD Bioscience, San Jose, CA, USA
KLRG1	IgG2a, κ	13A2	Alexa-488	1/400	Human	Dr H. Pircher, U of F, Freiburg, Germany
OB-Rb	IgG2b, κ	52263	FITC	1/300	Human	R&D systems, Minneapolis, MN, USA

3.5.2. Antigen-specific T-cells staining

Identification and numeration of viral-specific CD8⁺ T-cells as presented in the *Chapter 7* was completed by flow cytometry analysis. Antigen-specific CD8⁺ T-cells from seropositive donors were labeled with 5 μ L of undiluted Allophycocyanin(APC)-conjugated CMV-specific MHC class I pentamers or EBV-specific MHC class I pentamers (pp65: A*201 NLVPMVATV and BMLF-1: A*201 GLCTVAML respectively, Proimmune, Oxford, UK) for 15 minutes at room temperature in the dark. The labeled antigen-specific CD8⁺ T-cells were then labeled according to the protocol described above.

3.5.3. Flow cytometers

PBMCs phenotypes in the study presented in *Chapter 4* were assessed on two different flow cytometer depending on the testing location site. The FACSCalibur flow cytometer (BD Biosciences, San Jose, CA USA) used at Edinburgh Napier University was equipped with a 15-mW argon ion laser emitting light at a fixed wavelength of 488 nm whereas the Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) used at the University of Houston was equipped with a blue laser emitting light at a fixed wavelength of 488nm and a red laser emitting light at a fixed wavelength of 640nm. Fluorescein (FITC) or Alexa488 fluorescence were detected in the FL-1 filter centred at 533nm with a 30nm half-peak bandpass and PE fluorescence was detected in the FL-2 filter centred at 585nm with a 42nm bandpass (FACSCalibur) or 40nm bandpass (C6). The FL-3 and FL-4 filter were respectively used to detect the PerCP Cy-5.5 fluorescence centered at wavelength over 670nm longpass and Allophycocyanin (APC) fluorescence at 661 nm with a 16 nm bandpass (FACSCalibur) or at 675nm with a

25nm bandpass (C6). Data were analyzed using the BD FacsCalibur cell analysis software or the Accuri Cflow v17. software depending on the testing site. The results presented in *Chapter 5* and *Chapter 7* were entirely obtained at the University of Houston using the Accuri C6 flow cytometer, whereas the study presented in *Chapter 6* was conducted at Edinburgh Napier University using the FACSCalibur flow cytometer.

3.5.4. Flow cytometer comparison

The study presented in *Chapter 4* was conducted on two testing sites using two different flow cytometers. To control for potential systematic differences in the measured cell frequencies, two samples were collected from the same individuals at both testing sites and labeled with the same antibody panel. The first sample was obtained in Edinburgh in June 2008 (using the FACSCalibur) and the second sample was collected in Houston (using the Accuri C6) in September 2009. Representative flow cytometry dotplots for the expression of KLRG1 and CD57 on CD8⁺ T-cells obtained using the FACSCalibur and the Accuri C6 are presented in Figure 3.3. Although the time elapsed from one sample collection to the next was 15-months, the values obtained from both instruments on this same individual are not significantly different, confirming the lack of inter-flow cytometer variability.

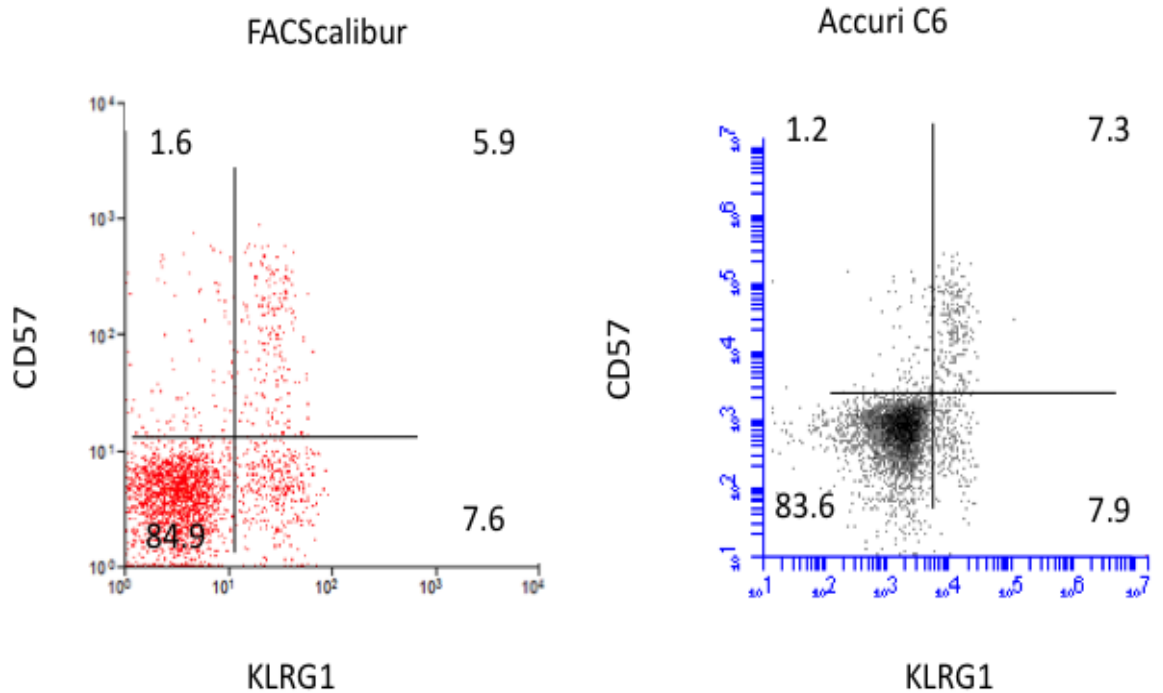


Figure 3.3. Representative flow cytometry dotplots of CD8⁺ T-cells expressing KLRG1 and CD57 obtained from the same individual and analysed using the FACScalibur flow cytometer or the Accuri C6 flow cytometer.

3.5.5. Flow cytometry gating procedures

The cells were identified and electronically gated using the forward and side light-scatter mode. The light emitted by the laser were either diffracted in function of the cell size, into a Forward scatter (Fsc) detector, or reflected/refracted in function of the cell granularity, into a Side scatter (Ssc) detector (Figure 3.4). For each sample, 100,000 cells of interest were collected. The signals were collected in the logarithmic mode (4 or 6 decade logarithmic amplifier depending on the flow cytometer).

The Fsc was used to eliminate the debris and dead cells based on their small size. An electronic gate was placed around the lymphocytes (Figure 3.4 and 3.5), which is

critical for subsequent analysis in order to select the population, free of remaining debris and unrelated cells.

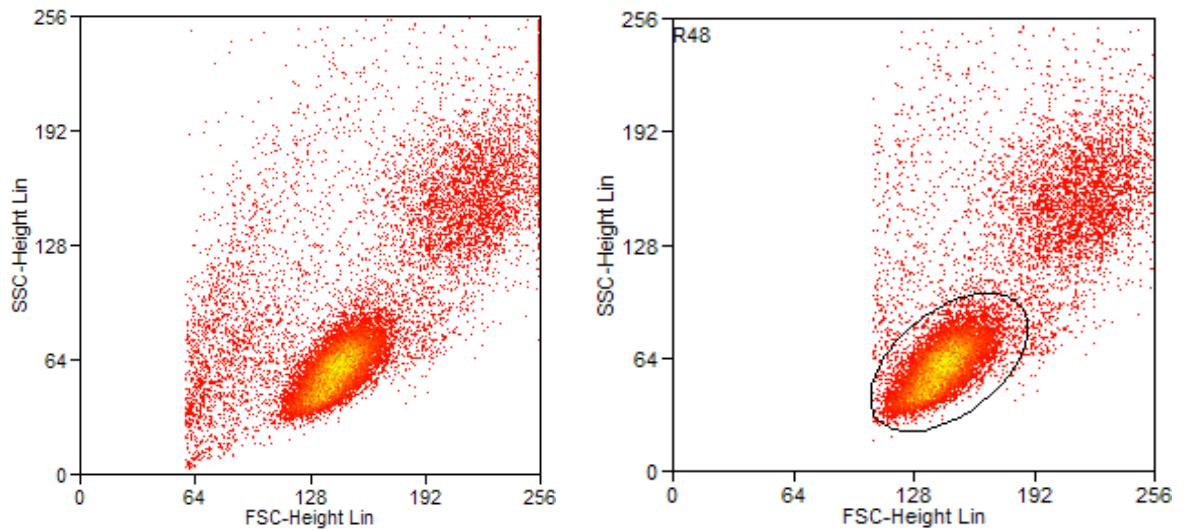


Figure 3.4. Reduction of the background noise by increasing the Fsc threshold and gating around the lymphocyte population.

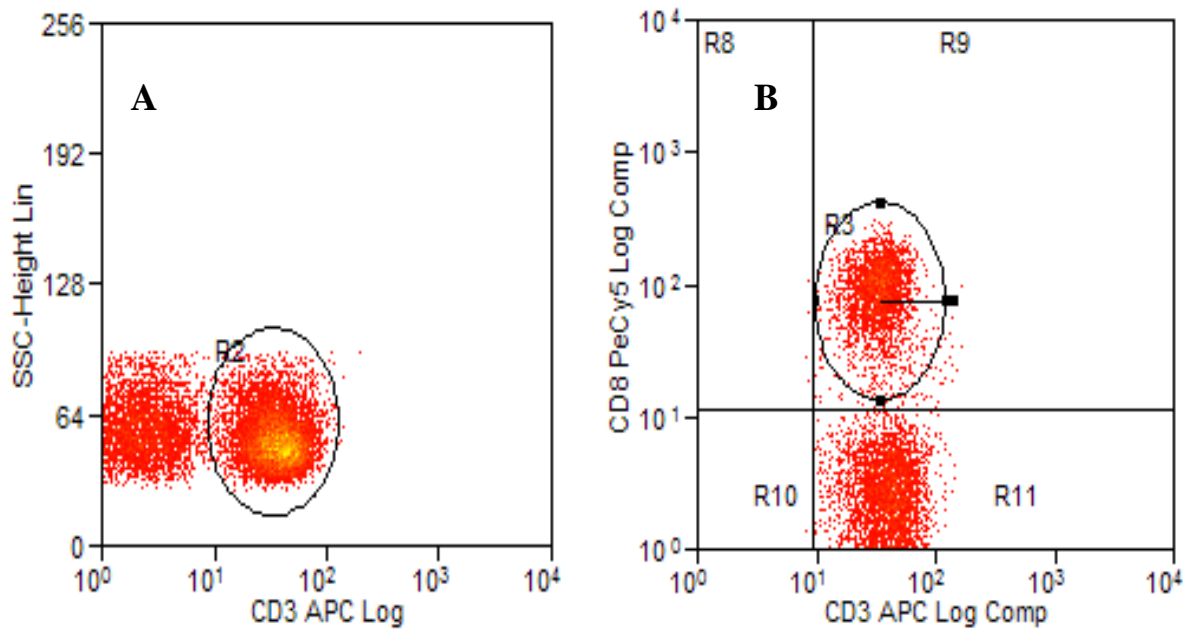


Figure 3.5. Selection of T-cells (A) and cytotoxic T-cells (B). The T-cells were selected by backgating on populations highly expressing the CD3 receptor. Another gate was placed to select the cells expressing the CD4 co-receptor or CD8 co-receptor, in order to isolate the different subpopulations of T-cells.

The use of different fluorochromes coupled to the use of electronic gates were essential to separate cell populations co-expressing cell surface markers of senescence (KLRG1, CD28, and CD57), naïve (CD45RA) and memory (CD45RO) T-cell on each CD4+ and CD8+ subset (Figure 3.6).

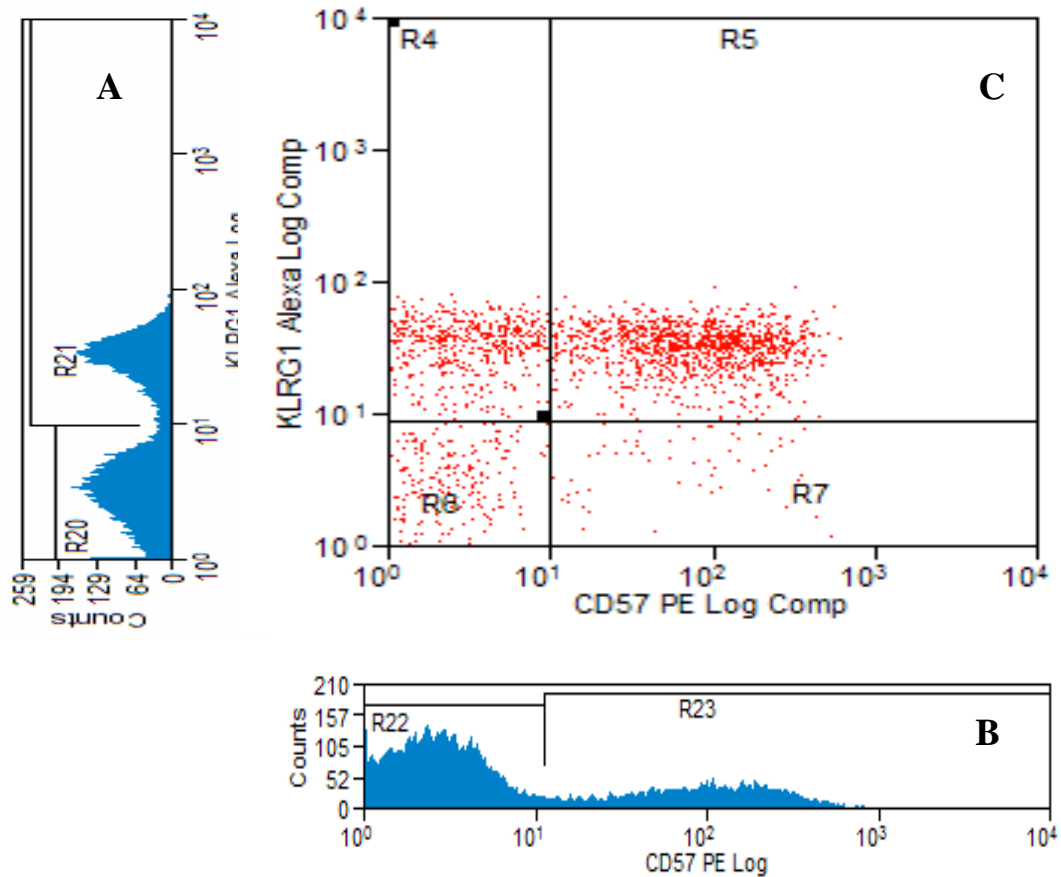


Figure 3.6. Cytotoxic CD8+ T-cells expressing KLRG1 (A), CD57 (B) or both markers (C) on their surface.

Several filter and fluorochromes emitting at different wavelength were used to characterise the co-expression of cells surfaces molecules. Monoclonal antibodies labeled with Alexa488 or FITC molecules emit green radiations detected in FL1, whereas mAbs labeled with Phycoerythrin (PE) molecules emit orange-red radiations detected in FL2. However, the important width of the emission spectra from fluorescent antibody labels induces overlaps between the different fluorochromes. It results in

detection of fluorescence from both fluorochrome in the same filter, and consequently results in the apparition of false positive populations. These overlaps were corrected by electronic compensation, by which the excess of fluorescence originating from a fluorochrome other than the one specified on a specific Filter is subtracted as a proportion of the signal from the other detector.

3.5.6. T-cell subset phenotypic identification

Different monoclonal antibody combinations were used to identify and quantify the different T-cell populations in the different studies described within this thesis. The different combinations used are presented in the Table 3.2.

Table 3.2. Phenotypic identification of the blood T-cell subsets in the studies presented in the subsequent chapters

Chapter	Phenotypes	T-cell subsets	References
Chapter 4	KLRG1-/CD28⁺^a	Naïve T-cells	(Koch <i>et al.</i> 2008)
	KLRG1-/CD57-		
	CD45RA+/CD45RO⁺^d	Transitional T-cells	(Akbar <i>et al.</i> 1988; Sanders <i>et al.</i> 1988)
	CD45RA-/CD45RO⁺	Pan memory T-cells	
	KLRG1+/CD28⁺^c	Memory T-cells	(Ibegbu <i>et al.</i> 2005)
	KLRG1+/CD57-		
KLRG1+/CD28⁻^b	Senescent T-cells	(Voehringer <i>et al.</i> 2002; Ibegbu <i>et al.</i> 2005)	
KLRG1+/CD57⁺^b			
Chapter 5	CD28+/CD27⁺	Early Differentiated T-cells	(Appay <i>et al.</i> 2002)
	CD45RA+/CCR7⁺	Naïve T-cells	(Geginat <i>et al.</i> 2003)
	CD45RA-/CCR7⁺	Central-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)

Chapter 5	CD45RA-/CCR7-	Effector-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)
	CD28-/CD27+	Intermediate Differentiated T-cells	(Appay <i>et al.</i> 2002)
	CD28-/CD27-	Late Differentiated T-cells	(Appay <i>et al.</i> 2002)
	CD45RA+/CCR7-	TEMRA cells	(Geginat <i>et al.</i> 2003)
	CD28-/CD57+	Senescent T-cells	(Bandres <i>et al.</i> 2000)
Chapter 6	CD4+/CD69+	Activated T _H -cells	(Cosulich <i>et al.</i> 1987; Cebrian <i>et al.</i> 1988)
	CD8+/CD69+	Activated T _C -cells	
Chapter 7	KLRG1-/CD28+ ^a	Naïve T-cells	(Koch <i>et al.</i> 2008)
	KLRG1-/CD57-		
	CD45RA+/CCR7+		(Geginat <i>et al.</i> 2003)
	KLRG1+/CD28+ ^c	Memory T-cells	(Ibegbu <i>et al.</i> 2005)
	KLRG1+/CD57-		
	CD45RA-/CCR7+	Central-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)
	CD45RA-/CCR7-	Effector-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)
	CD45RA+/CCR7-	TEMRA cells	(Geginat <i>et al.</i> 2003)
	KLRG1+/CD28- ^b	Senescent T-cells	(Voehringer <i>et al.</i> 2002; Ibegbu <i>et al.</i> 2005)
	KLRG1+/CD57+ ^b		

^a Approximately 3–7% of all CD8+ T-cells displaying this phenotype may be central-memory T-cells (Koch *et al.*, 2008).

^b The term “senescent” has been used to describe cells with this phenotype, however, some KLRG1+/CD28- cells may be in a state of “exhaustion” and their proliferative capabilities may be restored under certain circumstances (Akbar and Henson, 2011). The relationship between the proportions of KLRG1+/CD28- and KLRG1+/CD57+ was $r = 0.762$ ($p < 0.001$) and $r = 0.814$ ($p < 0.001$) for CD4+ and CD8+ T-cells, respectively.

^c The variation in CCR7 expression (28–57%) among KLRG1+/CD28+ cells (Ibegbu *et al.*, 2005) indicates that this phenotype may overlap both central-memory and effector-memory T-cells.

^d Identifies naïve T-cells transitioning to a memory phenotype, or an effector-memory T-cell transitioning to a terminal phenotype.

3.6. T-cell subset enrichments from whole blood

The data presented in *Chapter 6* were partially generated after T-cell subset isolation and enrichment. Following whole blood isolation, pan CD3⁺ T-cells, CD4⁺ T-cells and CD8⁺ T-cells were separated from the PBMC. To limit undesirable cell activation resulting from the isolation procedure, a negative selection of the cell subsets of interest was preferred (BD Biosciences, Oxford, UK). The PBMC were washed in IMagTM buffer for 10 minutes at 250g and re-suspended in the same buffer to obtain a final concentration of 10×10^6 cells.mL⁻¹. Every 10^6 cells were put in contact with 5 μ L of different commercially available biotinylated enrichment cocktails (BD Biosciences, Oxford, UK) depending on the cell subset of interest (see appendix 3 for the different enrichment cocktails compositions) for 20 minutes at room temperature. The cells were then washed in large excess (10x) of IMagTM buffer at 250g for 10 minutes and the supernatant was removed. The cells were re-suspended in 5 μ L of IMagTM Streptavidin Particles Plus (BD Biosciences, Oxford, UK) per 10^6 cells and incubated for 30 minutes at room temperature to allow adequate binding of the streptavidin-conjugated magnetic beads to the previously added primary antibodies.

Following incubation, the labeled cells were washed in excess of IMagTM buffer for 7 minutes at 250g and re-suspended in the same buffer to obtain a final concentration of 50×10^6 cells.mL⁻¹. The cells were placed in the IMagnetTM for 7 minutes to magnetically separate the unwanted cell subsets labeled with specific antibodies present in the biotinylated enrichment cocktails. The supernatant composed of the unlabeled cell subsets of interest (negative fraction) was removed and the tube was removed from the magnet to re-suspend the positive fraction in IMagTM buffer before being replaced in the magnet. This operation was repeated twice and the remaining positive fraction was

discarded. The negative fraction was placed twice in the IMagnet™ for 7 minutes to ensure maximum purity of the isolated T-cells subsets. Enriched T-cell subsets were assessed for OB-Rb expression by flow cytometric analysis.

3.7. Cell storage in liquid nitrogen

Due to technical difficulties, RNA from the isolated PBMCs could not be extracted on freshly enriched samples and had to be performed on a later date. To ensure integrity of the genetic material, the samples were conserved in liquid nitrogen for a period inferior to 3 months.

The stimulated isolated PBMCs from 4 subjects were washed in PBS-BSA-NaN₃ for 10 minutes at 250g and slowly re-suspended in 1mL of freshly made cryomedium composed of 90% of Foetal Calf Serum (Invitrogen, Paisley, Scotland) and 10% of Dimethyl Sulfoxide (Sigma-Aldrich, Irvine, Scotland) on ice. Cells were then transferred in cryotubes (NUNC, Roskilde, Denmark) and placed overnight at -80 °C before being stored in liquid nitrogen.

3.8. Leptin receptor gene expression analysis

3.8.1. Total RNA extraction

The data presented in *Chapter 6* were obtained after total RNA was extracted and amplified by either Reverse Transcription Polymerase Chain Reaction (RT-PCR) or Real-Time Polymerase Chain Reaction (RT-PCR'). Samples were defrosted at room

temperature for 1 hour prior to RNA extraction. Once completely defrosted, they were transferred to Eppendorf tubes (Eppendorf, Hamburg, Germany), centrifuged at 200g for 5 minutes and the supernatant was removed. Every 5×10^6 cells were lysed in 1mL of TRIsure™ (Bioline, London, UK) for 5 minutes at room temperature. To ensure correct phase separation, 0.2mL of chloroform (Bioline, London, UK) were added for every milliliter of TRIsure, the tubes were vigorously agitated for 15 seconds and centrifuged at 12 000g for 15 minutes at 4°C after an incubation period of 2 minutes at room temperature. The upper aqueous phase of the supernatant in which the RNA was dissolved, was carefully pipette transferred into another eppendorf tube and precipitated by 0.5mL of isopropyl alcohol. After incubating the samples for 10 minutes at room temperature, they were centrifuged at 12 000g for 10 minutes at 4°C and washed in 1mL of 75% ethanol. Finally the extracted RNA was centrifuged at 7 500g for 5 minutes at 4°C, the pellet was air-dried and re-suspended in diethylpyrocarbonate -treated water (DEPC-water) (Bioline, London, UK).

3.8.2. RNA quantification

After isolation, RNA purity and concentration was determined in each sample, using UV absorption with the NanoVue™ Plus Spectrophotometer (GE Healthcare, Munich, Germany). The nucleotide nitrogenous bases contained in RNA have an absorption maximum at $\lambda=260\text{nm}$, whereas proteins have a UV absorption maximum of $\lambda=280\text{nm}$. Measures of RNA absorption were made at both wavelengths to measure RNA concentration in the samples, an optical density of 1 at $\lambda=260\text{nm}$ corresponding to $40 \mu\text{g.mL}^{-1}$ of RNA, but also the protein contamination by measuring the ratio of the

absorbance at 260nm/280nm. Samples were required to have a purity superior to 95% of RNA to be included in the analysis.

3.8.3. Leptin Receptor gene amplification and detection

The expression of the functional form of the leptin receptor (OB-Rb) mRNA in PBMCs was analysed in *Chapter 6*. Extracted total RNA was used to generate complimentary DNA (cDNA) prior to PCR amplification. Following brief centrifugation, 2 µg of total RNA was added to a sterile, nuclease-free, thin-walled PCR tube containing 2 µL of Random Hexamer Primer (Roche Diagnostic, Burgess Hill, UK) on ice. A sufficient volume of PCR-grade water was added to the tube to dilute the Hexamer primer down to 60 µM. The samples were placed in a thermal block cycler with heated lid (PCR sprint, Thermo Scientific, Waltham, MA, USA) at 65 °C for 10 mins to ensure RNA heat denaturation before being cooled down on ice. When the sample reached 4 °C, 10 units of Transcriptor high fidelity Reverse Transcriptase (Roche Diagnostic, Burgess Hill, UK) was added to the reaction tube, with 2 µL of Deoxynucleotide Mix® (Roche Diagnostic, Burgess Hill, UK) and 20 units of Protector RNase inhibitor (Roche Diagnostic, Burgess Hill, UK.). Samples were incubated in the PCR thermal cycler for 30 minutes at 50 °C before inactivation of the reverse transcriptase at 85 °C for 5 minutes. The cDNA samples were conserved on ice until amplification by PCR.

PCR was carried out in 50 µL reaction mixture using PCR master (Roche Diagnostic, Burgess Hill, UK) to amplify a specific region of the OB-Rb gene mRNA (237 bp) with the forward primer Ob-Rb forward primer: 5'-GCCAACAACACTGTGGTCTCTC-3' and the reverse primer Ob-Rb reverse primer: 5'-AGAGAAGCACTTGGTGACTG-3'. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as

an internal control to validate the RT-PCR reaction. The sequence of the forward primer used to amplify GAPDH gene was GAPDH forward primer: 5'-GCTTGGTAAAAAGATGCTCAATG-3' and the sequence of the reverse primer used was GAPDH reverse primer: 5'-GCTTGGTAAAAAGATGCTCAATG-3'. Negative control consisted of omitting the RT reaction, which led to a lack of band on the agarose gel after PCR amplification and electrophoresis.

The samples were denatured at 95 °C for 5 min and PCR was carried out for 40 cycles with 55 °C annealing temperature. A final 10 min extension step at 72 °C terminated the reaction. The PCR products were loaded on a 2% agarose gel electrophoresis along with the controls and a 100bp DNA molecular weight marker (Roche Diagnostic, Burgess Hill, UK) and visualised under UV light after ethidium bromide staining (Roche Diagnostic, Burgess Hill, UK).

3.8.4. Real Time Polymerase Chain Reaction

Prior to running Real-Time Polymerase Chain Reaction (RT-PCR'), complementary DNA (cDNA) was generated from the extracted RNA using reverse transcriptase as described above.

Leptin-receptor (Ob-Rb) levels contained in the samples were quantified using TaqMan® Real-Time PCR (LightCycler® 2.0, Roche Diagnostic, Burgess Hill, UK). TaqMan® probes are double-dyed oligonucleotides with a fluorophore attached to the 5' end of the probe and a dark quencher to the 3' end. When excited, the fluorophore transmits the energy to the quencher via Fluorescence Resonance Energy Transfer. While no light is emitted by the fluorophore when paired to the quencher, the 5'3' exonuclease of the Taq polymerase cleaves the 5' end of the probe, leading to

measurable light emission by the now free fluorophore. The probe used was labelled with fluorescein (FAM reporter) and Carboxytetramethylrhodamine (dark quencher TAMRA) (Roche Diagnostic, Burgess Hill, UK).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) being stably expressed in T-cells, it was used as the housekeeping gene to normalize gene expression. The sequence of the probe used to amplify the leptin receptor gene was Ob-Rb_{probe}: 5'-AAACCACATAGAATTTCTGACACATCATCTTTC-3', the sequence of the forward primer used was Ob-Rb_{forward primer}: 5'-GCCAACAACTGTGGTCTCTC-3' and the sequence of the reverse primer used was Ob-Rb_{reverse primer}: 5'-AGAGAAGCACTTGGTGACTG-3'. The sequence of the probe used to amplify the GAPDH gene was GAPDH_{probe}: 5'-GCTTGGTAAAAAGATGCTCAATG-3', the sequence of the forward primer used was GAPDH_{forward primer}: 5'-GCTTGGTAAAAAGATGCTCAATG-3' and the sequence of the reverse primer used was GAPDH_{reverse primer}: 5'-GCTTGGTAAAAAGATGCTCAATG-3'. The Master Mix was composed of 4µL of Lightcycler TaqMan MasterMix® (Roche Diagnosis, Burgess Hill, UK) and 2 µL of the primers and probes. Finally 5 µL of 1/10 diluted amplified cDNA was added to the Master Mix prior to be loading in 20 µL glass capillaries (Roche Diagnosis, Burgess Hill, UK). Samples were amplified using the Lightcycler® 2.0 and data were analysed with the Lightcycler® software 4.05. Relative levels of Ob-Rb expression was determined by normalizing the OB-Rb level to the endogenously expressed housekeeping gene (GAPDH) and the $\Delta\Delta C_t$ method, based on the threshold cycle (C_T), was used to estimate OB-Rb expression as described previously. (Livak & Schmittgen 2001):

$$\Delta\Delta C_T = (C_{T \text{ OB-Rb}} - C_{T \text{ GAPDH}})_{\text{stimulation}} - (C_{T \text{ OB-Rb}} - C_{T \text{ GAPDH}})_{\text{control}}$$

3.9. Statistical analysis

Data were analysed statistically using the software “Statistical Package for the Social Sciences” (SPSS, Chicago, IL, USA) and appropriate statistical tests for the experiments used. Specific details of the different statistical methods used to analyse the data comprised within this thesis are presented in the subsequent chapters to which they are relevant. All data in the different chapters of this thesis are presented as mean \pm SEM, unless stated otherwise. Statistical significance has been set at $p < 0.05$.

CHAPTER 4

**IMPACT OF AEROBIC FITNESS ON THE
PROPORTIONS OF SENESCENT BLOOD T-CELLS IN
MAN**

4.1. Introduction

Human ageing is associated with a progressive decline in the function of the immune system, which is commonly referred to as immunosenescence. This is characterised by poor vaccine efficacy, increased incidence of opportunistic infections, and high morbidity and mortality among the elderly (Koch *et al.* 2007). Immunosenescence is thought to be a consequence of an accumulation of multiple exposures to external pathogens and persistent viral infections throughout the lifespan, although certain lifestyle factors (i.e. smoking, inactivity, socio-economic status) have also been shown to accelerate telomere shortening and thus age-associated immune dysfunction (Valdes *et al.* 2005). This manifests as increased prevalence of infectious disease due to an impaired ability to recognize and respond to newly evolving pathogens (i.e. influenza, rhinovirus, respiratory syncytial virus). Although immunosenescence refers to a dysfunction in systemic immunity, it is the cellular components of the immune system, particularly of the adaptive arm (i.e. T-cells, B-cells and their products), that appear to diminish most with increasing age (Pawelec 2006; Pawelec *et al.* 2006). Indeed, age-related changes within the T-cell compartment are hallmark features of the immune risk profile (IRP), which is an amalgam of immune biomarkers that have been used to predict morbidity and mortality in seniors (Pawelec & Gouttefangeas 2006).

T-cell clonal expansion in response to an antigenic stimulus is a fundamental process of adaptive immunity, allowing for the formation of antigen specific effector T-cells to combat invading pathogens. However, this clonal expansion is not unending, and after repeated and excessive rounds of cell division, T-cells undergo cell cycle arrest and become senescent (Spaulding *et al.* 1999). In this state, T-cells will no longer clonally expand upon further antigenic stimulation, but will still retain effector cell properties

(i.e. recognizing and killing virally infected cells) and are still capable of producing large amounts of pro-inflammatory cytokines, such as TNF- α , IL-2 and TNF- γ (Effros *et al.* 2003; Vescovini *et al.* 2007) Senescent T-cells express a number of signature cell-surface proteins, such as the killer cell lectin-like receptor G1 (KLRG1) and/or CD57 (Voehringer *et al.* 2002; Brenchley *et al.* 2003) while also lacking surface expression of the co-stimulatory molecule CD28 (Brzezinska *et al.* 2004). With advancing age, there is a progressive reduction in the number of fully functional naïve antigen-virgin T-cells and a concomitant increase in the frequency of senescent T-cells within the peripheral blood compartment. This occurs due to the age-associated atrophy of the thymus and a consequential reduced output of naïve T-cells, coupled with the homeostatic proliferation and differentiation of activated/memory cells due to life-long exposure to various pathogens and persistent viral infections (Simpson 2011). In this instance, the accumulated senescent T-cells, which have limited antigen specificity, occupy the “immune space” at the expense of naïve cells leading to a severely restricted T-cell repertoire. This, in turn, contributes to higher rates of morbidity and mortality as a result of infectious disease.

Reconstituting the immune system by increasing thymic mass and output or removing excess clones of antigen-specific senescent T-cells, is of interest to those working in the immunotherapy fields as a means to maintain a diverse T-cell repertoire and reduce the incidences of infectious disease in the elderly. However, many of these therapeutic interventions (i.e. gene, cytokine, hormone and monoclonal antibody therapy) are expensive, risky and bear a large number of undesired side-effects, not to mention that the implementation of these invasive procedures in the elderly, who are not diseased per se, has ethical complications. As such, there has been recent interest in the manipulation of certain lifestyle factors that could be used as effective countermeasures against

immunosenescence within the ageing population. In particular, the beneficial effects of regular exercise on immunity have been well-documented in older adults, although the mechanisms that underpin enhanced immunity with regular exercise are not well understood (Shinkai *et al.* 1995; Simpson & Guy 2010). Nevertheless, many biomarkers associated with the IRP are positively displayed in physically active elderly compared to their sedentary counterparts (Simpson & Guy 2010), which include longer leukocyte telomere lengths, enhanced *in vitro* T-cell responses to mitogens (Ferguson *et al.* 1995), elevated *in vivo* immune responses to vaccines and recall antigens (Targonski *et al.* 2007), and increased IL-2 synthesis and expression of the IL2 receptor (Flurkey *et al.* 1992; Ferguson *et al.* 1995).

The maximal oxygen uptake ($\dot{V}O_{2\max}$) is a “gold standard” measure of aerobic capacity that is known to decline with age and is associated with risk of morbidity, mortality and quality of life (Jackson *et al.* 2009). Long-term follow-up studies have shown that individuals with a lower $\dot{V}O_{2\max}$ are more likely to develop hypertension, diabetes and metabolic syndrome as well as higher mortality rates due to cardiovascular disease and cancer (Blair *et al.* 1996; Lynch *et al.* 1996; Evenson *et al.* 2003; Barlow *et al.* 2006; Kodama *et al.* 2009). Increased proportions of highly differentiated effector-memory (TEMRA) and senescent T-cells are signature properties of an ageing immune system and a hallmark of the immune risk profile (IRP), which, in turn, is predictive of morbidity and mortality in the elderly. It is not known, however, if $\dot{V}O_{2\max}$ is associated with the ageing-induced accumulation of these functionally impaired T-cells. The aim of the study presented in this chapter, therefore, is to examine the impact of estimated $\dot{V}O_{2\max}$ on the age-associated accumulation of highly differentiated effector-memory and senescent T-cells in a population of healthy adult men. It is hypothesized that

estimated $\dot{V}O_{2\max}$ will offset the increased proportions of highly differentiated effector-memory and senescent T-cells that are seen with advancing age.

4.2. Materials and Methods

4.2.1. Subjects

A total of 102 healthy non-smoker males of various ethnicities aged 18-61 years old (Mean \pm SD: age= 39 ± 6 yrs; BMI= 25.5 ± 1.8 kg/m²) volunteered for this study. All subjects were recruited from the higher education-based community at Edinburgh Napier University, Scotland UK (n = 66; Mean \pm SD: age: 40 ± 5.5 yrs; BMI: 25.0 ± 1.7 kg/m²) or the University of Houston, Houston, TX, USA (n = 36; Mean \pm SD: age: 37 ± 6.5 yrs; BMI: 26.5 ± 1.8 kg/m²) and consisted of either students or academic members of staff. The percentage age distribution of the participants was: 18-31yrs: 29%; 32-41yrs: 20%; 42-51yrs: 31%; 52-61yrs: 20%. All subjects completed a health and lifestyle questionnaire prior to participating in this study. Subjects with excessive alcohol intake (>14 drinks per week), taking medication affecting the immune system, routinely using ibuprofen and/or aspirin, anti-depressants, medications designed to alter blood pressure or cardiovascular function and hormone replacement therapy were excluded from the subject pool. Subjects reporting major affective disorders, HIV infection, hepatitis, chronic/debilitating arthritis, central or peripheral nervous disorders, previous stroke or cardiac events, were bedridden in the past three months, suffer from known cardiovascular disease or autoimmune diseases were all excluded from the study.. Before their visit, subjects were instructed to refrain from any form of exercise

considered as “vigorous” (Jackson *et al.* 1990) for 24 h prior to their arrival at the laboratory. All volunteers gave written informed consent and ethical approval was obtained from the School of Life Sciences, Edinburgh Napier University and the Committee for Protection of Human Services at the University of Houston. The physical characteristics of the participants are presented in Table 4.1.

Table 4.1. Subjects’ characteristics (mean \pm SD)

	Mean	Standard deviation	Range
Age (yrs)	39	± 6	18 - 61
Body Mass (kg)	81	± 6	53 - 110
BMI (kg/m²)	25.5	± 1.8	20.1 - 35.1
% Body Fat	19.9	± 3.2	6.6 - 38
Physical Activity Rating (PA-R)	5	± 2	1 - 7
$\dot{V}O_{2max}$ (mL.kg⁻¹.min⁻¹)	41.7	± 3.7	27.8 - 58.5
Waist (cm)	87	± 5.8	64 - 117
Hips (cm)	92.8	± 4.6	77 - 111
Waist/Hips ratio	0.94	± 0.04	0.80 - 1.09

4.2.2. Anthropometric measurements

On arrival to the laboratory between 08:00 and 10:00 local time, subjects were seated for 5 minutes before anthropometric measurements were taken as per the general materials and methods *Chapter 3.1.2*. Bioelectrical impedance analysis was used to measure percentage body fat using a single-frequency (50 kHz) electric current produced by a tetrapolar bodystat 1500 (Bodystat, Douglas, UK) as described previously. (Ghosh *et al.* 1997).

4.2.3. Blood Sampling and PBMC isolation

Blood samples were drawn in 6 mL collection tubes (BD vacutainer™, Franklin Lakes, NJ, USA) containing a serum gel or spray-coated with lithium heparin to prevent coagulation and Peripheral Blood Mononuclear Cells (PBMC) were immediately isolated as described in the general materials and methods *Chapter 3.3*.

4.2.4. Serological Testing for latent viral IgG Antibodies

Anti-CMV, anti-EBV and anti-HSV-1 antibody titers were determined as described in the general materials and methods *Chapter 3.4*.

4.2.5. Submaximal Exercise Test to Estimate Maximal Oxygen Uptake and Physical Activity Rating (PA-R)

The $\dot{V}O_{2\max}$ of each subject was estimated using a submaximal cycling exercise protocol (Astrand 1960) as described in general materials and methods *Chapter 3.2.3*. Prior to exercise, each participant also provided his physical activity score using a 7-level questionnaire over the last 8 weeks, which assigns numerical value (Physical Activity Rating; PA-R) for infrequent (0-1), moderate (2-3) and vigorous (4-7) exercisers (Jackson *et al.* 1990). This PA-R was also used to estimate the participants' $\dot{V}O_{2\max}$ (Jackson *et al.* 1990). The relationship between $\dot{V}O_{2\max}$ estimated from the non-exercise questionnaire and the $\dot{V}O_{2\max}$ estimated from the submaximal cycling test was $r = 0.88$ ($p < 0.001$).

4.2.6. Labelling of cell surface antigens

Aliquots of 1×10^6 isolated PBMCs were labelled with a combination of directly conjugated monoclonal antibodies (mAbs) against the cell surface antigens KLRG1, CD57, CD28, CD45RA and CD45RO as described in the general materials and methods *Chapter 3.5.1*. The monoclonal antibodies were freshly diluted in PBS-BSA to their optimal concentration as determined by antibody titration before a volume of 50 μ L was added to the cell suspension. The monoclonal antibody panel used and the phenotypes of interest are presented in Table 3.2 and Table 4.2.

Table 4.2. Phenotypic identification of the blood T-cell subsets

Phenotypes	T-cell subsets	References
KLRG1-/CD28+	Naïve T-cells	(Koch <i>et al.</i> 2008)
KLRG1+/CD28- KLRG1+/CD57+	Senescent T-cells	(Voehringer <i>et al.</i> 2002; Ibegbu <i>et al.</i> 2005)
KLRG1+/CD28+	Memory T-cells	(Ibegbu <i>et al.</i> 2005)
CD45RA-/CD45RO+	Pan memory T-cells	(Akbar <i>et al.</i> 1988; Sanders <i>et al.</i> 1988)
CD45RA+/CD45RO+	Transitional T-cells	(Akbar <i>et al.</i> 1988; Sanders <i>et al.</i> 1988)

4.2.7. Flow cytometric analysis

PBMC phenotypes were assessed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA USA) at Edinburgh Napier University or on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) at the University of Houston as per the general materials and methods *Chapter 3.5.5*.

4.2.8. Statistical Analysis

All data were assessed for assumptions of normality and constant error variance prior to formal statistical testing. Skewed cell-surface phenotypic data was then normalised by logarithmic transformation prior to statistical analysis. Initial analysis was performed by linear regression to examine the association between age and the proportion of T-cell subsets expressing a particular cell surface phenotype in accordance with Table 4.2. When significant associations were found, additional variables (i.e. Age, estimated $\dot{V}O_{2max}$, BMI and % Body Fat) were systematically added to subsequent blocks of the hierarchical multiple regression models to evaluate their impact on the proportion of T-cell subsets (Table 4.3). In all models, standardized regression coefficients (B) and R^2 values are reported as a measure of association (Table 4.4). Linear regression models were created by including the centered independent variables as suggested by Aiken and West (1991) to eliminate doubts of potential multicollinearity between the different variables. No significant interactions were found between the independent variables tested so interaction effects were eliminated from the final analysis. A one-way ANOVA was also used to detect differences among age-adjusted $\dot{V}O_{2max}$ classifications (Whaley *et al.* 2006). When significant group effects were found, student *t*-tests with Bonferroni correction were performed to detect location of significance. Independent *t*-tests were used to compare physical characteristics and T-cell phenotypes due to latent CMV, EBV or HSV-1 serostatus. All values are presented as the mean \pm Standard Deviation (SD) and all statistical analyses were performed using “Statistical Package for the Social Sciences” (SPSS v17.0, Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

4.3. Results

4.3.1. The effects of age and estimated $\dot{V}O_{2max}$ on T-cell phenotypes

The impact of age or estimated $\dot{V}O_{2max}$ on the T-cell subsets was initially determined by univariate regression analysis (Table 4.3). As expected, a positive relationship was found between age and the proportion of senescent (KLRG1+/CD57+) CD4+ and CD8+ T-cells ($r = 0.264$ and 0.363 respectively; $p < 0.05$). The proportion of senescent CD4+ and CD8+ T-cells defined as KLRG1+/CD28- were also positively associated with age ($r = 0.182$ and 0.377 respectively; $p < 0.05$ and $p < 0.001$) (Table 4.3). Similarly, a positive relationship between age and the proportion of CD4+ ($r = 0.436$; $p < 0.01$) and CD8+ ($r = 0.411$; $p < 0.01$) T-cells with a transitional (CD45RA+/CD45RO+) phenotype were observed (Table 4.3). Similar positive relationships were seen between estimated $\dot{V}O_{2max}$ and the frequencies of naïve (KLRG1-/CD28+) CD4+ and CD8+ T-cells ($r = 0.171$ and 0.224 respectively; $p < 0.05$), while estimated $\dot{V}O_{2max}$ was inversely associated with senescent (KLRG1+/CD57+) and transitional (CD45RA+/CD45RO+) CD4+ ($r = -0.340$ and -0.403 respectively; $p < 0.01$) and CD8+ T-cells ($r = -0.287$ and -0.336 respectively; $p < 0.01$). Only transitional T-cells were positively associated with BMI ($r = 0.255$ and 0.215 for CD4+ and CD8+ T-cells respectively; $p < 0.05$). The associations with T-cell phenotype and BMI were mirrored when percentage body fat was used instead of BMI, but for clarity only the BMI data is shown.

Table 4.3. Univariate regression analysis of age, estimated $\dot{V}O_{2max}$ and BMI on T-cell subsets.

T-cell subsets	Pearson's R (p value)			F statistic (p value)		
	Age	$\dot{V}O_{2max}$	BMI	Age	$\dot{V}O_{2max}$	BMI
CD4+ T-cells						
KLRG1+/CD57+ (Senescent)	0.264 (0.004)	-0.340 (0.000)	0.028 (0.388)	7.502 (0.007)	13.035 (0.000)	0.081 (0.776)
KLRG1+/CD28- (Senescent)	0.182 (0.034)	-0.221 (0.013)	0.073 (0.235)	0.745 (0.39)	5.091 (0.026)	0.528 (0.469)
KLRG1+/CD28+ (Memory)	0.052 (0.304)	-0.023 (0.410)	-0.102 (0.154)	0.264 (0.609)	0.052 (0.820)	1.046 (0.309)
KLRG1-/CD28+ (Naïve)	-0.275 (0.003)	0.171 (0.044)	0.073 (0.235)	8.120 (0.005)	2.977 (0.088)	0.538 (0.469)
CD45RA+/CD45RO+ (Transitional)	0.436 (0.000)	-0.403 (0.000)	0.255 (0.011)	18.267 (0.000)	15.086 (0.000)	5.414 (0.023)
CD8+ T-cells						
KLRG1+/CD57+ (Senescent)	0.363 (0.000)	-0.287 (0.002)	-0.043 (0.334)	15.139 (0.000)	8.961 (0.003)	0.186 (0.667)
KLRG1+/CD28- (Senescent)	0.377 (0.000)	-0.350 (0.000)	0.024 (0.405)	12.974 (0.000)	13.852 (0.000)	0.058 (0.810)
KLRG1+/CD28+ (Memory)	0.368 (0.000)	-0.137 (0.086)	-0.033 (0.371)	15.517 (0.000)	1.897 (0.172)	0.110 (0.741)
KLRG1-/CD28+ (Naïve)	-0.189 (0.029)	0.224 (0.012)	0.024 (0.405)	3.655 (0.059)	5.247 (0.024)	0.058 (0.810)
CD45RA+/CD45RO+ (Transitional)	0.411 (0.000)	-0.336 (0.001)	0.215 (0.027)	16.094 (0.000)	10.030 (0.002)	3.823 (0.054)

4.3.2. The impact of $\dot{V}O_{2max}$ on age-associated changes in T-cell phenotype

T-cell phenotypes found to be associated with age and/or estimated $\dot{V}O_{2max}$ were examined by hierarchical multiple regression analysis to determine if these associations were modified when adjusted for the other (Table 4.4). The potential confounders BMI, percentage body fat or hip/waist ratio were also added to the model, although for clarity only the BMI results are shown. Age was no longer associated with the proportions of senescent CD4+ (KLRG1+/CD57+) or CD8+ (KLRG1+/CD28-) T-cells when adjusted for estimated $\dot{V}O_{2max}$ ($p > 0.05$). Similarly, proportions of naïve (KLRG1-/CD28+) CD4+ or CD8+ T-cells were not associated with age after adjusting for estimated $\dot{V}O_{2max}$ ($p > 0.05$). A moderating effect of estimated $\dot{V}O_{2max}$ was also found for pan-

effector-memory (defined as KLRG1+) CD4+ and CD8+ T-cells ($B = -0.569$ and -1.186 respectively), although age was still significantly associated ($B = 0.542$; $p < 0.01$). The association between estimated $\dot{V}O_{2\max}$ and senescent CD4+ (KLRG1+/CD57+) and CD8+ (KLRG1+/CD28-) T-cells withstood adjustment for age (both $B = -0.953$; $p < 0.05$). Moreover, the association between estimated $\dot{V}O_{2\max}$ and naïve (KLRG1-/CD28+) CD8+ T-cells ($B = 1.042$; $p < 0.01$) but not CD4+ T-cells ($p > 0.05$) also withstood age adjustment. Similar results, albeit with smaller effect sizes, were observed when self-reported physical activity ratings were used in the models instead of estimated $\dot{V}O_{2\max}$ (data not shown). All moderating effects of estimated $\dot{V}O_{2\max}$ presented in Table 4.4 withstood adjustments for BMI and percentage body fat ($p < 0.05$). The associations between BMI and transitional (CD45RA+/CD45RO+) CD4+ and CD8+ T-cells were eliminated when adjusted for age and estimated $\dot{V}O_{2\max}$ ($p > 0.05$). Marginal but non-significant effects (i.e. $p = 0.05$ to $p = 0.10$) for BMI were found for senescent (KLRG1+/CD57+) CD8+ T-cells ($p = 0.07$) and memory (KLRG1+/CD28+) CD4+ T-cells ($p = 0.05$) after adjusting for age and estimated $\dot{V}O_{2\max}$.

Table 4.4. Hierarchical multiple regression statistics for age, estimated $\dot{V}O_{2max}$ and BMI on T-cell subsets after adjusting for the other factors.*indicates significant relationship between estimated $\dot{V}O_{2max}$ and T-cell subsets after age, BMI adjustment ($p < 0.05$).

T-cell subsets	Factors	CD4+ T-cells					CD8+ T-cells				
		R ²	B	SEB	F statistic (p value)	t statistic (p value)	R ²	B	SEB	F statistic (p value)	t statistic (p value)
KLRG1+ (Pan Effector-Memory)	Age		-0.071	0.075		-0.946 (0.347)		0.542	0.173		3.139 (0.002)
	$\dot{V}O_{2max}$	0.129	-0.569	0.207	3.599 (0.009)	-2.747 (0.007)*	0.359	-1.186	0.480	13.609 (0.000)	-2.473 (0.015)*
	BMI		-0.477	0.305		-1.566 (0.121)		-0.701	0.706		-0.994 (0.323)
KLRG1+/CD57+ (Senescent)	Age		1.000	1.007		0.023 (0.982)		0.204	0.152		1.345 (0.182)
	$\dot{V}O_{2max}$	0.147	-0.953	1.021	4.166 (0.004)	-2.375 (0.019)*	0.179	-0.690	0.421	5.282 (0.001)	-1.638 (0.105)
	BMI		-0.964	1.030		-1.207 (0.230)		-1.153	0.620		-1.861 (0.066)
KLRG1+/CD28- (Senescent)	Age		0.993	1.012		-0.734 (0.465)		1.005	1.007		0.690 (0.492)
	$\dot{V}O_{2max}$	0.048	-0.955	1.030	1.205 (0.314)	-1.529 (0.130)	0.178	-0.953	1.023	5.205 (0.098)	-2.247 (0.027)*
	BMI		-0.971	1.045		-0.701 (0.485)		-0.955	1.033		-1.456 (0.149)
KLRG1-/CD28+ (Naive)	Age		-0.998	1.002		-1.834 (0.070)		-1.002	1.005		0.576 (0.566)
	$\dot{V}O_{2max}$	0.077	1.000	1.005	2.015 (0.098)	0.092 (0.927)	0.116	1.042	1.016	3.135 (0.018)	2.665 (0.009)*
	BMI		1.002	1.007		0.359 (0.720)		1.038	1.023		1.581 (0.117)
CD45RA+/CD45RO+ (Transitional)	Age		0.009	0.003		2.752 (0.007)		0.010	0.004		2.901 (0.005)
	$\dot{V}O_{2max}$	0.238	-0.004	0.008	7.916 (0.000)	-0.540 (0.591)	0.200	0.000	0.008	6.420 (0.001)	-0.005 (0.996)
	BMI		0.012	0.013		1.086 (0.281)		0.016	0.013		1.188 (0.239)
KLRG1+/CD28+ (Memory)	Age		0.000	0.057		-0.008 (0.994)		0.410	0.452		3.580 (0.001)
	$\dot{V}O_{2max}$	0.045	0.033	0.158	1.137 (0.344)	0.208 (0.836)	0.157	0.452	0.316	4.478 (0.002)	1.427 (0.157)
	BMI		-0.452	0.226		-1.998 (0.050)		-0.192	0.455		-0.422 (0.674)

4.3.4. T-cell Phenotypes and Age-related $\dot{V}O_{2max}$ classifications

As age and estimated $\dot{V}O_{2max}$ were co-related ($r = -0.563$; $p < 0.001$), the impact of age-related estimated $\dot{V}O_{2max}$ classifications on the phenotype characteristics of CD4+ and CD8+ T-cells was investigated. All participants were divided into 3 groups using the age-adjusted $\dot{V}O_{2max}$ classifications provided by the American College of Sports Medicine (ACSM) (Whaley *et al.* 2006). Participants with an age-adjusted $\dot{V}O_{2max}$ ranked from the 10th to the 39th percentiles were classified as “Below Average”, whereas participants with an age-adjusted $\dot{V}O_{2max}$ ranked from the 40th to the 69th and 70th to the 99th percentiles were classified as “Average” and “Above Average” respectively. The physical characteristics of the participants divided into these groups are presented in Table 4.5. Participants among the $\dot{V}O_{2max}$ groups did not differ in terms of age ($p > 0.05$), although individuals in the below average group had a significantly greater BMI and % Body fat ($p < 0.05$) compared to the other subject groups. However, it was already established by linear regression that estimated $\dot{V}O_{2max}$ was associated with specific T-cell phenotypes even after BMI and % Body fat adjustment (Table 4.4) and were therefore not considered to be confounding factors.

Table 4.5. Subject's physical characteristics classified according to their age-adjusted $\dot{V}O_{2max}$. Values are mean \pm SD. Statistically significant difference from below average indicated by * ($p < 0.05$); Statistically significant difference from average indicated by # ($p < 0.05$)

	$\dot{V}O_{2max}$ below average	$\dot{V}O_{2max}$ average	$\dot{V}O_{2max}$ above average
n	36	25	41
Age (yrs)	40.7 \pm 2.3	40.2 \pm 1.9	37.1 \pm 1.9
BMI (kg/m²)	28.4 \pm 0.6	25.2 \pm 0.5 *	23.2 \pm 0.4 *#
% Body Fat	25.5 \pm 1	19.4 \pm 0.6 *	15.4 \pm 0.7 *#
$\dot{V}O_{2max}$	34.4 \pm 0.8	43 \pm 0.6 *	47.3 \pm 0.9 *#
PA-R	3.6 \pm 0.3	5.5 \pm 0.2	6.4 \pm 0.2

T-cell phenotype comparisons among the three $\dot{V}O_{2max}$ tertiles are presented in Figure 4.1. The above average $\dot{V}O_{2max}$ tertile had 21.1% and 22.6% less pan-effector-memory (KLRG1+) CD8+ T-cells compared to the below average and average tertiles respectively ($p < 0.05$) (Figure 4.1). The proportion of senescent (KLRG1+/CD57+) CD4+ T-cells in the above average tertile was 56.3% and 57.8% lower than the average and below average tertiles respectively ($p < 0.05$). The above average tertile had 37% less senescent (KLRG1+/CD57+) CD8+ T-cells compared to the average tertile ($p < 0.05$), but no differences were found between the below average and above average tertiles ($p > 0.05$). The above average tertile had a 16.4% and 17.5% higher proportion of naïve (KLRG1-/CD28+) CD8+ T-cells compared to the average and below average tertiles respectively ($p < 0.05$). Consistent with the regression analysis, no differences were found for transitional (CD45RA+/CD45RO+) or memory (KLRG1+/CD28+) T-cells within CD4+ or CD8+ subsets among the $\dot{V}O_{2max}$ classifications ($p > 0.05$). Fitted line plots with individual subject data for proportions of senescent (KLRG1+/CD57+) CD4+ and CD8+ T-cells and naïve (KLRG1-/CD28+) CD4+ and CD8+ T-cells in

accordance with age-related $\dot{V}O_{2\max}$ classification tertiles are presented in Figure. 4.2. A below average $\dot{V}O_{2\max}$ is associated with an increased rate of senescent (KLRG1+/CD28-) CD4+ and CD8+ T-cells accumulation with ageing, when compared to average and above average $\dot{V}O_{2\max}$. Furthermore, the decline in number of naïve (KLRG1-/CD28+) CD8+ T-cells is reduced in subjects with above average $\dot{V}O_{2\max}$ when compared to subjects with lower $\dot{V}O_{2\max}$.

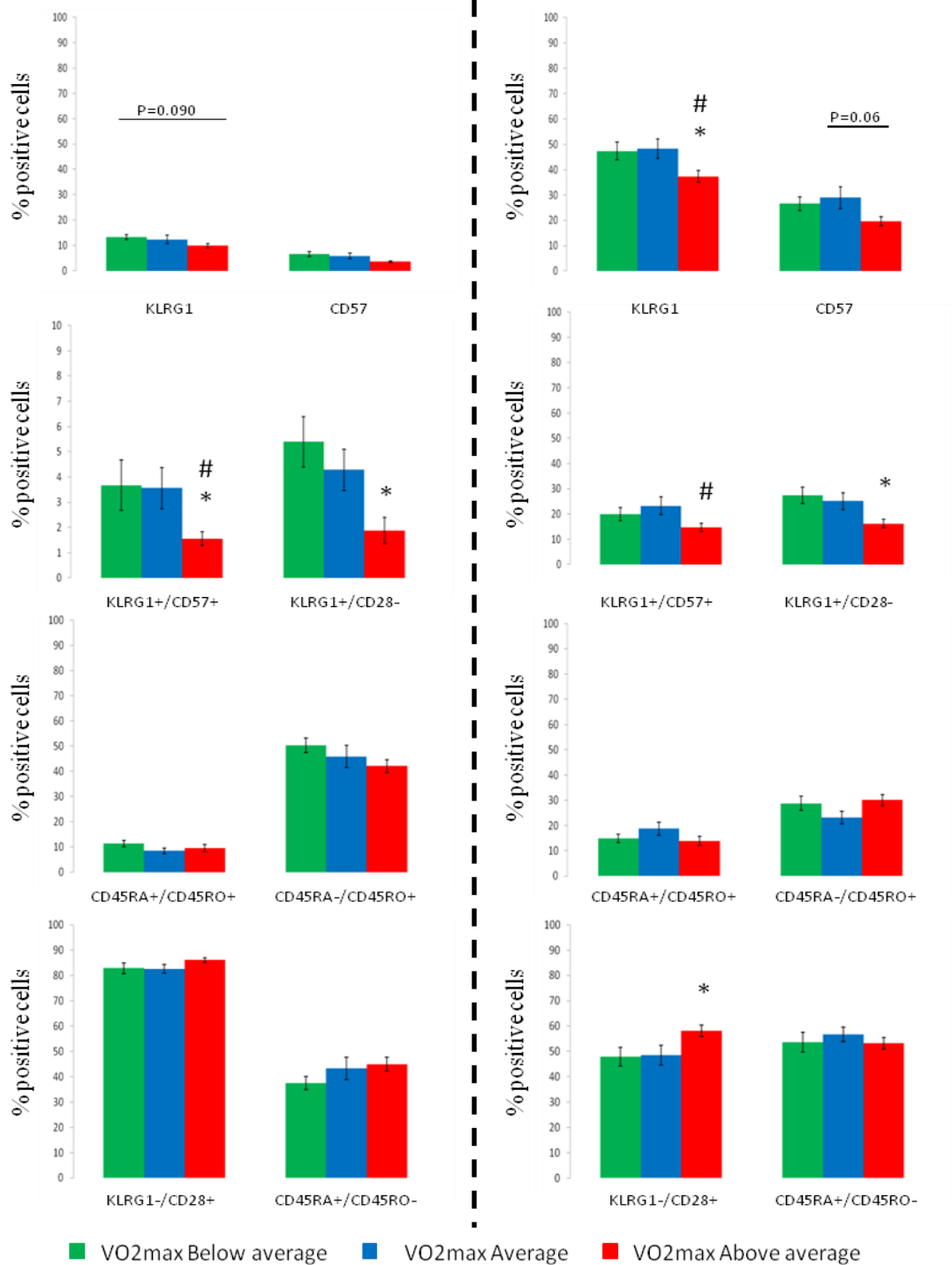


Figure 4.1. The percentage of all CD4+ (left column) and CD8+ (right column) T-cells expressing surface markers of naïve (KLRG1-/CD28+; CD45RA+/CD45RO-), memory (CD45RA-/CD45RO+), transitional (CD45RA+/CD45RO+) and senescent (KLRG1+/CD57+; KLRG1+/CD28-) T-cells among the different age-adjusted $\dot{V}O_{2max}$ classifications. Values are mean \pm SD. Statistically significant difference from below average indicated by * ($p < 0.05$); Statistically significant difference from average indicated by # ($p < 0.05$).

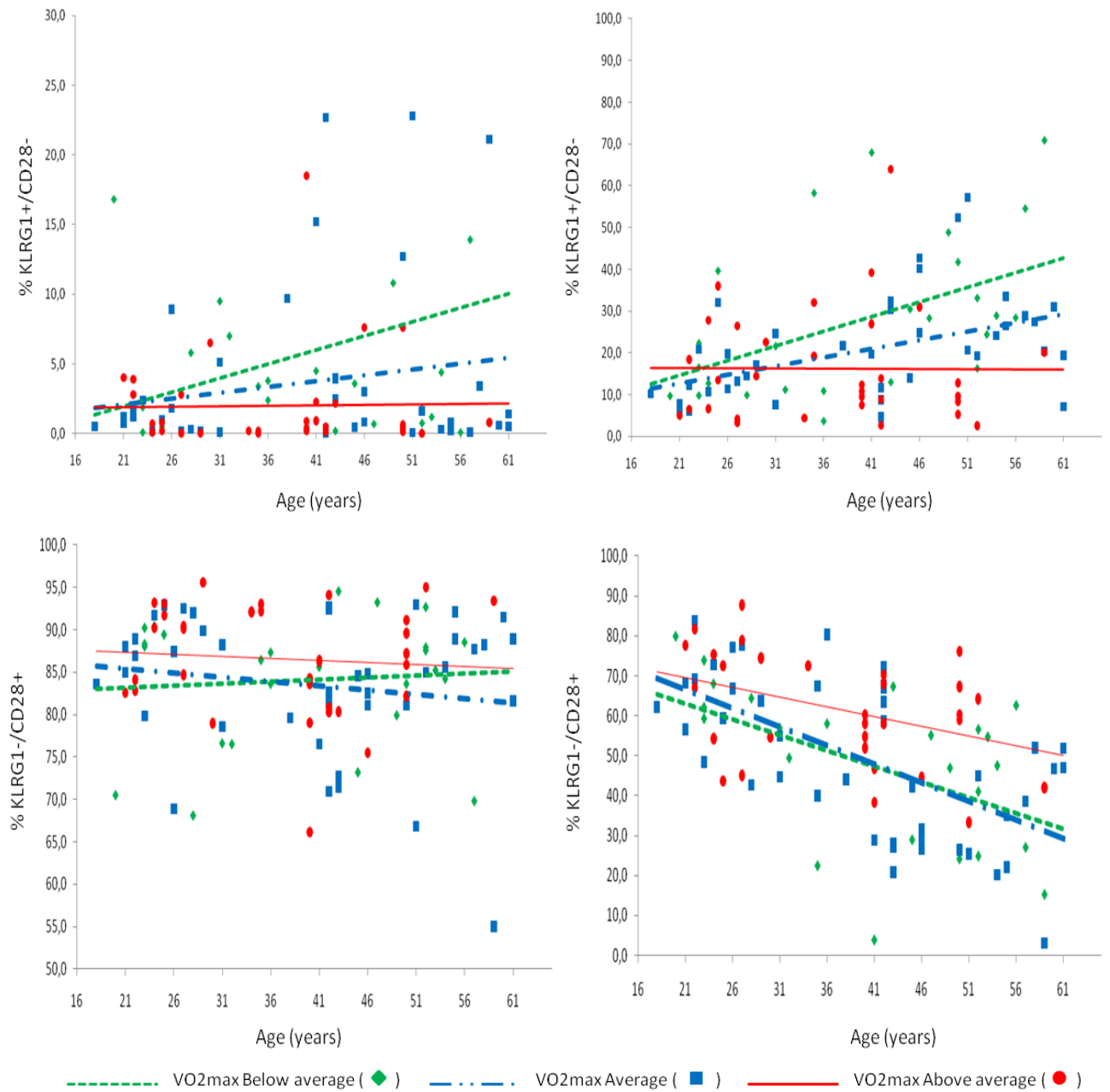


Figure 4.2. Changes in the percentage of all CD4+ (left column) and CD8+ (right column) T-cells expressing surface markers of naïve (KLRG1-/CD28+) and senescent (KLRG1+/CD28-) T-cells as a function of age. Changes with age in accordance with $\dot{V}O_{2max}$ classification are also shown.

4.3.5. Physical Activity Rating (PA-R) and T-cell phenotypes

To determine whether scores of physical activity, as opposed to estimated $\dot{V}O_{2\max}$, influenced blood T-cell phenotypes, all participants were divided into three groups in accordance with their self-reported PA-R following the procedures described by Jackson *et al.* (1990). The results could not be analysed using the multiple regression model analysis alone due to the lack of linearity between the PA-R scores. Indeed the difference of physical activity level between the PA-R 6 and 7 are much greater than the difference of physical activity level between the PA-R 1 and 2. Those with a score of 1-2, 3-4 and 5-7 were considered to have a “low PA-R”, “moderate PA-R” and “high PA-R” respectively. The physical characteristics of the participants divided into these groups are presented in Table 4.6. No differences were found among the groups for age, however while individuals with a greater PA-R had a lower BMI and % Body Fat ($p < 0.05$) than the individuals in the two other groups, the regression model analysis established the absence of impact of these factors on the different T-cells subsets. Those with a low PA-R had a 67.8% and 55.7% greater proportion of senescent KLRG1+/CD57+ CD4+ T-cells compared to average and high PA-R group respectively ($p < 0.05$). However, despite finding differences in senescent KLRG1+/CD57+ CD8+ T-cells among the $\dot{V}O_{2\max}$ groups, no significant differences were found among the three PA-R groups for this T-cell subset ($p > 0.05$). Interestingly individuals with a high PA-R had a 33.9% lesser proportion of KLRG1+/CD28- CD8+ T-cells, another senescent phenotype compared to individuals with a low PA-R ($p < 0.05$). Similarly, the differences in the proportion of KLRG1-/CD28+ naïve CD8+ T-cells that were associated with estimated $\dot{V}O_{2\max}$ were not seen among the different PA-R groups ($p > 0.05$), instead new statistical differences in the proportions of naïve

CD45RA+/CD45RO- CD4+ are being seen among the different PA-R groups (Figure. 4.3). Individuals with an average PA-R had a 29.9% greater proportion of naïve CD45RA+/CD45RO- CD4+ T-cells compared to individuals with a low PA-R ($p < 0.05$). Similarly, individuals with an average PA-R had 16% greater proportions of naïve CD45RA/CD45RO- CD8+ T-cells than the individuals with a high PA-R ($p < 0.05$).

Table 4.6. Subject's physical characteristics classified according to their PA-R. Values are mean \pm SD. Statistically significant difference from low PA-R indicated by * ($p < 0.05$); Statistically significant difference from medium PA-R indicated by # ($p < 0.05$).

	Low PA-R	Medium PA-R	High PA-R
n	19	28	57
Age (yrs)	41 \pm 3.5	36.4 \pm 2.7	39.8 \pm 1.4
BMI (kg/m²)	28.9 \pm 0.9	26.4 \pm 0.7 *	24.1 \pm 0.4 *#
% Body Fat	25.4 \pm 1.5	21.1 \pm 1.3 *	17.6 \pm 0.7 *#
$\dot{V}O_{2max}$	32.7 \pm 1.1	39.4 \pm 1.1 *	45.8 \pm 0.8 *#

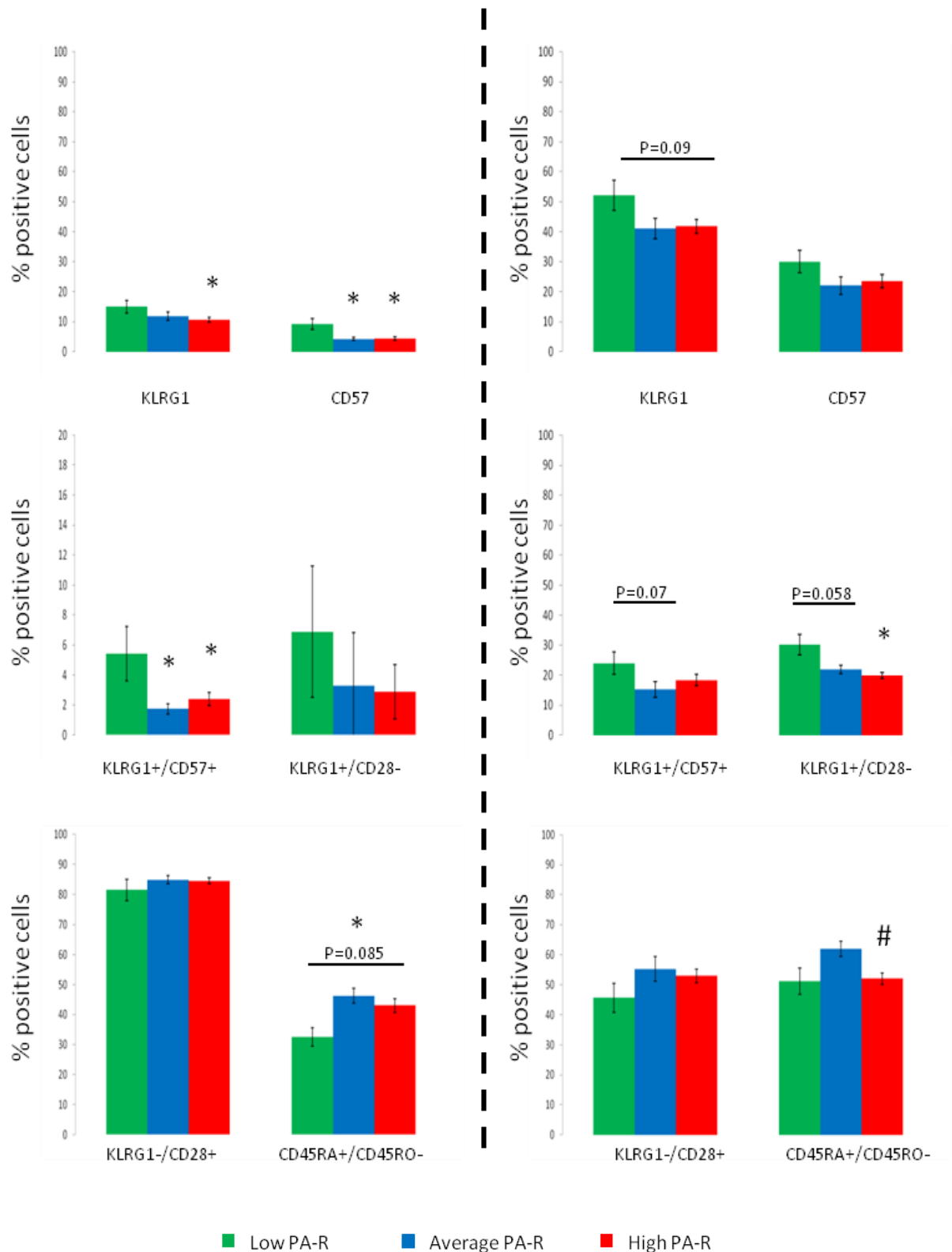


Figure 4.3. The percentage of all CD4+ (left column) and CD8+ (right column) T-cells expressing surface markers of naïve (KLRG1-/CD28+; CD45RA+/CD45RO-) and senescent (KLRG1+/CD57+; KLRG1+/CD28-) T-cells among the different level of physical activity (PA-R). Values are mean \pm SD. Statistically significant difference from below average indicated by * ($p < 0.05$); Statistically significant difference from average indicated by # ($p < 0.05$).

4.3.6. *The Impact of latent CMV, EBV and HSV-1 infections*

As the moderating effects of estimated $\dot{V}O_{2\max}$ on age-associated changes in T-cell phenotype may be confounded by latent viral infections, it was important to determine the CMV, EBV and HSV-1 serostatus on the Houston subject cohort (n=36). Due to the greater prevalence of these viruses with age, subjects were age-matched in accordance with their serostatus and compared for physical characteristics and T-cell phenotypes. Neither CMV, EBV or HSV-1 serostatus was associated with estimated $\dot{V}O_{2\max}$, PA-R, BMI or percentage body fat (data not shown; $p > 0.05$). Latent CMV infection was associated with a greater proportion of senescent (KLRG1+/CD57+; $20.2 \pm 1.9\%$ versus $10.9 \pm 0.8\%$) and lower proportion of naïve (KLRG1-/CD28+; $51 \pm 3.3\%$ versus $61 \pm 2.2\%$) CD8+ T-cells compared to the non-infected. Similarly, CMV infection was associated with a greater proportion of senescent (KLRG1+/CD57+; $3.4 \pm 0.4\%$ versus $1.9 \pm 0.3\%$) CD4+ T-cells. However, the frequencies of naïve (KLRG1-/CD28+; $82.5 \pm 1.3\%$ versus $82.8 \pm 1.2\%$) CD4+ T-cells were not affected by CMV infection. T-cell phenotypes were not influenced by latent EBV or HSV-1 infection (data not shown; $p > 0.05$).

4.4. Discussion

Increased proportions of effector-memory and senescent T-cells are striking features of an ageing immune system and a hallmark of the immune risk profile (IRP). The results presented in this study sought to examine for the first time the impact of estimated

maximal aerobic capacity ($\dot{V}O_{2max}$) as a measure of aerobic fitness on the proportions of naïve, memory, effector-memory and senescent blood T-cell populations that are known to undergo profound age-associated changes. As expected, the proportions of senescent CD4+ and CD8+ T-cells increased with advancing age at a respective rate of 10% and 10.2% per decade. This was accompanied by a per decade reduction in the proportions of naïve CD4+ and CD8+ T-cells of 10 % and 9.9 % respectively. Supporting the main hypothesis of the study presented in this chapter, we found that those with above average $\dot{V}O_{2max}$ scores had less senescent CD4+ and CD8+ T-cells and more naïve CD8+ T-cells than those with below average $\dot{V}O_{2max}$ scores, even after adjusting for age, body mass index and percentage body fat. Strikingly, it was found that the well-accepted association between age and senescent T-cells no longer existed when age was adjusted for $\dot{V}O_{2max}$, indicating that aerobic fitness may be a stronger determinant of T-cell phenotypic shifts than chronological age. This effect was limited to the senescent cells as estimated $\dot{V}O_{2max}$ was not associated with the memory (KLRG1+/CD28+) cells after adjusting for age. Interestingly, a similar effect of high PA-R on the proportions of senescent T-cells could not be observed. This can be explained by the lack of linearity within the PA-R rating scale, which is likely to have reduced the power of the statistical analysis. This is the first study to show that aerobic fitness moderates the natural age-related accumulation of senescent T-cells in peripheral blood, highlighting the beneficial effects of maintaining a physically active lifestyle on the ageing immune system.

Increased proportions of effector-memory and senescent T-cells have been found in blood due to ageing (Simpson *et al.* 2008), psychological adverse working conditions (Bosch *et al.* 2009) and persistent viral infections (Thimme *et al.* 2005). Although

recent studies indicate that a sedentary lifestyle promotes biological ageing and telomere shortening (Cherkas *et al.* 2008; Ludlow *et al.* 2008; LaRocca *et al.* 2010; Song *et al.* 2010), the study presented in this chapter is the first study to our knowledge to report a moderating effect of aerobic fitness on the age-related accumulation of senescent T-cells. The results show that, on average, a difference in estimated $\dot{V}O_{2\max}$ of $10\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, which corresponds to a transition from the below average to the above average $\dot{V}O_{2\max}$ category, was associated with a 9.5% difference in the proportion of senescent CD8+ T-cells independently of age. As the average proportion of senescent CD8+ T-cells was found to increase by 10% per decade, this indicates that aerobic fitness could play a major role in shaping the ageing T-cell compartment. Indeed, senescent T-cells are known to have critically short telomeres (Olovnikov 1973; Karlseider *et al.* 2002) and have been associated with poor vaccine efficacy, impaired immune vigilance and greater morbidity and mortality as a result of infectious disease (Wikby *et al.* 1998). As such, the data presented in this chapter bolsters the intuition that regular physical activity may exert preventative and/or rejuvenating properties on the ageing immune system by dampening the age-related accumulation of senescent T-cells and preventing early transition to the IRP category in later life (Simpson & Guy 2010).

Aerobic fitness also moderated the proportions of naïve CD8+ T-cells, which were positively associated with estimated $\dot{V}O_{2\max}$ scores even after adjusting for age. Remarkably, the associations observed between age and naïve and senescent T-cells did not withstand adjustment for estimated $\dot{V}O_{2\max}$, indicating that ageing may be secondary to changes in aerobic fitness at shaping T-cell phenotypic shifts. This seems plausible as $\dot{V}O_{2\max}$ is also known to decline with age, although not conspicuously so until after 40yrs of age (Jackson *et al.* 2009). It is plausible to postulate that maintaining high

levels of aerobic fitness during the natural course of ageing may help prevent the accumulation of senescent T-cells that have limited antigenic specificity, whilst also maintaining adequate numbers of naïve T-cells capable of recognizing and responding to novel pathogens. Moreover, because many of the currently available therapeutic interventions (i.e. gene, cytokine, hormone and monoclonal antibody therapy) that have been proposed to negate ageing immunity (Greenstein *et al.* 1987; Kendall *et al.* 1990; Phillips *et al.* 2004; Rosenberg *et al.* 2006) are risky and bear a large number of undesired side effects and ethical complications, physical exercise could be considered a safer alternative strategy to combat age-related immunosenescence. It is acknowledged, however, that the presently reported links between estimated $\dot{V}O_{2\max}$ and T-cell phenotypes have been obtained using cross-sectional data and a longitudinal study is required to determine if increases in $\dot{V}O_{2\max}$ are accompanied by changes in the proportions of naïve and senescent T-cells.

The impact of estimated $\dot{V}O_{2\max}$ on the T-cell subsets withstood adjustment for, BMI, percentage body fat and waist/hip ratio, suggesting that the moderating effects of estimated $\dot{V}O_{2\max}$ were not secondary to differences in body composition. However, previous studies have documented negative associations linking BMI, % Body fat, and biological markers of adiposity such as leptin with leukocyte telomere length (Valdes *et al.* 2005; Lee *et al.* 2010) and reduced thymic output (Yang *et al.* 2009). Although the measures of adiposity presented in this study were not related with immunosenescence, this was not surprising as very few subjects (7%) in the present cohort were classified as obese (BMI > 30 kg/m²) as the aim of the study presented in this chapter was to determine the association of aerobic fitness on blood T-cell phenotypes independently of BMI and % Body fat, which are known confounders. It remains possible, and very

likely, therefore, that excess adiposity will be related with increased senescent blood T-cells when more severe classifications of obesity are examined. Indeed, BMI was marginally associated with the proportions of memory (KLRG1+/CD28+) CD4+ T-cells and senescent (KLRG1+/CD57+) CD8+ T-cells after age and estimated $\dot{V}O_{2\max}$ adjustment.

The mechanisms by which regular exercise exerts positive effects on the ageing immune system remain to be established. It has been suggested that the beneficial effects of exercise may come from either prevention and/or rejuvenation perspectives (Simpson & Guy 2010). Firstly, from a prevention standpoint, exercise may elicit secondary effects on the immune system due to its well-known stress reducing properties (Salmon 2001), thus limiting the potential for stress-induced latent viral reactivation and telomere erosion. Secondly, from a rejuvenation standpoint, regular exercise may lead to the destruction of excess viral specific T-cell clones via apoptosis, freeing up “immune space” for naïve T-cells to occupy and expand the antigenic T-cell repertoire (Simpson 2011). Furthermore, it was shown recently that exercise elicits Interleukin-7 release from active skeletal muscle (Haugen *et al.* 2010). This cytokine is known to play an important role in naïve T-cell homeostasis and the output of recent thymic emigrants (Schluns *et al.* 2000) and may therefore be involved in the aetiology of exercise-induced immune enhancement.

As latent herpesvirus infections, particularly CMV, are associated with a greater number and proportion of senescent T-cells (Ouyang *et al.* 2004), CMV, EBV and HSV-1 serostatus were considered as potential confounders of the moderating effects of estimated $\dot{V}O_{2\max}$. Although CMV, but not EBV or HSV-1, serpositivity was associated

with a greater proportion of senescent CD4⁺ and CD8⁺ T-cells, none of these latent viruses influenced $\dot{V}O_{2\max}$ scores. It is therefore possible to consider estimated $\dot{V}O_{2\max}$ to be associated with blood T-cell phenotypes independently of these persistent viral infections. It is acknowledged, however, that the sample size used for the serostatus determination was relatively small and the possibility that relationships exist between aerobic fitness and latent viral infections cannot be ruled out if a larger cohort is examined. Furthermore, another limitation lies in the $\dot{V}O_{2\max}$ determination. Indeed to limit the physical stress to be endured by the older volunteers, individuals' $\dot{V}O_{2\max}$ were estimated from a submaximal cycling protocol. Although the protocol created by Astrand *et al.* (196) has been validated and used by the scientific community for the past 50 years, and although a validated non-exercise questionnaire (Jackson *et al.* 1990) was also used to compare the estimated $\dot{V}O_{2\max}$ value obtained by the cycling protocol (Pearson's R = 0.88), small inaccuracies could have occurred. It is however important to highlight that such inaccuracies would have been more likely to happen in an athletic population, than in the normal population of subject used. Additional limitations of the present study include the non-inclusion of women and the elderly (i.e. over 65yrs), and, because the subject recruited to be part of the study presented in this chapter was limited to students and members of academic staff at institutions of higher education, participants of lower socioeconomic standing were less likely to be recruited and may explain why estimated $\dot{V}O_{2\max}$ was not related to latent viral infections in our study.

In conclusion, aerobic fitness is associated with a lower proportion of senescent and a higher proportion of naïve cells, particularly within the CD8⁺ T-cell compartment in healthy adult men. These findings withstood adjustment for a number of potential confounders (i.e. age, BMI, % body fat, latent viral infections) indicating that high

levels of aerobic fitness could independently exert protective effects on the ageing immune system. Future longitudinal studies are required to determine if changes in $\dot{V}O_{2\max}$ due to exercise training will be accompanied by changes in the composition of naïve and senescent T-cells. It will be of particular interest to determine if exercise can improve immunity in those displaying biomarkers associated with the IRP, as this would help to establish if regular exercise has immune restorative properties. Furthermore, the results presented in this chapter were obtained on a relatively lean cohort (less than 7% of the subjects had a BMI above 30 kg/m²), potentially explaining the lack of confounding effect of BMI and % Body fat on the T-cell repertoire. The plausible implication of excess body mass and adiposity on the accumulation of senescent T-cells and the premature onset of immunosenescence is consequently required to be further investigated in an age-controlled obese population.

CHAPTER 5

THE IMPACT OF CHILDHOOD OBESITY AND PERSISTENT VIRAL INFECTION ON THE T-CELL DIFFERENTIATION ASSOCIATED WITH IMMUNOSENESCENCE

5.1. Introduction

Reduced physical activity and sustained excessive energy intake, amongst other obesity-related behavior and genetic factors, have led to the fast increasing number of overweight and obese adults observed in both industrialized and developing countries (Mokdad *et al.* 2001; Deitel 2003; Chaput & Tremblay 2009). This is not limited to adults as childhood obesity has more than tripled in the past 25 years, rising from 5% of the 12-19 years old who were obese in 1980 to 18% in 2008 (Skelton *et al.* 2009). Alarming, the proportion of overweight adolescents in the United States is even greater, with almost a third of the 12-19 years old (31.7%) with an excess body mass (Ogden *et al.* 2010). Childhood obesity affects adolescents of various socio-economical status and ethnicities, with the greatest prevalence of obesity observed amongst Afro-Americans and Mexican-Americans (Skelton *et al.* 2009) of low socio-economical status (Strauss & Pollack 2001). The wide-ranging detrimental effect of adult obesity on health have been well-documented (Zimmet *et al.* 2001; Poirier *et al.* 2006; Su *et al.* 2011), raising concerns on the impact of childhood obesity on health.

The development of childhood obesity has been linked with the apparition amongst teenagers of disease formerly affecting adults, such as type 2 diabetes mellitus (American Diabetes Association 2000; Daniels *et al.* 2005) and metabolic syndrome (Cook *et al.* 2003). Moreover, in addition to direct health effect, childhood obesity has long-term effects on adult health. Indeed, it has been shown to predispose to adult obesity (Guo & Chumlea 1999; Freedman *et al.* 2005), and could consequently fasten the onset of obesity-related disorders in later life. This is supported by evidence linking childhood obesity with lasting detrimental cardiac structural changes, sleep apnea, and thromboembolic disease in adults (Dhuper *et al.* 2011), but also psychological disorders such as depression and poor quality of life (Pine *et al.* 2001; Strauss & Pollack 2003).

Similarly to adult obesity, childhood obesity appears to promote a pro-inflammatory state with increased levels of circulating C-reactive protein and pro-inflammatory cytokines such as TNF- α and IL-6 (Giordano *et al.* 2011) concomitant with reduced levels of circulating anti-inflammatory cytokines such as IL-10 (Arslan *et al.* 2010).

The persistent pro-inflammatory state and the resulting increased oxidative stress and reactive oxygen species formation seen in obese adolescents could potentially induce DNA damage and lead to replicative senescence (von Zglinicki *et al.* 2000a; Chen *et al.* 2001; von Zglinicki *et al.* 2001; von Zglinicki 2002). The results detailed in *Chapter 4* suggest that a lack of physical activity precipitates the onset of immune senescence and the accumulation of dysfunctional senescent T-cells in adults, in addition to promoting obesity. Consequently, the early emergence of obesity and obese-related behaviors such as reduced physical activity could accelerate the development of immune senescence in teenagers, in turn aggravating the proportions of senescent T-cells in later life. Although the results presented in *Chapter 4* failed to show an impact of BMI and body fat composition on the proportions of senescent cells in healthy non-obese adults, it is plausible to hypothesize that these factors would enhance immune senescence in obese adults and to lesser extent obese adolescents.

Other indirect mechanisms have been proposed as potential sources for the chronic pro-inflammatory state seen in obese patients. Although controversial results exist in the literature, one of these factors is believed to be the elevated serum concentration of adipocytes-derived hormones such as leptin (Loffreda *et al.* 1998; Hukshorn *et al.* 2004). Indeed reductions in serum leptin concentrations as observed during weight loss intervention in middle-aged women, have led to the concurrent reduction in obesity-associated pro-inflammatory cytokines concentrations (Rosenbaum *et al.* 1997; Ziccardi *et al.* 2002). There is however no study to our knowledge, analyzing the impact of leptin

concentrations on the proportions of senescent and highly differentiated T-cells in adolescent.

Enhanced T-cell activation and proliferation are a consequence of repeated antigenic stimulation, as seen in the context of latent viral reactivations. Indeed recurring activations of the prevalent latent herpes viruses Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are believed to induce immune cell division leading to the premature terminal differentiation of T-cells. Although CMV and EBV seropositivity are hallmarks of the “immune risk profile” and are known to influence the proportions of terminally differentiated T-cells and increase infection risk in adults (Wikby *et al.* 2002; Wikby A. 2003; Koch *et al.* 2007), it is not known if CMV or EBV infections impact on the proportions of these cells in a young subject cohort. We decided to measure latent viral infections as a potential confounding variable to the impact of obesity and the subsequent circulating serum leptin on the T-cell subsets.

The accumulation of effector-memory and senescent/exhausted T-cells, along with CMV and EBV infections, are part of the immune risk profile and have been shown to partially predict morbidity and mortality in the elderly (Wikby *et al.* 2002; Wikby A. 2003). However the exact mechanisms and factors driving immune senescence remain unclear and the influence of normal chronological ageing on the accumulation of senescent T-cells needs to be isolated from other potential causes. No study to date has examined the impact of obesity on the different T-cell subsets, and although obesity is believed to accelerate the onset of immunosenescence, further studies are required. The aim of the study presented in this chapter was to examine relationships between adiposity and plasma leptin concentrations and the proportions of blood T-cell subsets in a population of Mexican-American adolescents, while adjusting for potential confounding factors such as latent CMV and EBV infections and quality of life. It was

hypothesized that increasing age- and sex-adjusted body mass and plasma leptin concentration would be positively associated with the proportions of senescent/exhausted CD8⁺ T-cells in peripheral blood independently of latent viral status.

5.2. Materials and Methods

5.2.1. Subjects

All subjects for this study were recruited by Dr. John P. Foreyt and Craig A. Johnston (Baylor College of Medicine). A total of 123 Mexican-American children (67 females) aged 10 to 14 (mean 12.3 ± 0.7 years) volunteered for this study. Subjects were recruited from an urban charter school in the city of Houston, with a 95% prevalence of Mexican-Americans. The physical characteristics of the participants are presented in Table 5.1. Intravenous blood samples were drawn as described in the general materials and methods *Chapter 3.3*. None of the subjects were on any medication, and were free of any infectious illness for 6 weeks prior to the study. Prior to the inclusion of their child in the study, parents signed a written consent form on behalf of their child along with a written assent provided by the subjects. Ethical approval was granted by the Institutional Review Board for Human Subjects at Baylor College of Medicine.

Table 5.1. Physical characteristics of the participants (mean \pm SD)

	Mean	Standard deviation	Range
Age (yrs)	12.3	± 0.7	10.8 – 14.7
Body Mass (kg)	52.5	± 14.5	30.1 – 115.2
BMI (kg/m²)	22.6	± 5.3	14.3 – 41.9
zBMI	0.9	± 1.1	-2.2 – 2.7
BMI percentile	73.0	± 27.6	1.4 – 99.7
% Body Fat	26.5	± 11.1	6.2 – 63.6
Serum Leptin (ng.mL⁻¹)	27.2	± 22.0	3.7 - 113.9

5.2.2. Anthropometric measurements

Body weight and height were obtained using a digital scale and a stadiometer respectively. Percentage of body fat was assessed by measuring the subcutaneous fat over the right triceps muscle equidistant between the acromion and the olecranon (Jackson *et al.* 1978). Measurements were made in triplicates on all subjects by an individual technician from Baylor College of Medicine to ensure inter-measurements reliability. Childhood obesity characterization diverges from the classical diagnosis of adult obesity as a consequence of the continuing skeletal and muscular development of the child or adolescent. The use of age-matched and sex-matched Body Mass Index (BMI) percentile along with the transformation of BMI into a standardized score (zBMI) are consequently preferred to the use of traditional BMI (Ogden *et al.* 2002). Weight classifications were determined using the Center for Disease Control guidelines growth charts (Centers for Disease Control and Prevention 2000). Subjects were classified as obese when their BMI was at or above the 95th percentile, and at risk of obesity when their BMI was comprised between the 86th to the 94th percentile. A BMI

below the 85th percentile and above the 5th percentile was considered as appropriate weight. The use of the 85th percentile as a cut-off value was supported by studies linking the 85th percentile with adult obesity and obesity-related health disorders (Reilly 2005).

5.2.3. Lifestyle Scores

Socio-economical status and quality of life are known to impact chronic-health conditions in both adults and children (Eiser 2004; Poterico *et al.* 2011; Pudrovska & Anikputa 2011). In order to assess the impact of health-related quality of life of the volunteers on the proportions of the different T-cell subsets the subjects were asked to fill the Pediatric Quality of Life Inventory (PedsQL 4.0) (Pediatric Quality of Life Inventory™). Physical health scores along with Psychosocial health scores and total quality of life scores were obtained from the self-reported itemized questionnaire. Items were graded on a 5-points scale, from 0 corresponding to “never a problem” to 4 “always almost a problem”. Finally, items were reverse-scored and lineary transformed to a 0-100 scale where higher scores attest of a greater health related quality of life (Varni *et al.* 2006).

5.2.4. Serum Leptin and viral antibodies titration

Serum leptin concentrations were measured in freshly thawed serum samples, using enzyme-linked immunosorbent assays (Invitrogen, Carlsbad, California, USA). Measures were done in duplicate, according to the manufacturer’s guidelines. Anti-CMV and anti-EBV antibody titers were determined as described in the general materials and methods *Chapter 3.4.*

5.2.5. PBMC isolation

Mononuclear cells were isolated from freshly drawn peripheral blood as per the general materials and methods *Chapter 3.3*.

5.2.6. Flow Cytometric analysis

Isolated mononuclear cells were labeled with a combination of four antibodies including anti-CD28 FITC, anti-CD45RA FITC, anti-CD27 PE, anti-CD57 PE, anti-CCR7 PE, anti-CD3 APC, and either with anti-CD4 PEcy5.5 or anti-CD8 PEcy5.5 depending on the cell population of interest as described in general materials and methods *Chapter 3.5.6*, Table 3.2. The exact combinations used to detect the different cell types are presented in Table 5.2. Once labeled, flow cytometric analysis was performed using an Accuri C6 flow Cytometer in combination with Cflow software.

Table 5.2. Combination of monoclonal antibodies used in the characterization of the different T-cell phenotypes

Tube	FL-1	FL-2	FL-3	FL-4	T-cell phenotypes	T-cell subtypes
1	CD28	CD27	CD4	CD3	CD28+/CD27+	Early dif.
2	CD28	CD27	CD8	CD3	CD28-/CD27+ CD28-/CD27-	Intermediate dif. Late dif.
3	CD28	CD57	CD4	CD3	CD28+/CD57-	Senescent
4	CD28	CD57	CD8	CD3	CD45RA+/CCR7+ CD45RA-/CCR7+	Naïve Central-memory
6	CD45RA	CCR7	CD8	CD3	CD45RA-/CCR7- CD45RA+/CCR7-	Effector-memory TEMRA

5.2.7. Statistical analysis

All data were assessed for assumptions of normality using the Shapiro-Wilk test and constant error variance prior to formal statistical testing. Chi Square tests were used to

determine the impact of weight classification on both gender and viral status; while its impact on the participant's physical characteristics were determined using a one-way ANOVA. As normalization of some skewed cell-surface phenotypic data were not obtainable by numerical transformation, the Kruskal-Wallis non-parametric test was used to determine the impact of weight classification on the proportions of the different T-cell subsets. When a significant impact of the weight classification on the proportions of a T-cell subset was observed, further non-parametric post-hoc pairwise group comparisons were performed as suggested by Sprent and Smeeton (Sprent 2001).

Generalized linear model were used to detect potential impacts of the different variables on the proportions of T-cell subsets. By creating fractional logit model, initial univariate associations between each individual variable and the proportions of the different T-cell subsets in accordance with Table 3.2 were analysed. All the variables being shown to have a significant effect on the T-cell repertoire were included in multivariate models to evaluate their independent impact on the proportion of T-cell subsets while adjusting for the other significant factors. The effects size of each variable on the T-cell subsets were reported as an average change in T-cell proportions for a single-unit change of the independent variable.

Statistical significance was set at $P < 0.05$. All values are presented as the mean \pm Standard Error of the mean (SEM). All statistical analyses were performed using "Statistical Package for the Social Sciences" (SPSS v17.0, Chicago, IL, USA).

5.3. Results

The physical characteristics of all subjects are presented in Table 5.3. Physical characteristic differences were seen amongst the weight groups ($p < 0.001$), however no significant differences in age or PEDS QL scores were observed amongst the three categories ($p > 0.05$). No statistical differences in CMV or EBV seroprevalence amongst the different weight classifications were seen ($\chi^2_{\text{CMV}}(2) = 1.346$, $p > 0.05$; Chi $\chi^2_{\text{EBV}}(2) = 3.110$; $p > 0.05$). Male and Female subjects were equally distributed within the different weight classifications ($\chi^2(1) = 1.180$; $p > 0.05$) and no statistical difference in CMV or EBV seroprevalence were seen between Male and Female subjects.

Table 5.3. Physical characteristics of the participants in relation to their weight classifications (mean \pm SEM). Significant differences from the appropriate weight classification (* $p<0.001$) and the obese classification (# $p<0.01$).

	All Subjects (n=123)	Appropriate Weight (n=65)	At risk of obesity (n=22)	Obese (n=36)
Age (yrs)	12.3 \pm 0.7	12.4 \pm 0.7	12.2 \pm 0.6	12.1 \pm 0.6
Female frequency (%)	54 %	58.5 %	54.5 %	47.2 %
Body Mass (kg)	52.5 \pm 14.5	43.1 \pm 7.3 #	54.5 \pm 6.7 * #	69.0 \pm 12.7
BMI (kg/m ²)	22.6 \pm 5.3	18.7 \pm 1.9 #	23.3 \pm 1.0 * #	29.2 \pm 4.2
zBMI	0.9 \pm 1.1	0.1 \pm 0.7 #	1.4 \pm 0.1 * #	2.1 \pm 0.3
BMI percentile	73.0 \pm 27.6	53.5 \pm 24.6 #	91.3 \pm 2.3 *	97.7 \pm 1.4
% Body Fat	26.5 \pm 11.1	19.6 \pm 6.8 #	27.9 \pm 6.8 * #	38.6 \pm 10.2
% Overweight	24.5 \pm 29.7	2.7 \pm 10.4 #	28.8 \pm 4.5 * #	62.4 \pm 22.7
Serum Leptin (ng.mL ⁻¹)	27.0 \pm 22.0	15.4 \pm 7.7 #	24.5 \pm 11.5 #	49.5 \pm 26.8
CMV seropositivity	27.4 %	28.6 %	29.4 %	18.2 %
EBV seropositivity	50.9 %	47.3 %	35.3 %	60.6 %
PEDS QL Physical health score	86.7 \pm 16.3	87.8 \pm 16.2	88.8 \pm 12.4	83.4 \pm 18.1
PEDS QL Psychosocial health score	80.6 \pm 19.3	82.0 \pm 23.0	79.9 \pm 15.2	78.8 \pm 13.9
PEDS QL Total score	82.8 \pm 20.1	83.9 \pm 23.0	83.0 \pm 13.2	80.5 \pm 18.0

5.3.1. Univariate associations between the different factors and the T-cell subsets

We sought to determine the associations between the different factors presented in Table 5.3 and the proportions of different T-cell subsets. Although we hypothesized that excess body mass, and consequently high circulating serum leptin, would be associated with increased proportions of senescent T-cells in adolescents, other factors such as gender, age or latent viral infection could play a role in the development of premature immune senescence. The independent effect of single variables on the proportions of the different T-cell subsets was analysed before creating multivariate models where the

impact of each significant individual variable on the T-cell proportions was controlled for the other variables. The effects of the following variables on the T-cell subsets were assessed independently: Gender, CMV status, CMV titer, EBV status, EBV titer, Weight classification, Leptin concentration, Age, zBMI, Body composition (ie. % Body Fat), PEDS QL Physical health, PEDS QL Psychological health, PEDS QL Total score). The variables shown to have a significant effect ($p < 0.05$) or an effect close to significance ($p < 0.10$) on the proportions of early, intermediate, late and senescent CD4+ and CD8+ T-cell subsets are presented on Tables 5.4 and 5.5.

5.3.1.1. Naïve and Early differentiated T-cells

The univariate effects of the different variables on the naïve and early differentiated T-cell subsets are presented in the Table 5.4 and 5.5 respectively. As previously discussed, the univariate analysis shows an effect of weight classification on the naïve or early differentiated T-cell proportions. Subjects at risk of obesity had less naïve CD8+ T-cells (-4.08 %, $p = 0.05$) than the subjects with an appropriate weight (Table 5.4.) Obese subjects have in average 4.01 % less early differentiated ($p = 0.05$) CD4+ T-cells than subjects with an appropriate weight. Furthermore, obese subjects also had less early differentiated CD8+ T-cells (-7.60 %, $p < 0.05$) than subject with an appropriate weight. Subjects at risk of obesity had less early differentiated (-12.05 %, $p = 0.001$) CD8+ T-cells than the subjects with an appropriate weight (Table 5.5).

Latent viral infections were also associated with lower proportions of naïve or early differentiated T-cells. CMV seropositivity was associated with a lower proportions of naïve CD8+ T-cells (-6.13 %, $p < 0.05$) (Table 5.4.) and EBV seropositivity was associated with a lower proportions of early differentiated CD8+ T-cells (-5.93 %, $p <$

0.05) (Table 5.5). Similarly, a difference of 1-unit in anti-CMV antibody titer was inversely associated with the proportions of naïve (-3.63 %, $p < 0.05$) and early differentiated (-5.29 %, $p < 0.01$) CD8+ T-cells (Table 5.4. and Table 5.5).

The univariate analysis also showed that female subjects have significantly higher proportions of naïve and early differentiated (CD28+/CD27+, CD28+/CD57- and CD45RA+/CCR7+) CD8+ T-cells than males (+6.54 %, $p < 0.05$ and +7.78 %, $p < 0.01$ respectively) (Table 5.4 and Table 5.5).

5.3.1.2. Central-memory, Effector-memory and intermediate differentiated T-cells

The univariate effects of the different variables on the central-memory, effector-memory and intermediate differentiated T-cell subsets are presented in the Table 5.4 and Table 5.5.

Body weight also appeared to have an impact on the proportions of memory T-cells. Obese subjects had a significantly greater proportion of effector-memory (+3.37 %, $p < 0.05$) and intermediate differentiated (+4.51 %, $p < 0.001$) CD8+ T-cells than subjects with an appropriate weight (Table 5.4 and 5.5 respectively). Subjects at risk of obesity also exhibited an increased proportions of effector-memory CD8+ T-cells (+6.04 %, $p < 0.001$) when compared to subjects with an appropriate weight. In addition, body composition, in the form of an increase in 1% of Body fat, was also associated with a modest but significantly greater proportion of effector-memory (+0.17%, $p < 0.05$) CD4+ T-cells, along with intermediate differentiated CD8+ T-cells (+0.06 %, $p < 0.01$). Finally an increase of 1-unit in zBMI was significantly associated with an increase in intermediate differentiated CD8+ T-cells (+ 1.66%, $p < 0.01$) and non-significantly

associated with an increase in effector-memory CD4+ and CD8+ T-cells (+1.49%, $p = 0.071$ and +1.15 %, $p = 0.083$ respectively) (Table 5.4 and 5.5).

Similarly to the association between CMV seropositivity and naïve CD8+ T-cells, latent CMV infection was associated with reduced proportions of intermediate differentiated CD8+ T-cells. Indeed, CMV seropositive subjects had an average of 3.64% ($p < 0.05$) less intermediate differentiated CD8+ T-cells than non-infected subjects (Table 5.5). Finally, a yearly age-increase was positively associated with the proportions of central-memory CD8+ T-cells (+4.47 %, $p < 0.001$) (Table 5.4).

5.3.1.3. Late differentiated, TEMRA and senescent T-cells

The univariate effects of the different variables on the late differentiated, TEMRA and senescent T-cell subsets are presented in the Table 5.4 and 5.5. Increasing body mass had an effect on the proportions of senescent and late differentiated T-cell subsets. Indeed obese subjects had in average 0.97 % and 2.00 % more senescent ($p < 0.05$) and late differentiated ($p < 0.01$) CD4+ T-cells respectively than subjects with an appropriate weight (Table 5.4 and 5.5). Subjects at risk of obesity also had higher proportions of senescent and late differentiated CD8+ T-cells (+5.42 %, $p < 0.05$ and +10.10%, $p < 0.01$ respectively). CMV seropositivity was also associated with the greater proportions of TEMRA and senescent T-cells. CMV seropositive subjects had in average 5.42 % more senescent CD8+ T-cells ($p < 0.05$) and 5.70 % more TEMRA CD8+ T-cells ($p < 0.05$) when compared to their seronegative counterpart. The univariate analysis highlighted sex-associated differences in TEMRA and senescent T-cell proportions. Female subjects had in average 5.16 % ($p < 0.01$) less senescent CD8+

T-cells and 4.80 % ($p < 0.05$) less TEMRA CD8+ T-cells than males (Table 5.4 and 5.5).

Table 5.4. Univariate Associations: Unadjusted direct relation between the different factors and the proportions of T-cell subsets.

T-cell subtype	Factors	Effect Size*	p value
Naïve CD4+ (CD45RA+/CCR7+)	EBV status (Positive)	-4.09 %	0.100
	Age	-3.54 %	0.050
Naïve CD8+ (CD45RA+/CCR7+)	Gender (Female)	+6.35 %	0.024
	CMV status (Positive)	-6.13 %	0.049
	At risk	-4.09 %	0.050
	CMV titer	-3.63 %	0.050
Central-memory CD4+ (CD45RA-/CCR7+)	PEDS QL Psychological health score	-0.09 %	0.075
	PEDS QL Total score	-0.11 %	0.061
Central-memory CD8+ (CD45RA-/CCR7+)	Age	+4.47 %	0.000
Effector-memory CD4+ (CD45RA-/CCR7-)	Age	+4.89 %	0.000
	zBMI	+1.49 %	0.071
	% Body Fat	+0.17 %	0.029
Effector-memory CD8+ (CD45RA-/CCR7-)	At risk of obesity	+6.04 %	0.001
	Obese	+3.37 %	0.025
	Age	-1.73 %	0.090
	zBMI	+1.14 %	0.083
TEMRA CD4+ (CD45RA+/CCR7-)	None	--	--
TEMRA CD8+ (CD45RA+/CCR7-)	Gender (Female)	-4.80 %	0.041
	CMV status (Positive)	+5.70 %	0.031
	CMV titer	+3.13 %	0.045
	Age	-4.58 %	0.007

*Effect size is the average difference (+/-) in % of the phenotype for a 1-unit difference in the respective factor.

Table 5.5. Univariate Associations: Unadjusted direct relation between the different factors and the proportions of T-cell subsets.

T-cell subtype	Factors	Effect Size*	p value
Early Differentiated CD4+ (CD28+/CD27+)	At risk	-4.43 %	0.084
	Obese	-4.01 %	0.050
	zBMI	-1.80 %	0.038
	% Body Fat	-0.15 %	0.083
Early Differentiated CD8+ (CD28+/CD27+)	Gender (Female)	+7.78 %	0.004
	CMV status (Positive)	-5.24 %	0.098
	EBV status (Positive)	-5.93 %	0.031
	At risk of obesity	-12.05 %	0.001
	Obese	-7.60 %	0.011
	CMV titer	-5.29 %	0.006
	zBMI	-3.46 %	0.008
Intermediate Differentiated CD4+ (CD28-/CD27+)	None	--	--
Intermediate Differentiated CD8+ (CD28-/CD27+)	CMV status (Positive)	-3.64 %	0.013
	Obese	+4.51 %	0.001
	CMV titer	-1.76 %	0.053
	Leptin Concentration	+0.05 %	0.094
	Age	-4.16 %	0.000
	zBMI	+1.66 %	0.006
	% Body Fat	+0.06 %	0.004
Late Differentiated CD4+ (CD28-/CD27-)	At risk of obesity	+1.95 %	0.020
	Obese	+2.00 %	0.003
	CMV titer	+1.21 %	0.022
	zBMI	+0.66 %	0.023
	% Body Fat	+0.05 %	0.068
Late Differentiated CD8+ (CD28-/CD27-)	Gender (Female)	-6.23 %	0.012
	CMV status (Positive)	+5.61 %	0.049
	At risk of obesity	+10.10 %	0.003
	CMV titer	+5.02 %	0.000
	Age	-3.77 %	0.040
Senescent CD4+ (CD28-/CD57+)	At risk of obesity	+0.97 %	0.096
	Obese	+0.97 %	0.037
	CMV titer	+0.53 %	0.073
Senescent CD8+ (CD28-/C57+)	Gender (Female)	-5.16 %	0.005
	CMV status (Positive)	+4.41 %	0.037
	At risk of obesity	+5.42 %	0.038
	CMV titer	+3.75 %	0.003
	EBV titer	+1.56 %	0.086
	Age	-2.96 %	0.030

*Effect size is the average difference (+/-) in % of the phenotype for a 1-unit difference in the respective factor.

When variables were shown to have a significant effect on the T-cell repertoire, they were included in multivariate generalized linear model analysis for proportional

dependent variables, creating fractional logit models and thus controlling each significant factor for the other variables in the model.

5.3.2. Multivariate associations between the different factors and the T-cell subsets.

5.3.2.1. Naïve and Early differentiated T-cells

When multiple variables were shown to have a significant effect on T-cell proportions in the univariate analysis, they were included in multivariate models to control for potential confounding effects and to characterise the effect of each variable, independently from the other significant variables. The independent effects of the different variables on the proportions of naïve and early differentiated T-cells are presented on the Table 5.6 and 5.7. When controlled for all other significant variables, subjects at risk of obesity still had on average 8.81% less early differentiated CD8+ T-cells ($p < 0.05$) than subjects with an appropriate weight, however obese subjects and subjects with an appropriate weight appeared to have similar proportions of cells with this phenotype ($p > 0.05$) (Table 5.7). Subjects infected with EBV also had less early differentiated CD8+ T-cells (-5.76 %, $p < 0.05$) than seronegative subjects (Table 5.7) and similar non-significant trends were observed between EBV seropositivity and the proportions of naïve CD4+ T-cells (-4.03 %, $p = 0.093$) (Table 5.6). While CMV seropositivity was not associated with the proportions of early differentiated CD8+ T-cells when controlled for the other factors, anti-CMV antibodies titer within the CMV seropositive subjects was negatively associated with the proportions of early differentiated CD8+ T-cells (-3.86 %, $p < 0.05$) (Table 5.7). Gender was still associated with the proportions of naïve and early differentiated CD8+ T-cells when controlled for all the other variables. Female subjects had an average of 5.31 % ($p < 0.05$) more naïve

CD8+ T-cells and 6.87 % ($p < 0.01$) more early differentiated CD8+ T-cells than male subjects. Naïve T-cell proportions were also associated with age. Yearly increase in age was associated with an average decrease of 3.50 % ($p < 0.05$) naïve CD4+ T-cells as expected (Table 5.6). While Weight classification, zBMI and Body composition were shown to have significant effect on the early differentiated CD4+ T-cell proportions in the univariate analysis, they did not have an effect when controlled for each other, suggesting confounding effects of among those factors (Table 5.7).

5.3.2.2. Central-memory, Effector-memory and intermediate differentiated T-cells

The independent effects of the different variables on the proportions of central-memory, effector-memory and intermediate differentiated T-cells are presented on the Table 5.6 and 5.7. Age-adjusted body mass was associated with greater proportions of effector-memory CD8+ T-cells as subjects at risk of obesity and obese subjects had on average 7.53 % ($p < 0.001$) and 5.49 % ($p < 0.05$) more effector-memory CD8+ T-cells respectively than subjects with an appropriate weight (Table 5.6). When controlled for the other variables, body weight classification was not associated with the proportions of intermediate differentiated CD8+ T-cells ($p > 0.05$) (Table 5.7). A significant positive effect of body composition, characterised by variable-adjusted percentage body fat was found for proportions of effector-memory CD4+ T-cells (+0.21 %, $p < 0.01$ respectively) (Table 5.6).

Subjects with CMV infections had an average of 3.11% ($p < 0.05$) less intermediate differentiated CD8+ T-cells than their infection-free counterparts (Table 5.7). After controlling for the individual effects of CMV status and anti-CMV antibodies titer, age

was also shown to be negatively associated with the proportions of intermediate differentiated CD8+ T-cells (-3.81 %, $p < 0.001$) (Table 5.7). A yearly increase was however associated with 5.29% more effector-memory CD4+T-cells ($p < 0.001$) (Table 5.6).

5.3.2.3. Late differentiated, TEMRA and senescent T-cells

The independent effects of the different variables on the proportions of late differentiated, TEMRA and senescent T-cell proportions are presented on the Table 5.6 and 5.7. Subjects at risk of obesity and obese subjects had more late differentiated CD4+ T-cells (+2.27 %, $p < 0.05$ and +2.60%, $p < 0.05$ respectively) than subjects with an appropriate weight. The impact of weight classification was also seen on the proportions of late differentiated CD8+ T-cells and senescent CD4+ T-cells. Indeed subjects at risk of obesity had in average 8.72% more senescent cells than subjects with an appropriate weight ($p < 0.01$) and obese subjects exhibited increased proportions of 0.98 % senescent CD4+ T-cells ($p < 0.05$) when compared to subjects with an appropriate weight (Table 5.7).

Anti-CMV antibody titers within the CMV seropositive subject group were associated with increased proportions of senescent CD4+ (+0.52 %, $p < 0.05$) and CD8+ T-cells (+4.58 %, $p < 0.05$) along with increased proportions of late differentiated CD8+ T-cells (+ 7.13 %, $p < 0.05$). Similarly, an increase in 1-unit of anti-EBV antibody titer was associated with an increase of 1.73 % of senescent CD8+ T-cells ($p < 0.05$) after controlling for the other significant variables (Table 5.7). Female subjects had consistently less senescent and late differentiated CD8+ T-cells (-5.40 %, $p < 0.01$ and - 5.13 %, $p < 0.05$ respectively) (Table 5.7) and less TEMRA CD8+ T-cells (-4.63 %, $p < 0.05$) (Table 5.6.) than males.

Table 5.6. Multivariate Associations: all factor effects controlled for the other significant factors shown for the proportions of T-cell subsets.

T-cell subtype	Factors	Effect Size*	p value
Naïve CD4+ (CD45RA+/CCR7+)	EBV status (Positive)	-4.03 %	0.098
	Age	-3.50 %	0.050
Naïve CD8+ (CD45RA+/CCR7+)	Gender (Female)	+5.31 %	0.050
Central-memory CD4+ (CD45RA-/CCR7+)	None	--	--
Central-memory CD8+ (CD45RA-/CCR7+)	None	--	--
Effector-memory CD4+ (CD45RA-/CCR7-)	Age	+5.29 %	0.000
	% Body Fat	+0.21 %	0.003
Effector-memory CD8+ (CD45RA-/CCR7-)	At risk of obesity	+7.53 %	0.001
	Obese	+5.49 %	0.025
	Age	-1.56 %	0.098
TEMRA CD4+ (CD45RA+/CCR7-)	None	--	--
TEMRA CD8+ (CD45RA+/CCR7-)	Gender (Female)	-4.63 %	0.036
	Age	-4.78 %	0.003

*Effect size is the average difference (+/-) in % of the phenotype for a 1-unit difference in the respective factor.

Table 5.7. Multivariate Associations: all factor effects controlled for the other significant factors shown for the proportions of T-cell subsets..

T-cell subtype	Factors	Effect Size*	p value
Early Differentiated CD4+ (CD28+/CD27+)	None	--	--
Early Differentiated CD8+ (CD28+/CD27+)	Gender (Female)	+6.87 %	0.005
	EBV status (Positive)	-5.76 %	0.018
	At risk of obesity	-8.81 %	0.039
	CMV titer	-3.86 %	0.029
Intermediate Differentiated CD4+ (CD28-/CD27+)	None	--	--
Intermediate Differentiated CD8+ (CD28-/CD27+)	CMV status (Positive)	-3.11 %	0.014
	Age	-3.81 %	0.000
Late Differentiated CD4+ (CD28-/CD27-)	At risk of obesity	+2.27 %	0.031
	Obese	+2.60 %	0.031
Late Differentiated CD8+ (CD28-/CD27-)	Gender (Female)	-5.13 %	0.025
	At risk of obesity	+8.72 %	0.005
	CMV titer	+7.13 %	0.020
	Age	-3.88 %	0.018
Senescent CD4+ (CD28-/CD57+)	Obese	+0.98 %	0.032
	CMV titer	+0.52 %	0.049
Senescent CD8+ (CD28-/CD57+)	Gender (Female)	-5.40 %	0.002
	CMV titer	+4.58 %	0.043
	EBV titer	+1.73 %	0.021
	Age	-3.19 %	0.009

*Effect size is the average difference (+/-) in % of the phenotype for a 1-unit difference in the respective factor.

5.3.3. Impact of body mass on the different T-cell subset proportions

To further characterise the impact of obesity on the proportions of the different T-cell subsets, we categorized the subjects in three groups, according to their BMI percentile. Subjects with a BMI below the 85th percentile were categorized as “Appropriate weight”, subjects with a BMI comprised between the 86th to the 94th percentile were classified as “At risk of obesity” and finally subjects with a BMI above the 95th percentile were classified as “Obese” (Centers for Disease Control and Prevention 2000). The impacts of increasing body mass on the proportions of the different T-cell subsets are presented in Figures 5.1. to Figures 5.3.

The proportions of naïve and early differentiated CD4+ and CD8+ T-cells in the different weight groups are presented in Figure 5.1. Subjects with an appropriate weight had significantly higher proportions of early differentiated CD4+ T-cells than the subjects at risk of obesity (4.4 % more, $p < 0.05$) and obese (+3.8 % more, $p < 0.05$) (Figure. 5.1). The same difference was observed in proportions of naïve CD4+T-cells between the subjects with an appropriate weight and the obese subjects (+8.1 % more, $p < 0.05$). However no differences in the proportions of those cell types were detected between the subjects with an appropriate weight and those at risk for obesity ($p > 0.05$). Subjects with an appropriate weight also presented higher proportions of early differentiated CD8+ T-cells than subjects at risk of obesity (17.6 % more, $p < 0.001$) and obese subjects (8.2 % more, $p < 0.05$). While no significant differences were observed between the subjects with an appropriate weight and the obese subjects in the proportions of naïve CD8+ T-cells, subjects at risk of obesity had less naïve CD8+ T-cells than the subjects with an appropriate weight (17.1 % less, $p < 0.01$) (Figure. 5.1).

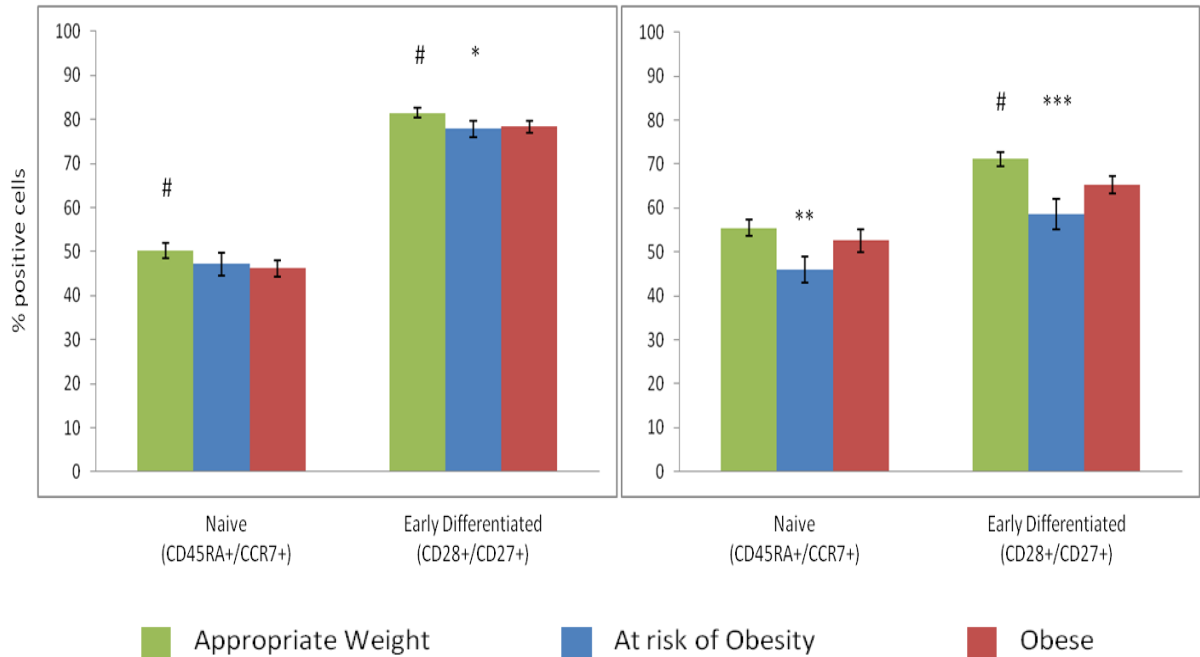


Figure 5.1. The impact of weight classification on the proportions of naïve CD45RA+/CCR7+ and early differentiated CD28+/CD27+ cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences from the “Appropriate weight” group is indicated by * $p < 0.05$; ** $p < 0.01$ and * $p < 0.001$. Statistically significant differences from the “Obese” group is indicated by # $p < 0.05$.**

The proportions of central-memory, effector-memory (CD45RA-/CCR7-) and intermediate differentiated CD4+ and CD8+ T-cells in the different weight groups are presented in the Figure 5.2. Obese subjects had significantly higher proportions of intermediate differentiated CD8+ T-cells than subjects with an appropriate weight (33.2 % more, $p < 0.01$). A non-significant identical trend was observed between the obese subjects and those at risk of obesity ($p = 0.060$). Subjects with an appropriate weight had significantly less effector-memory CD8+ T-cells than the subjects at risk of obesity and obese (43.7 % less and 32.1 % less respectively, $p < 0.001$). No difference in proportion of central-memory CD4+ and CD8+ T-cells could be seen among the different weight classification ($p > 0.05$) (Figure. 5.2).

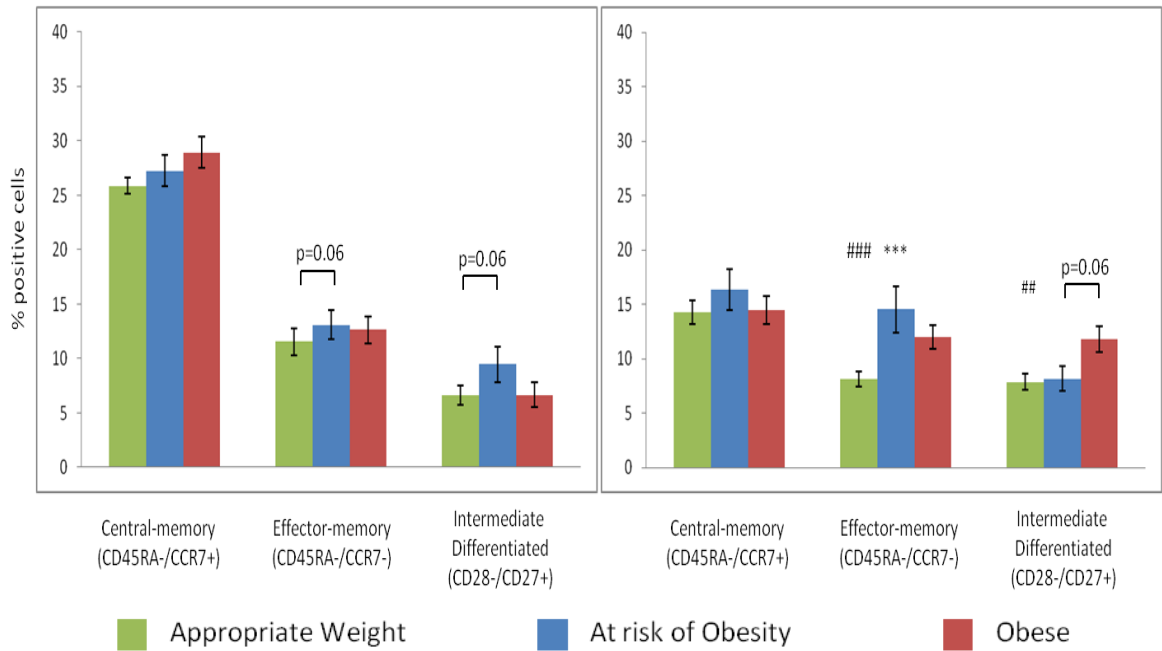


Figure 5.2. The impact of weight classification on the proportions of central-memory CD45RA-/CCR7+, effector-memory CD45RA-/CCR7- and intermediate differentiated CD28-/CD27+ cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences from the “Appropriate weight” group is indicated by *** $p < 0.001$. Statistically significant differences from the “Obese” group is indicated by ## $p < 0.01$ and ### $p < 0.001$.

The proportions of TEMRA, late differentiated and senescent CD4+ and CD8+ T-cells in the different weight groups are presented in the Figure 5.3. Obese subjects had significantly higher proportions of late differentiated CD4+ T-cells than subjects with an appropriate weight (41.1 % more, $p < 0.01$). A non-significant identical trend was observed between the subjects with an appropriate weight and those at risk of obesity ($p = 0.060$). Subjects with an appropriate weight had less senescent CD4+ T-cells than the subjects in the two other weight classification (at risk of obesity : 34.0 % less and obese : 41.8 % less, $p < 0.05$). Subjects at risk of obesity also exhibited increased proportions of senescent and late differentiated CD8+ T-cells when compared with the subjects with an appropriate weight (41.7 % more and 43.9 % more respectively, $p < 0.01$), while obese subjects had only a non-significant increased proportions of senescent CD8+ T-cells compared to subjects with an appropriate weight ($p = 0.070$). No difference in the

proportion of TEMRA CD4+ and CD8+ T-cells could be seen among the different weight classification ($p > 0.05$) (Figure. 5.3).

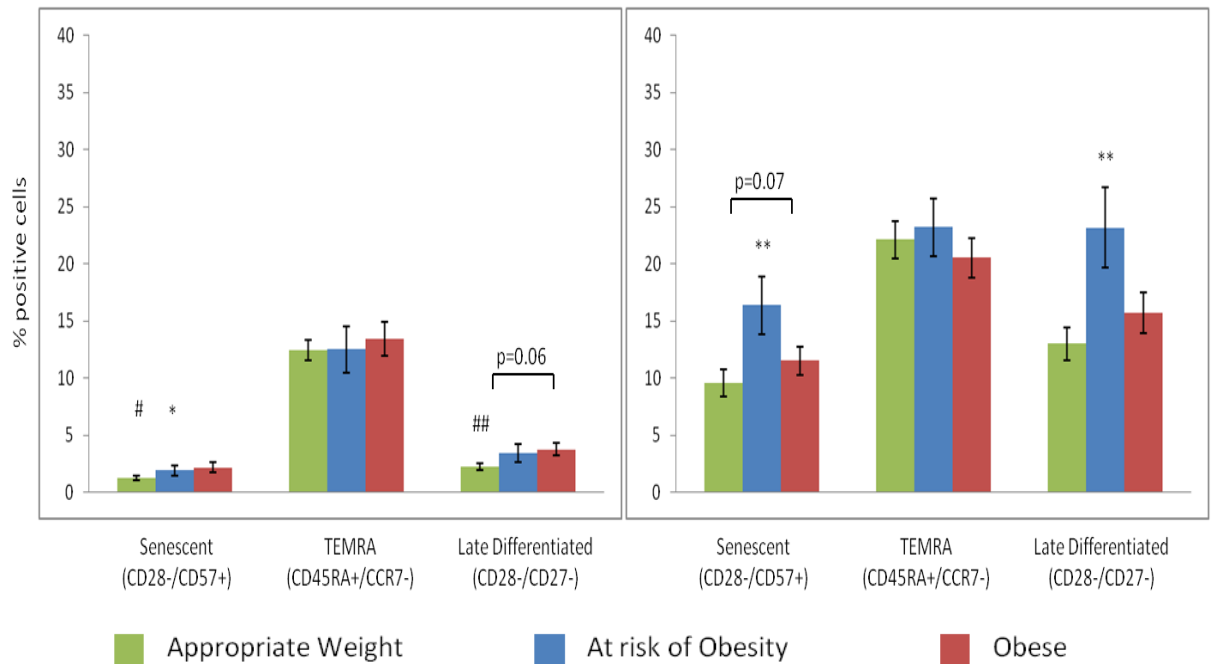


Figure 5.3. The impact of weight classification on the proportions of senescent CD28-/CD57+, TEMRA CD45RA+/CCR7- and late differentiated CD28-/CD27- cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences from the “Appropriate weight” group is indicated by * $p < 0.05$ and ** $p < 0.01$. Statistically significant differences from the “Obese” group is indicated by # $p < 0.05$ and ## $p < 0.01$.

5.3.4. Effect of Body Mass and its associated factors on serum leptin concentration

The direct correlation between serum leptin concentration and body mass is widely accepted in the literature (Blum *et al.* 1997; Brannian *et al.* 2001; Chow & Phoon 2003), it was consequently important to verify that the subject cohort tested in this study did not differ from the general population. The relation between serum leptin concentration and Body mass, BMI or zBMI are represented in Figure 5.4. Serum leptin concentration was positively correlated with body mass and BMI. Although exponential, this relationship was also observed between serum leptin concentrations and age- and sex-adjusted zBMI (Figure. 5.4).

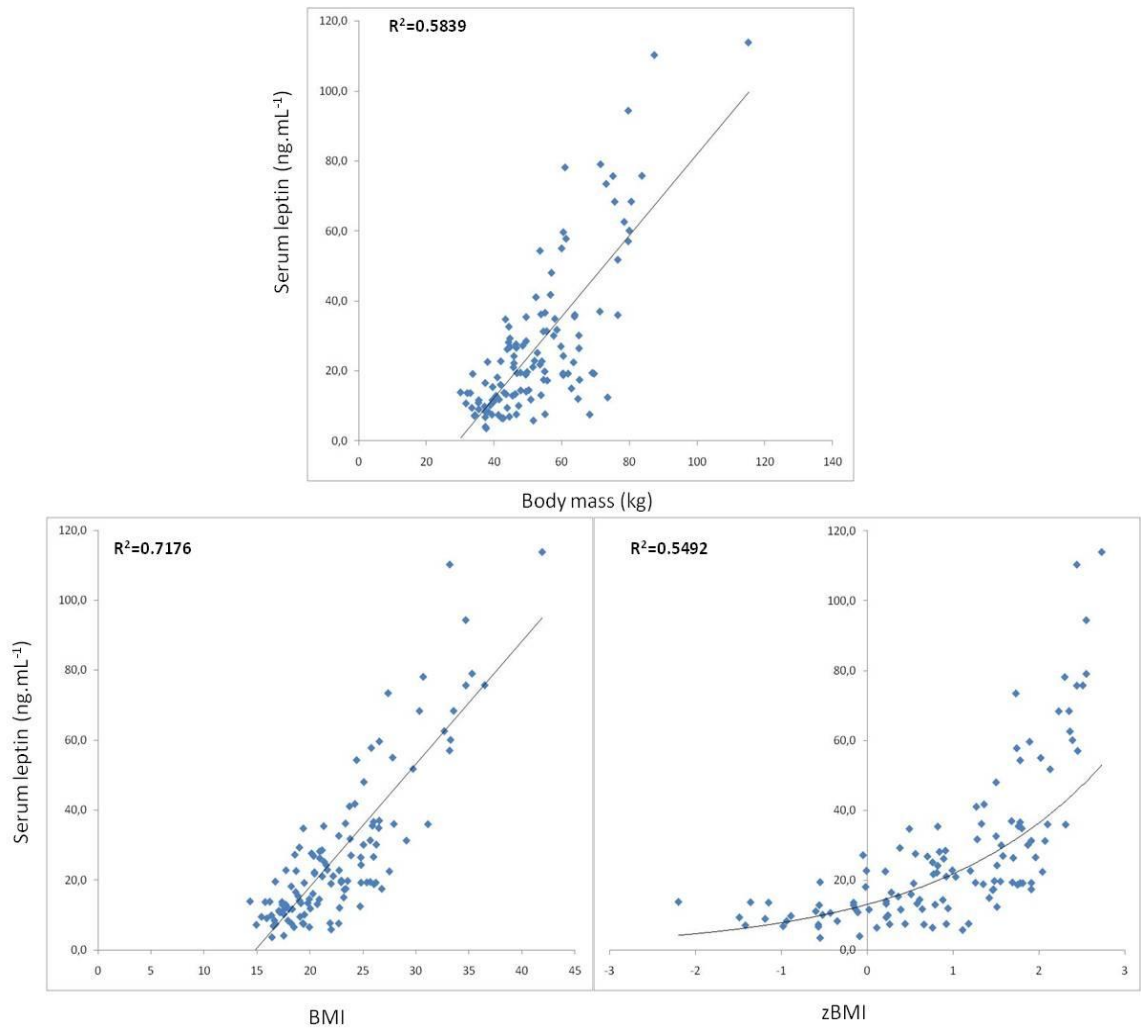


Figure 5.4. The impact of Body mass (kg) (top), BMI (bottom left) and zBMI (bottom right) on the circulating serum leptin concentration (ng.mL⁻¹) in adolescent Mexican-Americans.

5.3.5. Impact of serum leptin concentration on the different T-cell subset proportions

Serum leptin concentration was directly correlated with body mass (kg) and BMI (kg/m²) in our subjects. All participants were subsequently grouped into tertiles for plasma leptin concentration and comparisons among the three groups were made for the different T-cell subsets. Results are presented in Figure 5.5, 5.6 and 5.7. The proportion of early differentiated and naïve CD4⁺ and CD8⁺ T-cells in the different leptin tertiles are presented in the Figure 5.5. Subjects with low circulating leptin concentrations had higher proportions of early differentiated CD4⁺ T-cells than the subjects grouped in the

average (5.3 % more, $p < 0.05$) and highest leptin tertiles (4.6 % more, $p < 0.05$) (Figure. 5.5). Similarly, the proportions of early differentiated CD8+ T-cells were higher in subjects from the lowest leptin tertile than the subjects from the highest tertile (8.9 % more, $p < 0.05$) and although non-significant, a similar trend was observed between subjects from the lowest leptin tertile and subjects from the average leptin tertile ($p = 0.057$) (Figure. 5.5). No impact of leptin concentrations was observed on the proportions of naïve T-cells.

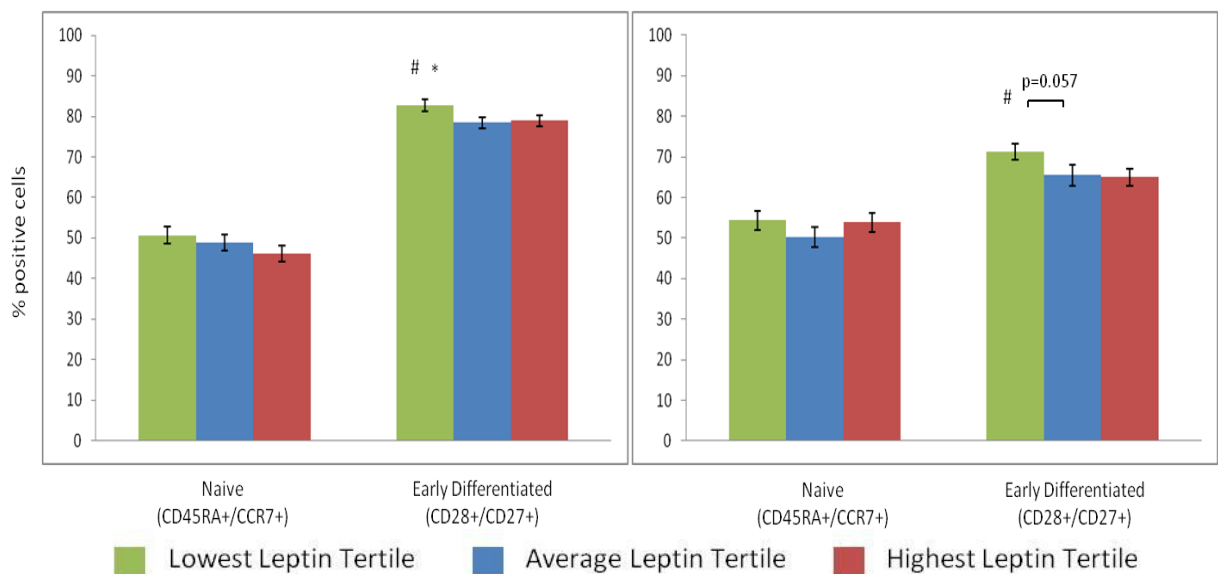


Figure 5.5. The impact of serum leptin concentration on the proportions of naïve CD45RA+/CCR7+ and early differentiated CD28+/CD27+ cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean ± SEM. Statistically significant differences from the average leptin tertile is indicated by * $p < 0.05$. Statistically significant differences from the highest leptin tertile is indicated by # $p < 0.05$.

The proportions of central-memory, effector-memory and intermediate differentiated CD4+ and CD8+ T-cells in the different leptin tertiles are presented in the Figure 5.6.

A greater proportion of intermediate differentiated CD4+ T-cells were present in subjects grouped in the average leptin tertile than in the lowest leptin tertile (38.3 % more, $p < 0.05$) but not in the highest leptin tertile ($p > 0.05$). Intermediate differentiated CD8+ T-cells were also found in greater proportions in subjects from the average and

highest leptin tertile (32.2 % more and 41.2 % more respectively, $p < 0.05$). While no significant difference in central-memory CD8+ T-cell proportions amongst the three tertiles were seen ($p > 0.05$), higher proportions of effector-memory CD8+ T-cells was observed in the highest leptin tertile when compared to the lowest leptin tertile (21.1 % more, $p < 0.05$) (Figure. 5.6). A similar trend, although non-significant, was observed in the proportions of effector-memory CD4+ T-cells between the lowest and highest leptin tertile ($p=0.076$).

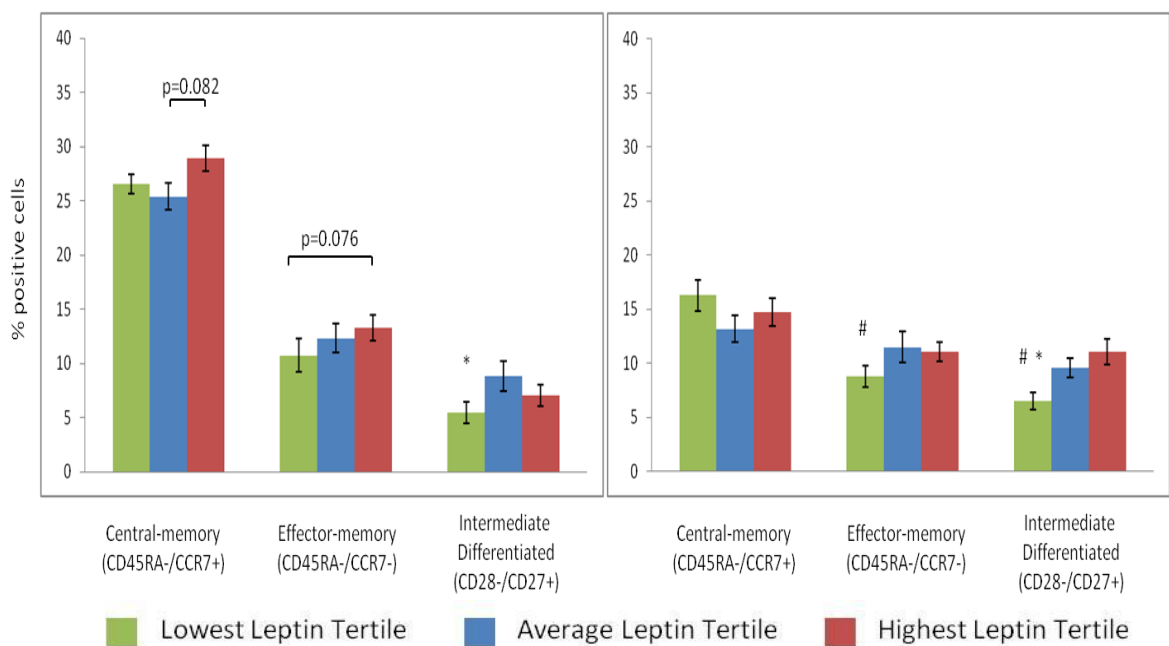


Figure 5.6. The impact of serum leptin concentration on the proportions of central-memory CD45RA-/CCR7+, effector-memory CD45RA-/CCR7- and intermediate differentiated CD28-/CD27+ cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences from the average leptin tertile is indicated by * $p < 0.05$. Statistically significant differences from the highest leptin tertile is indicated by # $p < 0.05$.

The proportions of TEMRA, late differentiated and senescent CD4+ and CD8+ T-cells in the different leptin tertiles are presented in the Figure 5.7.

Although no difference in late differentiated CD4+ T-cells proportions were seen between the lowest and the average leptin tertile ($p > 0.05$), their proportions were greater in the highest leptin tertile when compared to the average and lowest tertiles

(23.9 % more, $p < 0.05$ and 38.4 % more, $p < 0.01$ respectively). Non-significant greater proportions of senescent CD4+ T-cells were seen in the highest leptin tertile compared to the lowest tertile ($p = 0.068$). No differences in TEMRA CD4+ T-cells proportions were observed among the different leptin tertiles. Greater proportions of late differentiated and senescent CD8+ T-cells proportions were found in subjects grouped in the average leptin tertile when compared to those grouped in the lowest tertile (35.1 % more and 33.8 % more respectively, $p < 0.05$) (Figure. 5.7).

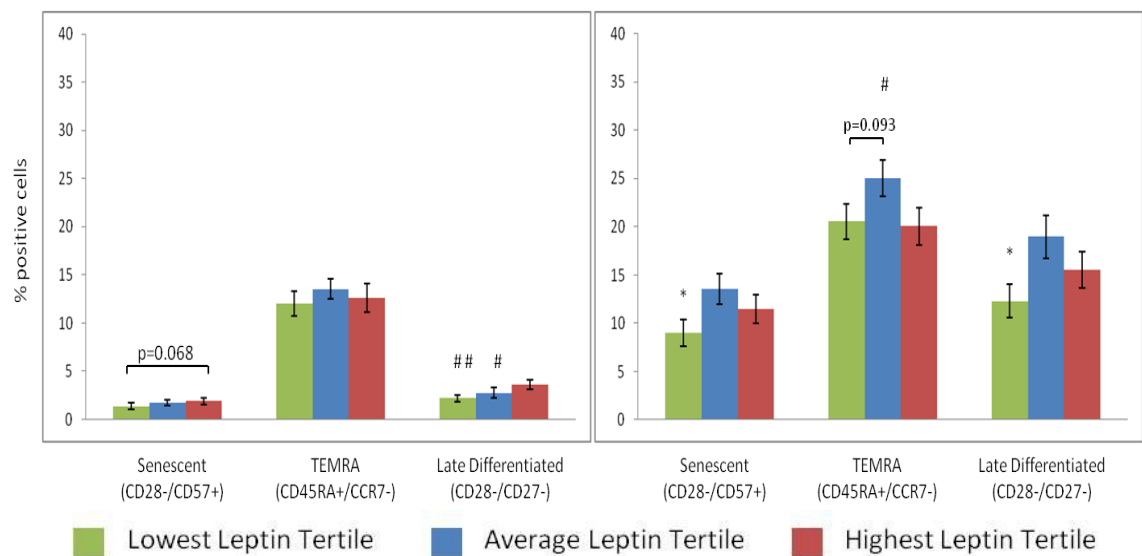


Figure 5.7. The impact of serum leptin concentration on the proportions of senescent CD28-/CD57+ TEMRA CD45RA+/CCR7- and late differentiated CD28-/CD27- cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences from the average leptin tertile is indicated by * $p < 0.05$. Statistically significant differences from the highest leptin tertile is indicated by # $p < 0.05$ and ## $p < 0.01$.

5.4. Discussion

This study sought to determine the associations between childhood obesity and T-cell differentiation indicative of immunosenescence. Although chronological ageing is believed to be the main factor responsible for the accumulation of senescent T-cells and the concomitant reduction in the number of naïve T-cells, this study was conducted to

determine the impact of obesity and serum leptin concentrations, in an age-controlled population, while adjusting for potential confounders including latent viral status and quality of life scores. The major findings in this study were: (1) Increasing body mass had a negative impact on the proportions of naïve and early differentiated T-cells. Furthermore excess body mass was associated with greater proportions of CD8+ effector-memory T-cells and CD4+ late differentiated T-cells. (2) Serum leptin concentration was also associated with altered proportions of blood T-cell subsets. High serum leptin concentrations were associated with reduced proportions of early differentiated T-cells and greater proportions of effector-memory and intermediate differentiated CD8+ T-cells. High leptin concentrations were also associated with greater proportions of late differentiated CD4+ T-cells. (3) When controlled for all the potential factors significantly affecting the level of T-cell differentiation, age-adjusted body mass was still correlated with reduced early differentiated T-cell proportions, and a concomitant increase in effector-memory CD8+ T-cells and senescent CD4+ T-cells. (4) Although the deleterious effect of latent herpesviruses on the “immunological space” was believed to be dependent on repeated reactivation cycles, implying prolonged exposure to the viruses (Almanzar *et al.* 2005), it was found in this study that latent CMV and EBV infections were associated with a reduction in naïve and early differentiated T-cell proportions even at a very early age. Furthermore in seropositive subjects, anti-CMV antibody titer was associated with increased proportions of late differentiated and senescent T-cells and anti-EBV antibody titer was associated with increased proportions of senescent CD8+ T-cells.

5.4.1. Impact of weight classification on the different T-cell subsets

Although no study to date has assessed the impact of excess body mass and obesity on the accumulation of senescent T-cells, a few studies have proposed mechanisms by which obesity could play a role in immunosenescence. Body mass index (BMI) has been shown to be inversely correlated with mean telomere length in leukocytes, a marker of biological ageing (Valdes *et al.* 2005). Furthermore, while both telomere length (Nakashima *et al.* 2004) and obesity (Grundy 2007; Mathieu *et al.* 2010) are known predictors of cardiovascular diseases, senescent T-cells have been shown to accumulate during the development of cardiovascular disease (Brouillette *et al.* 2003; Brouillette *et al.* 2007; Starr *et al.* 2008) suggesting potential links between obesity and immunosenescence. This is the first study to show that excess body weight is associated with T-cell differentiation indicative of immunosenescence, independently of age and persistent viral infections.

The beneficial effects of regular physical activity and aerobic fitness on the proportions of naïve, memory and senescent T-cells have been discussed previously (*Chapter 4*). While a sedentary lifestyle can lead to obesity (Martinez-Gonzalez *et al.* 1999; Mortensen *et al.* 2006; Chaput & Tremblay 2009) and is also associated with an increased proportion of senescent T-cells (*Chapter 4*), it was found in *Chapter 4* that increasing body mass was not associated with the proportions of senescent T-cells. However, the study presented in *Chapter 4* only included very few obese subjects (7%) so the potential link between body mass and the proportions of senescent T-cells could not be assertively excluded. Consequently, in order to accurately assess the impact of excess body mass on T-cell differentiation indicative of immunosenescence, this study was designed to test subjects with a wider BMI (ranging from 14.3 kg/m² to 41.9 kg/m²

and from the 1.4th to the 99.7th percentile), including 36 obese adolescents. Furthermore, only young subjects participated to the study presented in this chapter, to limit the normal age-associated immune changes observed in older populations.

The results highlighted clear differences in T-cell differentiation among the three weight classifications. Subjects categorized as having an appropriate weight, had greater proportions of early differentiated T-cells than subjects classified as obese or at risk of obesity. Furthermore, while no difference could be observed for the proportions of naïve CD4⁺ T-cells between subjects with an appropriate weight and subjects at risk of obesity, obese subjects had significantly fewer cells exhibiting a naïve phenotype. Similarly, obesity was associated with an accumulation of intermediate differentiated CD8⁺ T-cells and both obese and subjects at risk of obesity presented elevated proportions of effector-memory CD8⁺ T-cells when compared to lean subjects. Finally, weight classification was associated with differences in senescent T-cells proportions and an increasing age-adjusted and sex-adjusted body mass was synonym of a greater proportion of senescent CD4⁺ T-cells. Interestingly however, being at risk of obesity was associated with increased proportions of senescent CD8⁺ T-cells, but the difference in senescent CD8⁺ T-cells between obese subjects and lean subjects was not sufficiently ample to be significant ($p=0.070$). Those results are consistent with the main hypothesis of this stud, stating that excess body mass has a deleterious effect on T-cell differentiation, independently of chronological ageing.

The deleterious impact of obesity on the T-cell level of differentiation are also consistent with previous work investigating the impact of high BMI on leukocyte telomere length in women (Valdes *et al.* 2005) and in physically inactive subjects (Cherkas *et al.* 2008). Although these studies did not assess the impact of body mass on the individual T-cell subsets, they have shown that a higher BMI was associated with

shortened telomeres in mixed blood leukocytes. Telomere length is believed to be a good marker of biological ageing, as these non-coding nucleoproteins located at the chromosome ends are being shortened at each cell division and will lead to cellular cycle arrest when reaching a critical size (Hayflick & Moorhead 1961; Hayflick 1965). However, cellular senescence can also be triggered by non-telomeric dependent pathways during a phenomenon termed stress or aberrant signaling-induced senescence (STASIS) (Drayton & Peters 2002). Various stressors can induce STASIS such as the prolonged exposure to γ -radiations (Di Leonardo *et al.* 1994), dysregulated oncogenes (Herbig & Sedivy 2006) , but the most common stressor consist in transient or chronic increases in oxidative stress (Chen & Ames 1994; Chen *et al.* 2000), as seen during obesity. Consequently studying mean telomere length alone as a measure of immune ageing in the context of obesity is likely to minimize the deleterious impact of obesity on immunosenescence. Therefore, analyzing the proportions of the different T-cell subsets characterised by their level of differentiation as described in this study yields more accurate representations of obesity-associated immune changes.

Some results were however unexpected, and whereas subjects at risk of obesity had significantly greater proportions of late differentiated CD8⁺ T-cells than the subjects with an appropriate weight, the same difference was not observed between obese subjects and lean subjects. Similarly, subjects at risk of obesity had fewer naïve CD8⁺ T-cells than the subjects with an appropriate weight, while no differences in naïve CD8⁺ T-cells could be seen between the obese subjects and the subjects with an appropriate weight. These unforeseen results could be explained by the intermediate level of circulating leptin in subjects at risk of obesity. Indeed, while the very high concentrations of serum leptin observed in obese subjects are known for enhancing reactive oxygen species production, increasing oxidative stress (La Cava *et al.* 2004; La

Cava & Matarese 2004), and directly impacting T-cell activation and proliferation (Fantuzzi & Faggioni 2000; Martin-Romero *et al.* 2000), the effects of moderate serum leptin concentrations observed in subjects at risk of obesity are unknown. Constant elevated leptin concentration such as the level seen in obese subjects have been shown to induce leptin resistance, or in other words to down-regulate leptin receptors on various cell types (Matarese *et al.* 2005; Ricci *et al.* 2006; Nascimento *et al.* 2011) limiting its effects. It is plausible that such a down-regulation would occur on T-cells if serum leptin concentration was excessive, reducing its activation effects on T-cells in obese subjects, but not in subjects at risk of obesity. If this were to be the case, T-cells in subjects at risk of obesity would be extensively activated, and could become more differentiated than T-cells in contact with very high concentrations of leptin, such as the one seen in obese subjects. In order to characterise the impact of serum leptin on the various T-cell subsets, the plasma concentrations were measured in the participants.

5.4.2. Impact of leptin concentration on the different T-cell subsets

The adipokine leptin has for primary function to restrict food consumption, it was however recently discovered to also play a role on the immune system. Leptin has been shown to enhance naïve T-cell activation (identified by their expression of CD45RA) while restricting the anti-CD3 activation of memory T-cells (identified by their expression of CD45RO (Lord *et al.* 2002; De Rosa *et al.* 2006). Furthermore, it has been shown to elicit a shift from a T_{h2} to a pro-inflammatory T_{h1} profile (Loffreda *et al.* 1998; Lord *et al.* 1998), further establishing the obesity-associated state of chronic inflammation. In light of these effects on the immune system, serum leptin concentrations were measured in order to identify any association with the different T-

cell subsets. The subjects were divided into tertiles, according to their serum leptin concentrations. The average leptin concentration of each tertile corresponded to the average serum leptin concentration of the different weight groups. The average leptin concentration of the subjects from the lowest, intermediate and highest leptin tertile were 9.9 ng.mL^{-1} ($\pm 0.5 \text{ ng.mL}^{-1}$), 20.3 ng.mL^{-1} ($\pm 0.6 \text{ ng.mL}^{-1}$) and 51.2 ng.mL^{-1} ($\pm 3.6 \text{ ng.mL}^{-1}$) respectively, values corresponding to the representative serum leptin concentrations of lean, at risk of obesity and obese subjects (Prolo *et al.* 1998).

Leptin appears to have a direct effect on T-cells by binding to its receptor, OB-Rb, that belongs to the class I cytokine receptor superfamily (Chen *et al.* 1996). Leptin has been shown to affect the CD4⁺ balance by inducing a shift towards a T_{h1} pro-inflammatory subset (Loffreda *et al.* 1998; Lord *et al.* 1998). Furthermore, leptin can act as an early acute-phase adipokine, like IL-1 or IL-6 (Landman *et al.* 2003) and can stimulate the production of TNF- α by monocytes (Zarkesh-Esfahani *et al.* 2004) and IFN- γ -inducible proteins by monocytic cells (Meier *et al.* 2003). Taken together, those observations suggest that leptin, in addition to its neuroendocrine properties, also acts as pro-inflammatory adipokine. The chronic inflammatory status seen in obesity could be enhanced by the pro-inflammatory environment generated by high physiological concentrations of human leptin. The resulting increased oxidative stress could explain the accumulation of late differentiated CD8⁺ T-cells and senescent CD4⁺ T-cells (von Zglinicki *et al.* 2000a; Chen *et al.* 2001; von Zglinicki *et al.* 2001; von Zglinicki 2002). In turn, as late differentiated and senescent T-cells have also been shown to produce greater amounts of pro-inflammatory cytokines when compared to their less differentiated counterparts (Effros *et al.* 2003), their accumulation may further contribute to the obesity-associated inflammatory environment. This was confirmed by the data presented in this study showing associations between increasing leptin

concentrations and lowered proportions of naïve CD4⁺ and CD8⁺ T-cells. Furthermore, increasing leptin concentration was also associated with increased proportions of intermediate differentiated CD8⁺ T-cells and subjects with the highest leptin levels had increased effector-memory CD8⁺ T-cells compared to subjects with lower leptin concentrations. Finally, the greatest proportions of late differentiated CD4⁺ T-cells were observed in subjects with the highest concentration of leptin. These results tend to suggest that, as hypothesized, high physiological concentration of serum leptin may promote T-cell differentiation eventually driving them to become senescent. While the impact of leptin concentration on the proportions of late differentiated and senescent T-cells appear to be relatively moderate in young subjects, a sustained exposure to high concentrations of leptin is likely to induce greater deleterious effects on the T-cell level of differentiation in adults.

Interestingly, while differences between subjects at risk of obesity and obese subjects could be seen in the proportions of effector-memory CD8⁺ T-cells, these differences disappeared when the subjects were classified according to their leptin concentrations. Average leptin concentration was consequently not the factor leading to greater proportions of effector-memory CD8⁺ T-cells in subjects at risk of obesity than in obese subjects. However, when studying the senescent and TEMRA CD8⁺ T-cell subsets, only subjects with an average leptin concentrations had increased proportions of senescent and TEMRA CD8⁺ T-cells, compared to the two other groups. These surprising results suggest that although average serum leptin concentrations could have a greater impact on senescent CD8⁺ T-cells than high serum leptin concentrations, the lack of difference between average serum leptin concentrations and high serum leptin concentrations tend to disprove this hypothesis.

A great number of young subjects took part in this study, and as a consequence of their young age, they were at different stages of pubertal maturation, promoting high levels of diversity in the results. Differences in pubertal maturation will lead to hormonal differences between the different subjects, with greater differences between male and female subjects. While subjects were in a narrow age-range, the impact of biological ageing on the level of T-cell differentiation could not be dissociated from the impact of chronological ageing. The observed diversity could also be the reflection of the existence of a metabolically healthy but obese (MHO) group in the participants of this study. Individuals classified as MHO have been shown to have a metabolically healthy profile including high levels of insulin sensitivity and a clinically normal lipid and inflammation profiles (Brochu *et al.* 2001; Karelis *et al.* 2005; Stefan *et al.* 2008). These individuals are not subject to the same immunosuppression and cardiovascular risk as obese individuals with metabolic disorder (Meigs *et al.* 2006) and could explain the observed discrepancies in the results.

Other factors, such as age, latent viral infections or psychosocial health, may influence the level of T-cell differentiation and explain the differences seen between subjects at risk of obesity and obese subjects. Consequently, multivariate analyses were performed to assess the effect of each variable on the level of T-cell differentiation, independently from each other.

5.4.3. Multivariate analysis on the different T-cell subsets proportions.

The rate of accumulation of senescent T-cells is known to be highly influenced by many different factors, such as chronological ageing (Spaulding *et al.* 1997) , latent viral

infections (Thimme *et al.* 2005) including CMV and EBV infections (Wikby *et al.* 2002), sustained psychological stress due to adverse work conditions (Bosch *et al.* 2009) or low socioeconomic status, as suggested by studies that have used leukocyte telomere length as an outcome measure (Cherkas *et al.* 2006). It was therefore necessary to control for the impact of potential confounding factors in order to accurately characterise the effect of excess body mass and high serum leptin concentrations on the proportions of late differentiated and senescent T-cells.

After controlling for all potential confounding factors, excess body mass was still associated with altered T-cell subsets proportions. Obese subjects had lower proportions of early differentiated CD4⁺ T-cells than subjects with an appropriate weight, and subjects at risk of obesity had fewer early differentiated CD8⁺ T-cells than their lean counterparts. In addition, both subjects at risk of obesity and obese subjects had a greater number of effector-memory CD8⁺ T-cells than the subjects with an appropriate weight (+7.53% and +5.49% respectively) after controlling for all the confounding factors. Finally, excess body mass was also associated with increased proportions of senescent T-cells, as obese subjects had more senescent and late differentiated CD4⁺ T-cells than subjects with an appropriate weight and subjects at risk of obesity had greater amount of late differentiated CD4⁺ and CD8⁺ T-cells than lean subjects. These results confirm that, excess body mass has a deleterious impact on the composition of the T-cell population, by leading to the increase in late differentiated and senescent T-cell numbers, while reducing the naïve CD4⁺ T-cell pool. Although excess body mass was associated with changes in the level of T-cell differentiation indicative of immunosenescence, after controlling for the different confounding factors, no associations were seen with increasing leptin concentrations. While these results may suggest that increasing leptin concentration has no impact on T-cell differentiation and

on the proportions of the different T-cell subsets, the direct correlation seen between body mass and serum leptin is more likely to explain the apparent lack of association. Furthermore, as leptin concentration is known to increase exponentially with increasing body mass (Mantzoros 1999), categorizing subjects in tertiles according their serum leptin concentration is more sensitive than analyzing the effect of leptin concentrations using multivariate analysis. Consequently, the potential synergistic effect of excess body mass and high serum leptin concentration on the accumulation of late differentiated and senescent T-cells cannot be rejected.

It is interesting to observe that other factors, such as latent viral infections, were associated with alterations in the T-cell subsets proportions. Although studies have suggested that prolonged exposure to CMV and/or EBV were required to see modifications in T-cell differentiation level (Wikby *et al.* 2002; Almanzar *et al.* 2005; Thimme *et al.* 2005), it appears that CMV and/or EBV infections are associated with reductions in naïve and early differentiated T-cell proportions. In seropositive subjects, antiviral antibody titer was also positively associated with the proportions of late differentiated and senescent T-cells in young individuals. Whereas initial infections by the viruses were not determined, it is plausible to believe that the subjects were infected relatively recently due to their young age, or that they have been carrying the virus for a relatively shorter period, compare to adults. Consequently, latent viruses may have an immediate, or shortly delayed, deleterious impact on the immune system by promoting T-cell differentiation, thus accelerating the onset of immunosenescence. This is supported by studies conducted on Gambian infants, in which CMV infection was shown to induce T-cell differentiation and rapid shifts toward highly differentiated phenotypes (Miles *et al.* 2007; Miles *et al.* 2008). Those studies did not however characterise the impact of CMV infection on senescent T-cells and they failed to control

for potential confounding factors. Consequently, the study presented in this chapter is the first study to highlight an association between CMV and/or EBV infections and increased proportions of senescent T-cells in children, independently of body mass, age and psychosocial status.

While some gender-related differences in immune response have been well-documented, including greater humoral and cellular immune responses in women (Weinstein *et al.* 1984; Ansar Ahmed *et al.* 1985) and a greater response to mitogens (Santoli *et al.* 1976), only few studies have assessed the gender-associated differences in T-cell subset frequencies (Yan *et al.* 2010). The results presented in this study show that females have more naïve and early differentiated T-cells, while having less TEMRA, late differentiated and senescent CD8⁺ T-cells. The mechanisms behind the gender-associated difference remains to be identified, however it is possible to hypothesize that sexual hormones play a role in this immune protection. Indeed it has been shown that estrogen activates the gene coding for the telomerase reverse transcriptase leading to the up-regulation of telomerase and subsequently delay the onset of T-cell senescence (Kyo *et al.* 1999). Considering the potential protecting effect of sexual hormones, the difference in senescent T-cell proportions between males and females is likely to persist until the menopause. This hypothesis suggesting that female may have an increased immunocompetence prior to the menopausal phase when compared to their male counterpart is supported by various studies. Indeed females appear to be more protected than men against various diseases, such as CVD (Vassalle *et al.* 2012a; Vassalle *et al.* 2012b), before they reach the menopause, but are at similar risk after they entered the menopausal phase.

Finally, although we hypothesized that psychosocial status would impact the level of T-cell differentiation, no associations between physical and psychological PEDS QL and

the different T-cell subset proportions were observed. However, it is important to remember that no differences in PEDS QL scores were seen among the different groups. Subjects had high PEDS QL scores, suggesting that they were not psychosocially distressed. Further studies should investigate the difference in T-cell subsets frequencies among subjects emanating from opposed socioeconomic environments to potentially see an effect of PEDS QL scores. In addition, future studies should investigate the effect of body weight on the level of T-cell differentiation in children of different ethnic groups.

In conclusion, the study presented in this chapter highlights clear associations between excess body mass and reduced proportions of naïve T-cells, while being associated with increased proportions of effector-memory, late differentiated and senescent T-cells in young individuals. This is the first study to show an association between obesity and level of T-cell differentiation associated with immunosenescence independently of chronological ageing, raising clinical concerns. The accumulation of effector-memory and late differentiated or senescent T-cells has been associated with increased rate of morbidity and mortality in the elderly (Wikby *et al.* 2002; Wikby A. 2003), suggesting a state of immunodepression in obese children that may accelerate the onset of premature immunosenescence. Furthermore, the results presented in this chapter also highlighted similar deleterious impacts of CMV and/or EBV viral infections on the level of T-cell differentiation in children to the one seen in older adults, advocating for the important role played by latent viral infections on the accumulation of senescent T-cells, relatively shortly after primary infection. Finally, the study presented in this chapter highlighted a potential role of high serum leptin concentration in the level of T-cell differentiation, however a more detailed study should be conducted to underscore the mechanisms linking leptin concentration and T-cell differentiation. While the biological impact of excess body weight on the level of T-cell differentiation appears to be relatively modest

due to the small proportional differences between the different weight classifications, the young age of the volunteers greatly increase their clinical and biological differences. Indeed, the differences observed in teenagers, are likely to gradually and rapidly increase across the lifespan. Future studies should also examine the effect of weight loss interventions on the level of T-cell differentiation in children, in order to potentially restore a competent immune system and delay the onset of immunosenescence.

CHAPTER 6

**THE EFFECTS OF PHYSIOLOGICAL
CONCENTRATIONS OF LEPTIN ON T-CELL
ACTIVATION AND OB-Rb EXPRESSION**

6.1. Introduction

Excess body mass, and more specifically excess body fat has become a clear health-threatening condition to the general population when the World Health Organization classified obesity as a worldwide epidemic (World Health Organization 2000). Although obesity is not affecting countries equally, with higher prevalence in industrialized countries such as the USA with 33.8% of its population being obese (Flegal *et al.* 2010), than in developing countries such as Cambodia with only 1.5% of the population having a BMI over 30 kg/m² (UNICEF Cambodia 2009), it is expected to become increasingly prevalent. Besides having important deleterious impacts on psychological and mental health based on inabilities in identification with social standards (Martin-Lopez *et al.* 2011), obesity and excessive central adiposity have been linked to many physical chronic conditions such as heart diseases (Poirier *et al.* 2006), type 2 diabetes mellitus (Zimmet *et al.* 2001) and multiple cancers (Ceschi *et al.* 2007; Su *et al.* 2011). Recent studies have also found obesity to negatively impact the immune system.

Obesity has been shown to have deleterious effects on both the innate and adaptive arms of the immune system. Animal models of obesity have been used extensively to highlight some of these defects, such as a diminished tolerance towards bacterial and viral infections (Mancuso *et al.* 2002; Smith *et al.* 2007). On a cellular level, obesity is associated with reduced NK cells numbers (Mori *et al.* 2006), activation (Nave *et al.* 2008) and cytotoxicity (Lautenbach *et al.* 2009) along with impaired macrophages phagocytosis (Li *et al.* 2009b). Obesity has also been shown to be associated with a concomitant reduction in dendritic cells numbers and an attenuation of their antigen presentation capabilities (Macia *et al.* 2006; Smith *et al.* 2009). Although fewer studies

have been conducted in humans, similar immune alterations have been observed in obese individuals. Higher BMI has been associated with reductions in circulating T-cells (Tanaka *et al.* 2001) along with reductions in both naïve and memory T-cell functions (Nieman *et al.* 1999; Karlsson *et al.* 2010). The data presented in *Chapter 5* highlighted the deleterious impact of obesity on the “immune space” composition, by showing that obesity was associated with reduced naïve and early differentiated T-cells and a concomitant increase in late differentiated T-cells independently of chronological ageing. This suggests that the immunosuppression state associated with obesity may be resulting in the accumulation of dysfunctional senescent and late differentiated T-cells. The chronic pro-inflammatory state observed in obese individuals has been proposed to accelerate the rate of telomere attrition, and consequently promote the accumulation of senescent T-cells. In addition, high concentrations of serum leptin were shown in *Chapter 5* to be associated with increased proportions of late differentiated T-cells and reduced proportions of naïve and early differentiated T-cells.

Leptin is a 16 kDa protein, composed of 146 amino acids, transcribed from the *ob* gene in 3.5 kb mRNA in adipocytes. While essentially secreted by adipocytes, proportionally to the adipose tissue mass, leptin can also be secreted in small quantities by skeletal muscle cells, fundical epithelium and by the placenta (Friedman & Halaas 1998). Leptin is released in the bloodstream on a circadian rhythm, with a nadir at 0800-0900h succeeded by a progressive rise to reach a peak between midnight and 0200h (Licinio *et al.* 1997). Physiological serum leptin concentrations in healthy lean individuals range from 1 to 5ng.mL⁻¹ but can attain 50ng.mL⁻¹ in subjects with class IV obesity (super-obesity) (Prolo *et al.* 1998). This accumulation of serum leptin is likely to be a direct consequence of the down-regulation of leptin receptor on the hypothalamus observed in obese individuals (Chen *et al.* 2005). Leptin is primarily an energy homeostasis

adipokine, limiting excessive food intake (Korner *et al.* 2001; Konturek *et al.* 2002), but its structural similarities with certain pro-inflammatory cytokines, such as IL-6, IL-12 or IL-15 (Faggioni *et al.* 2001; La Cava *et al.* 2004), extends its action to immune cells. Thus, it has been shown to increase *in vitro* proliferation and functionality of innate immune cells, such as monocytes, macrophages and NK cells (Gainsford *et al.* 1996; Zhao *et al.* 2003; Mancuso *et al.* 2004) but also to participate in the reduction of sepsis-induced inflammation by reducing circulating pro-inflammatory cytokines and increasing secretion of anti-inflammatory IL-4 and IL1RA in animals (Gabay *et al.* 2001; Jaworek *et al.* 2002; Xiao *et al.* 2003). Although more research needs to be conducted to study the *in vivo* impact of high physiological concentrations of leptin in humans on inflammation and oxidative stress, a potential link between abnormal serum leptin concentration and premature immune ageing can be hypothesized. Indeed, in addition to the data presented in *Chapter 5* showing that leptin concentration was associated with increased proportions of late differentiated T-cells, studies have shown that both BMI and leptin concentrations were inversely correlated with leukocyte telomere length in human, a biomarker of immunosenescence (Valdes *et al.* 2005; Lee *et al.* 2010). Leptin-induced oxidative stress is however unlikely to be the only culprit of leukocyte telomere shortening precipitation, and a more direct action of the hormone on immune ageing is convincing.

As a consequence of a wide distribution of leptin receptor (Ob-R) expression amongst immune cells, serum leptin has a direct effect on circulating immune cells (Papathanassoglou *et al.* 2006). It has been shown to up-regulate the expression of late activation markers on CD4⁺ and CD8⁺ T-cells (Martin-Romero *et al.* 2000) and adhesion molecules such as ICAM-1, and very late antigen 2 (VLA-2) on CD4⁺ T-cells (Park *et al.* 2001). Moreover, supra-physiological doses of leptin have been shown to

enhance CD45RA⁺ CD4⁺ and CD8⁺ “naïve” T-cell activation and proliferation when cultured *in vitro* with a mitogen, while it tends to inhibit CD45RO⁺ “memory” T-cell proliferation (Martin-Romero *et al.* 2000; Lord *et al.* 2002; De Rosa *et al.* 2007).

Interestingly, while physiological concentrations of leptin corresponding to those found in lean individuals protects from stress-induced thymic atrophy (Gruver & Sempowski 2008) and thymic involution in mice (Hick *et al.* 2006; Dixit *et al.* 2007), elevated serum leptin concentrations appear to have an opposite effect by reducing thymic output in obese individuals (Yang *et al.* 2009). The stimulating effect of high doses of leptin on “pre-activated” T-cells is likely to result in a constant state of T-cell activation, leading to disregulated T-cell proliferation, and consequently promote the accumulation of late differentiated and senescent T-cells seen in obese individuals. However, while the stimulating effects of high concentrations of leptin on T-cells have been documented (Martin-Romero *et al.* 2000), the effect of physiological leptin concentrations, such as the one observed in lean and obese individuals, on T-cell activations remain to be investigated.

The aim of the study presented in this chapter was to examine the effect of physiological concentrations of plasma leptin on T-cell activation. In addition this chapter aimed to assess both the constitutional and inducible expression of OB-Rb receptor and mRNA in T-cells.

6.2. Materials and Methods:

6.2.1. Subjects

Volunteers were 10 lean healthy non-smoker Caucasian males (Mean \pm SD: age= 24.8 \pm 2.6 yrs; BMI= 22.6 \pm 2.1 kg/m²) Blood samples were drawn as described in the general materials and methods *Chapter 3.3*. Subjects with excessive alcohol intake (>14 drinks per week), with a BMI exceeding 25 kg/m² and taking medications affecting the immune system and/or their basal serum leptin concentration were excluded from the study. Height and weight measurements were used to determine their BMI.

6.2.2. T-cell stimulation

After isolation, 1x10⁶ PBMCs were cultured in 24 wells Iwaki flasks (Barloworld scientific, Stone, United Kingdom), with 1 mL of RPMI-1640 culture medium supplemented with 100 units.mL⁻¹ P/S and 2mM of L-Glutamine. As shown in the literature (Martin-Romero *et al.* 2000) leptin alone is not likely to trigger a sufficient activation signal on resting T-cells and the use of a mitogenic stimulation was thought to overcome the resting T-cells activation threshold. The optimal concentration of mitogen (PMA) was defined to be 0.25 ng.mL⁻¹ as described in the general material and methods *Chapter 3.3.2*.

The various physiological concentration of human recombinant leptin added to each well are reported in Table 6.1. These concentrations were obtained from 1mg of lyophilized human recombinant leptin (R&D systems, Mineapolis, MN, USA) after

being reconstituted in sterile 20 mM Tris-HCl (pH 8.0) and correspond to the physiological range of leptin found in humans (Niskanen *et al.* 1997). After 4 hours of incubation at 37°C with 5% of CO₂, the supernatant was removed from the wells, and centrifuged during 10 minutes at 250 Gs. The pellet was then washed twice with 500µL of PBS 0.5% BSA solution (Sigma-Aldrich, Irvine, Scotland).

Table 6.1 Concentrations of leptin used for the stimulation of T-cells.

	PMA concentration (ng.mL ⁻¹)	Leptin Concentrations (ng.mL ⁻¹)	Corresponding BMI (kg/m ²) (Zimmet <i>et al.</i> 1996; Chow & Phoon 2003)
Negative Control	0	0	NA
No leptin	0,25	0	NA
Well 1	0,25	0,5	BMI below 20
Well 2	0,25	1,5	20
Well 3	0,25	5	20-25
Well 4	0,25	25	30
Well 5	0,25	50	BMI above 40

6.2.3. Flow Cytometric analysis

After stimulation, PBMCs were labeled with diluted antibodies as described in the general materials and methods *Chapter 3.5.1*. A combination of four antibodies was used to assess T-cell levels of activation: CD8 FITC, CD69 PE, CD4 Pe-cy5 and CD3 APC. The samples were analysed by four-colour flow cytometry, as described in the general materials and methods *Chapter 3.5.5*.

6.2.4. T-cell enrichment

T-cells, CD4⁺ T-cells and CD8⁺ T-cells populations were negatively selected from PBMCs using commercially available enrichment kits (BD Biosciences, Oxford, UK) as described in the general materials and methods *Chapter 3.6* and assessed for OB-R expression by direct flow cytometric analysis.

6.2.5. Total RNA extraction and leptin receptor gene analysis RT-PCR'

RNA samples were isolated from stimulated PBMC (containing at least 80% of CD3⁺ T-cells) and the effect of leptin stimulation on Ob-Rb gene expression was determined using Real time Polymerase Chain Reaction as described in the general materials and methods *Chapter 3.8.4*.

6.2.6. Statistical analysis

All values are presented as mean \pm Standard Error (SE). Data were normally distributed and the effects of PMA and leptin on T-cell activation were respectively analysed by one-way ANOVA and pair samples t-tests. Mean fold changes in OB-Rb expression in PBMCs among the different stimulation conditions were analysed using one-way ANOVA. Statistical significance was set at $P < 0.05$. All values are presented as the mean \pm Standard Error of the mean (SEM) unless stated otherwise. All statistical analyses were performed using "Statistical Package for the Social Sciences" (SPSS v17.0, Chicago, IL, USA).

6.3. Results

6.3.1. Effect of leptin on T-cell activation

The proportions of CD3+, CD4+ and CD8+ T-cells subsets expressing the early activation marker CD69, after exposition to the mitogen PMA and increasing concentrations of leptin are presented in the Figure 6.1.

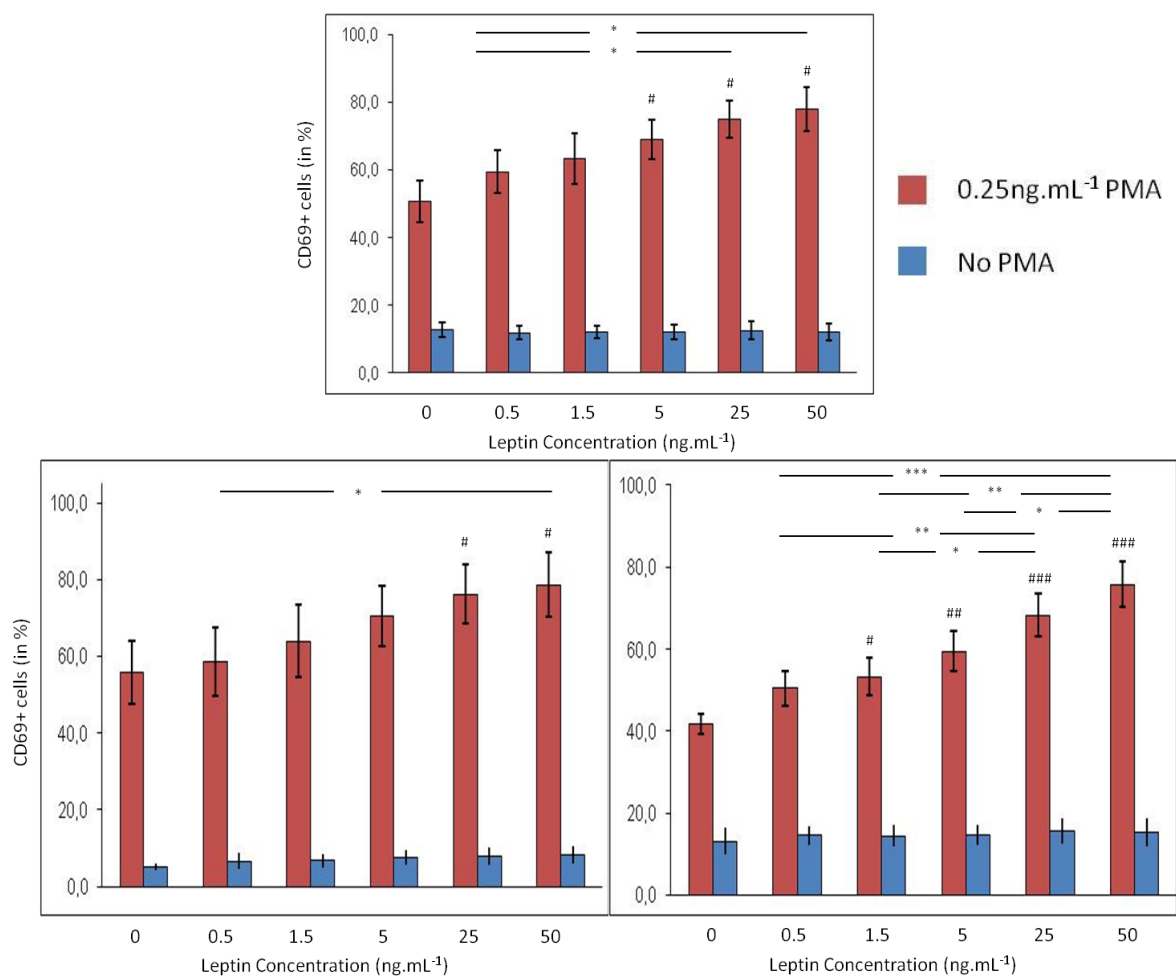


Figure 6.1 Proportions of all CD3+ (Top), CD4+ (Bottom left) and CD8+ (Bottom right) T-cells expressing CD69 in function of the leptin concentration with or without mitogenic stimuli. Mean values are represented \pm SEM. Statistical differences from the control condition, 0.25ng.mL⁻¹ PMA without leptin is indicated by # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$. Statistical differences between the different leptin concentrations is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

The proportions of CD3+, CD3+/CD4+ and CD3+/CD8+ T-cells expressing CD69 were significantly greater ($p < 0.001$), when stimulated with 0.25 ng.mL^{-1} of PMA. Proportions of T-cells expressing CD69 were further increased by the presence of human leptin. The amount of CD69+ CD3+ T-cells cultured with PMA and 5 ng.mL^{-1} , 25 ng.mL^{-1} or 50 ng.mL^{-1} of leptin were significantly greater than the amount of CD69+ cells cultured with lower concentrations or with an absence of leptin ($p < 0.05$). A greater proportion of cells stimulated with 25 ng.mL^{-1} and 50 ng.mL^{-1} of leptin expressed CD69 when compared to cells stimulated with 0.5 ng.mL^{-1} of leptin (Figure 6.1).

Greater proportions of CD69+ CD4+ T-cells were observed when the cells were stimulated with 25 ng.mL^{-1} and 50 ng.mL^{-1} compared with lower concentrations or with an absence of leptin ($p < 0.05$). The proportions of CD4+ T-cells expressing CD69 was also significantly higher when cells were stimulated with 50 ng.mL^{-1} than with 0.5 ng.mL^{-1} of leptin. However no differences were seen between the amount of CD4+ cells T-expressing CD69 in the control condition containing no leptin and when cells were exposed to 0.5 ng.mL^{-1} , 1.5 ng.mL^{-1} or 5 ng.mL^{-1} of leptin ($p > 0.05$) (Figure 6.1).

More CD8+ T-cells expressed CD69 when stimulated with PMA and 1.5 ng.mL^{-1} ($p < 0.05$), 5 ng.mL^{-1} ($p < 0.01$), 25 ng.mL^{-1} ($p < 0.001$) and 50 ng.mL^{-1} ($p < 0.001$) leptin, in comparison to the control condition ($p < 0.05$). Furthermore, cells cultured with 25 ng.mL^{-1} and 50 ng.mL^{-1} of leptin expressed greater levels of CD69 than cells cultured with 0.5 ng.mL^{-1} ($p < 0.05$) and 1.5 ng.mL^{-1} ($p < 0.01$) or 0.5 ng.mL^{-1} ($p < 0.001$), 1.5 ng.mL^{-1} ($p < 0.01$) and 5 ng.mL^{-1} ($p < 0.05$) of leptin respectively (Figure 6.1).

The CD69 Geometric Mean of Fluorescence Intensity (GMFI) of the CD3+, the CD4+ and the CD8+ T-cell populations after PMA stimulation and increasing concentrations of leptin are presented in the Figure 6.2.

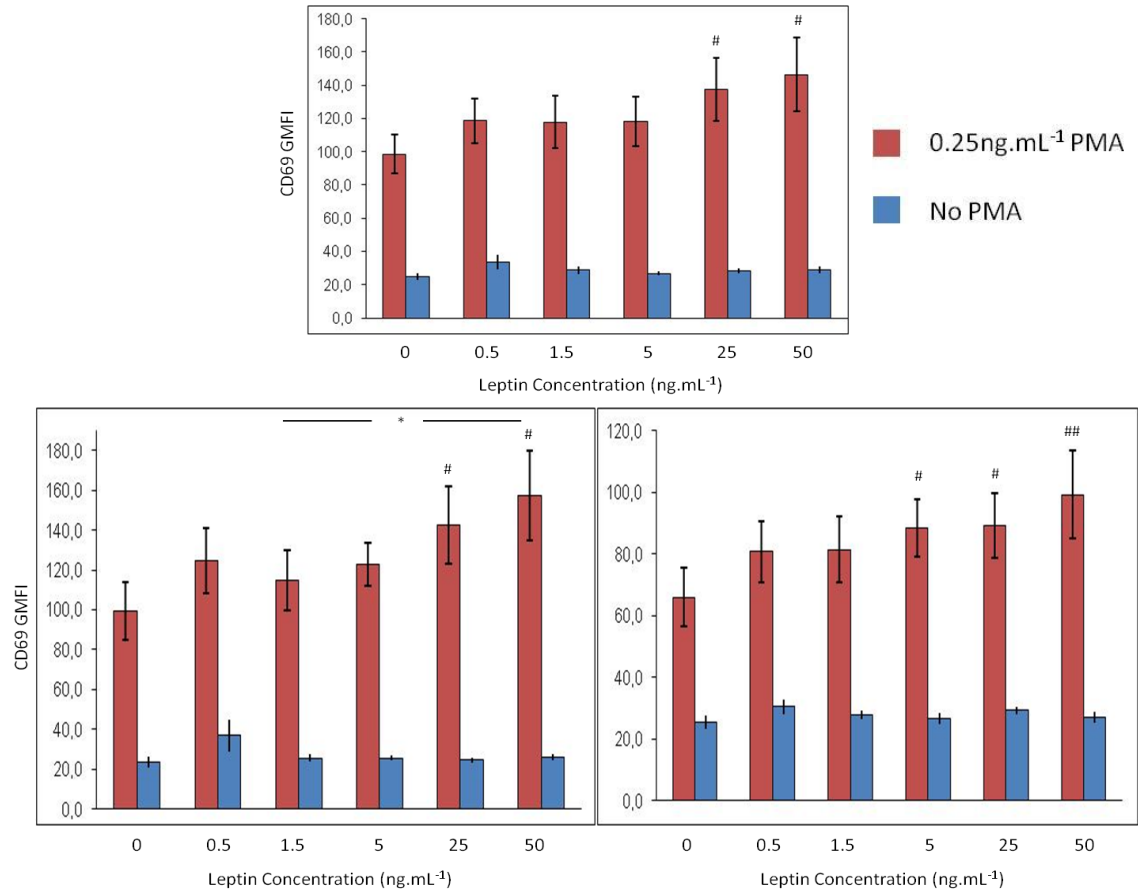


Figure 6.2 CD69 GMFI of all CD3+ (Top), CD4+ (Bottom left) and CD8+ (Bottom right) in function of the leptin concentration with or without mitogenic stimuli. Mean values are represented \pm SEM. Statistical differences from the control condition, 0.25ng.mL⁻¹ PMA without leptin is indicated by # $p < 0.05$ and ## $p < 0.01$. Statistical differences between the different leptin concentrations is indicated by * $p < 0.05$.

The CD69 GMFI expression was significantly increased in the CD3+, CD4+ and CD8+ T-cells by PMA stimulation ($p < 0.001$). The CD69 GMFI was significantly higher on CD3+ T-cells after stimulation with 25 ng.mL⁻¹ and 50 ng.mL⁻¹ of leptin stimulation when compared with lower concentrations or with an absence of leptin ($p < 0.05$). CD69 GMFI on CD4+ T-cells was greater when the cells were stimulated with 25 ng.mL⁻¹ and 50 ng.mL⁻¹ ($p < 0.05$) of leptin than the CD69 GMFI of control CD4+T-

cells and CD4⁺ T-cells expressed more CD69 when cultured with 50 ng.mL⁻¹ than 1.5 ng.mL⁻¹ of leptin ($p < 0.05$). No difference in CD69 GMFI could be observed between the control condition containing no leptin and when cells were stimulated with 0.5 ng.mL⁻¹, 1.5 ng.mL⁻¹ and 5 ng.mL⁻¹ of leptin ($p > 0.05$). An increased expression of CD69 was observed on CD8⁺ T-cells cultured with 5 ng.mL⁻¹ ($p < 0.05$), 25 ng.mL⁻¹ ($p < 0.05$), and 50 ng.mL⁻¹ ($p < 0.01$) when compared to the control conditions. Cells stimulated with 0.5 ng.mL⁻¹ and 1.5 ng.mL⁻¹ of leptin did not have a statistically different expression of CD69 GMFI than the cells from the control condition ($p > 0.05$) (Figure 6.2).

Representative histograms of CD69⁺ CD4⁺ and CD8⁺ T-cells after stimulation with increasing concentrations of human recombinant leptin and with or without mitogenic co-stimulation are presented in the Figures 6.3 and 6.4.

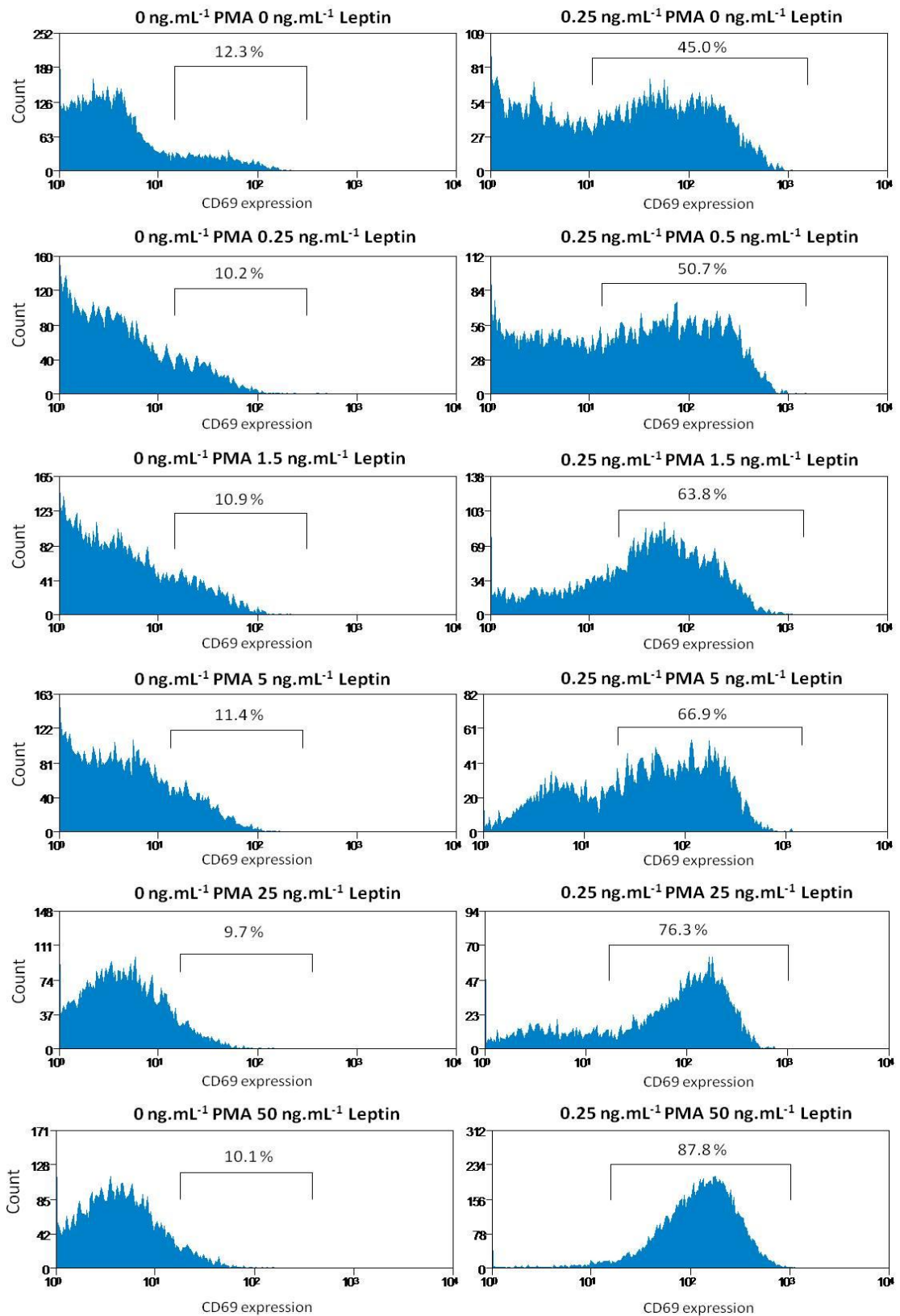


Figure 6.3. Representative flow cytometric histograms showing the proportions of CD69⁺ CD4⁺ T-cells cultured with different concentrations of leptin with or without mitogenic stimulation. The positive population is indicated by the marker gate. The y-axis is the log of the fluorescent intensity

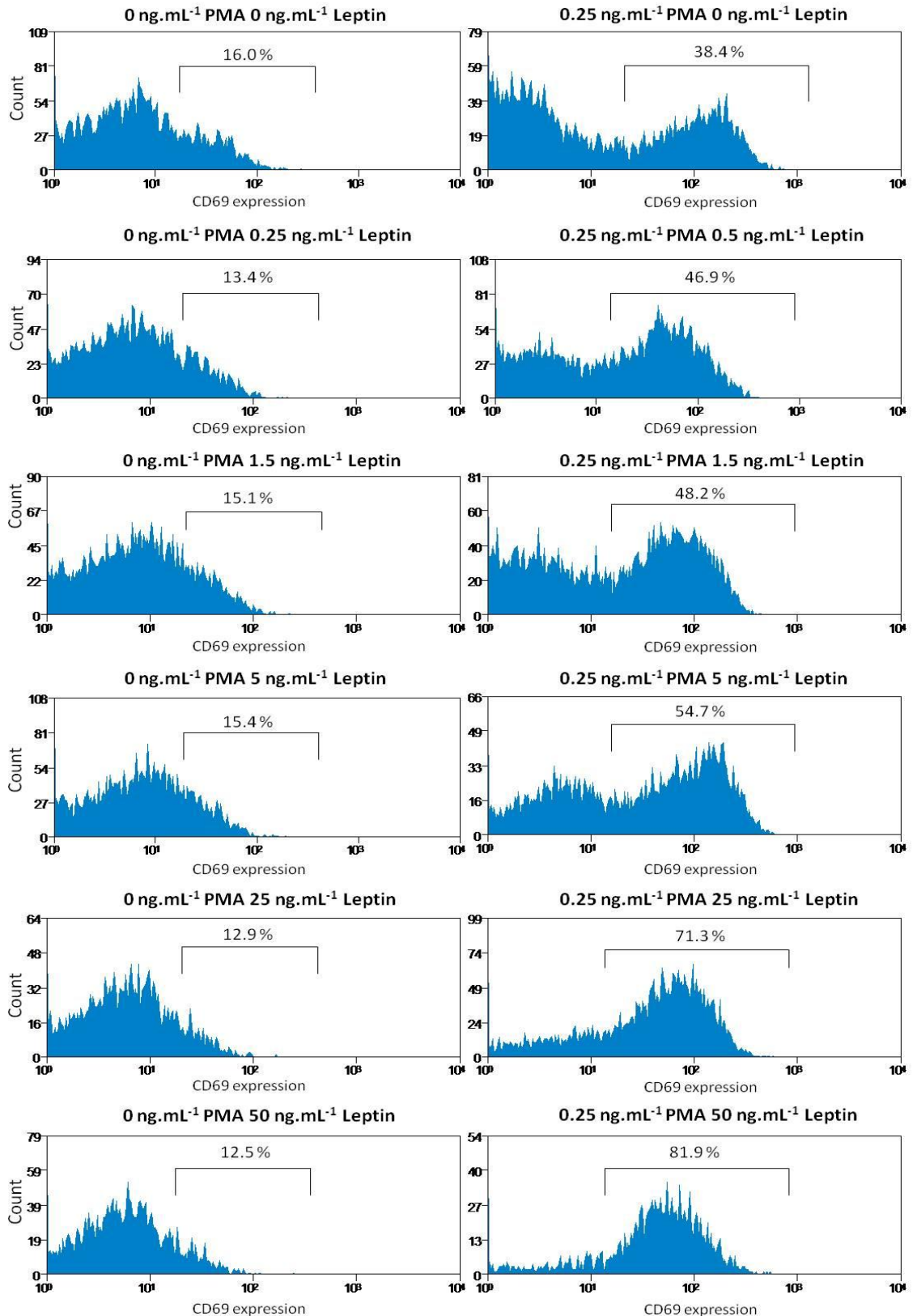


Figure 6.4. Representative flow cytometric histograms showing the proportions of CD69+ CD8+ T-cells cultured with different concentrations of leptin with or without mitogenic stimulation. The positive population is indicated by the marker gate. The y-axis is the log of the fluorescent intensity.

6.3.2. OB-Rb expression on T-cells

Increasing concentrations of leptin without simultaneous PMA stimulation did not induce T-cell activation, as demonstrated by the lack of CD69 expression on the different T-cell subsets. Consequently, we hypothesized that resting T-cells lacked OB-Rb expression, thus preventing their activation by serum leptin alone. We sought to characterise the surface expression of OB-Rb on the different T-cell subsets. The results are presented in the Figure 6.5.

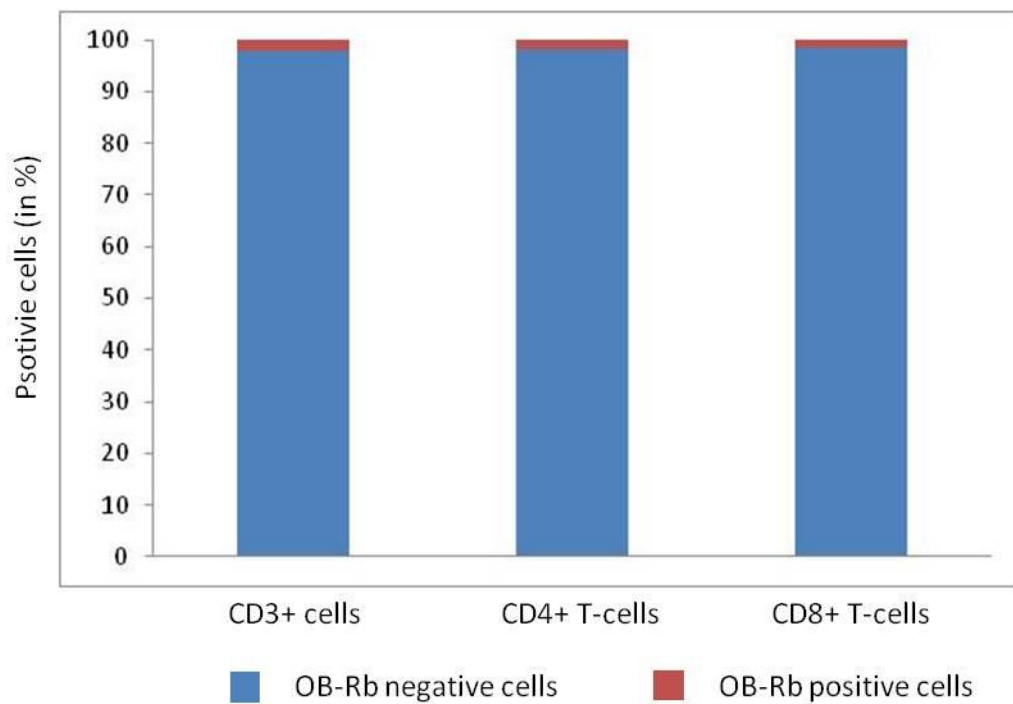


Figure 6.5. Proportions of enriched CD3+, CD4+ and CD8+ T-cell subsets expressing the leptin cell surface receptor (OB-Rb) analysed by three-colours flow cytometry.

Very low proportions of enriched CD3+, CD4+ and CD8+ resting T-cells expressed the leptin cell surface receptor (in average 2.2%, 1.9% and 2.1% respectively). Although those results highlight the limited expression of OB-Rb on the different resting T-cell subsets, it does not give information on the capacity of the different T-cell subsets to up-

regulate OB-Rb. Consequently OB-Rb gene expression was assessed on both resting and activated T-cells.

Total RNA was extracted from resting PBMCs and after PCR-amplification of OB-Rb, RNA gel electrophoresis was performed. The resulting gel is presented in Figure 6.6.

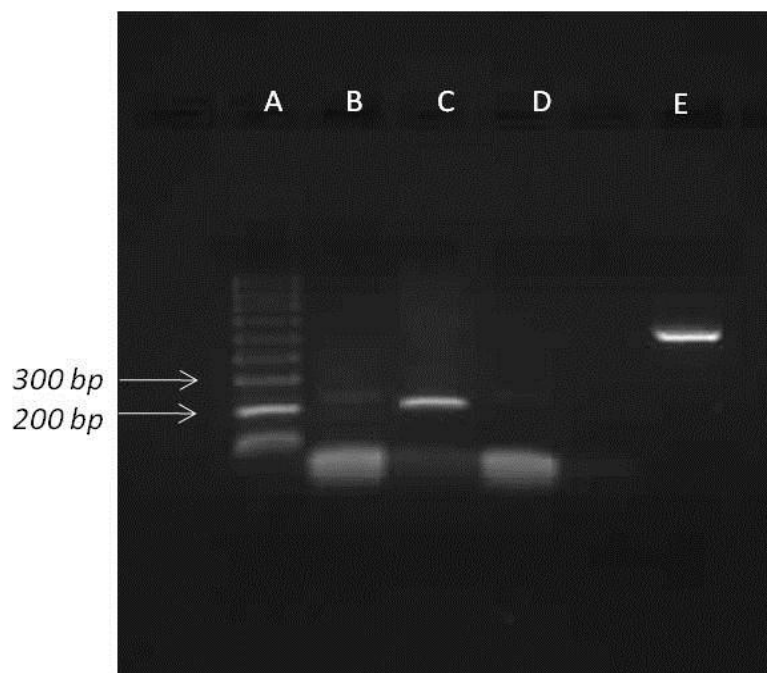


Figure 6.6. Ethidium-bromide stained agarose gel electrophoresis of OB-Rb cDNA isolated from resting PBMCs. The size of the cDNA produced (C) was estimated by comparing the mobility of the molecular weight markers (A). OB-Rb negative control (B and D) and positive control (GAPDH) (E) were used to validate the gel electrophoresis.

Using the migration of the molecular weight markers, the molecular weight of the generated mRNA is comprised between 200 bp and 300 bp, which corresponds to the expected molecular weight of OB-Rb mRNA (246 bp) (Sobhani *et al.* 2000). These results highlight the expression of OB-Rb in resting PBMCs at the mRNA level. Furthermore, in order to characterise the impact of mitogenic stimulation and leptin stimulation on OB-Rb gene expression, Real-Time PCR was performed. The average

relative change in OB-Rb expression in PBMCs after being stimulated by various concentrations of human leptin are presented in the Figure 6.7.

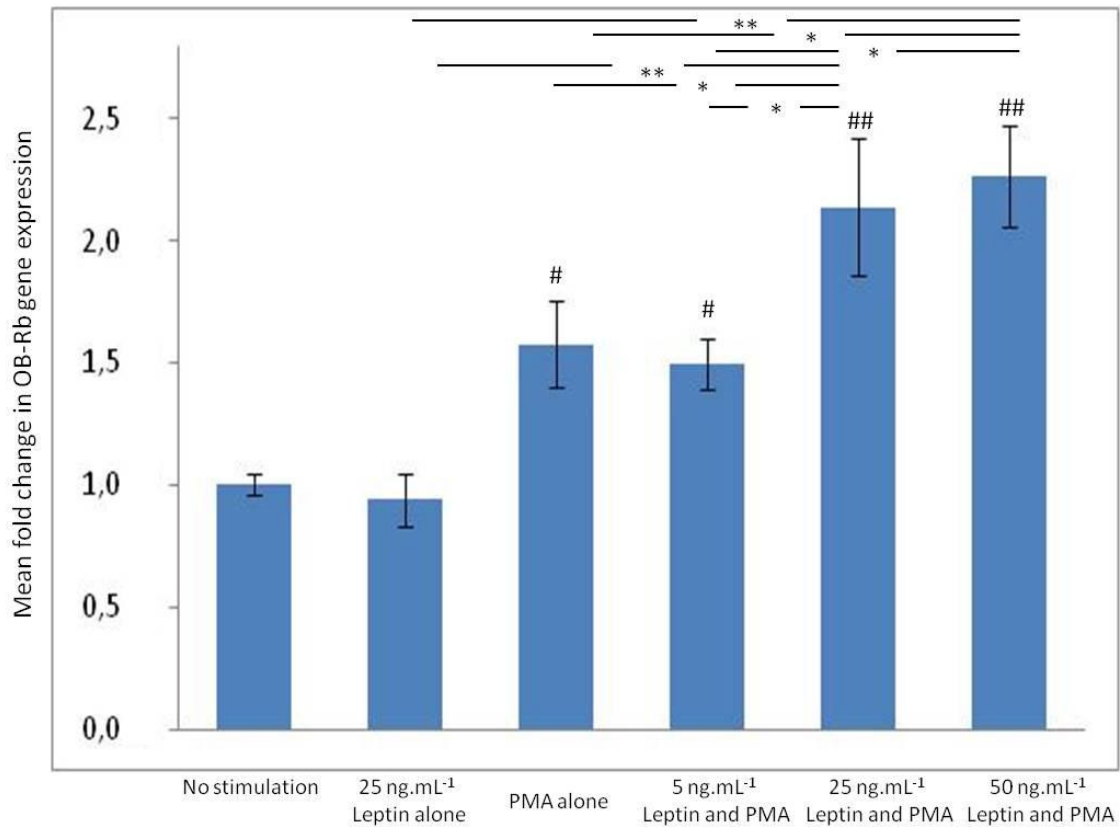


Figure 6.7. Mean fold change in OB-Rb mRNA expression in PMA and/or leptin stimulated PBMCs. Real-Time PCR was normalised by using the expression of the housekeeping gene GAPDH. Results represent mean \pm SEM value of 4 subjects (in duplicate) compared with control for each stimulation condition. Statistical differences from the unstimulated condition is indicated by # $p < 0.05$ and ## $p < 0.01$. Statistical differences between the different stimulations concentrations is indicated by * $p < 0.05$ and ** $p < 0.01$.

The level of OB-Rb mRNA expression was unchanged in unstimulated PBMCs when compared to the level of the housekeeping GAPDH mRNA expression, however when cells were stimulated with PMA, they up-regulated OB-Rb ($p < 0.05$). The up-regulation of OB-Rb mRNA expression was also observed in PBMCs stimulated with

PMA and 5 ng.mL⁻¹, 25 ng.mL⁻¹ and 50 ng.mL⁻¹ of human leptin (p < 0.05 and p < 0.01 respectively).

When cells were stimulated with 25 ng.mL⁻¹ and 50 ng.mL⁻¹ of leptin, they expressed more OB-Rb mRNA than cells stimulated with PMA alone (p < 0.05) or PMA and 5 ng.mL⁻¹ of leptin (p < 0.05). Indeed when cells were stimulated with PMA alone or PMA and 5 ng.mL⁻¹ of leptin the mean OB-Rb mRNA fold change observed was respectively 1.57 and 1.52 times, whereas when cells were stimulated with PMA and 25 ng.mL⁻¹ or 50 ng.mL⁻¹ of leptin (p < 0.05), the mean OB-Rb mRNA fold change was 2.13 and 2.12 times (p < 0.05).

The level of expression of OB-Rb mRNA remained unchanged when compared to the level of GAPDH mRNA expression in cells stimulated with 25 ng.mL⁻¹ of leptin alone.

6.4. Discussion

The main aim of the study presented in this chapter was to characterise the effect of various physiological concentrations of human leptin on the activation of peripheral blood T-cells. This study also sought to assess the expression of the leptin receptor (OB-Rb) on T-cells, both at the protein and genetic level, and the impact of leptin stimulation on its regulation. We have shown that although serum leptin alone was not able to activate peripheral blood T-cells, leptin increased mitogen-induced T-cell activation in a dose-dependent manner. It was also shown that, although only a very small proportion of resting T-cells expressed OB-Rb, they constitutively expressed OB-Rb mRNA after stimulation. Furthermore, mitogen-induced T-cell activation lead to the up-regulation of OB-Rb when cells were costimulated with high physiological doses of leptin typically

seen in obese individuals, but not with the low level of leptin typically seen in lean subjects.

The data presented in *Chapter 5* highlighted an association between excess body mass and increased proportions of late differentiated T-cells, along with reduced proportions of early differentiated and naïve T-cells. Furthermore, a similar effect of high physiological leptin concentrations was observed on these cell populations. The study presented in this chapter showed that high physiological leptin concentrations, such as the one seen in obese individuals, enhanced mitogen-induced T-cell activation. Although leptin alone was not able to activate resting T-cells, it amplified T-cell activation when costimulated with PMA. The immunological effects of leptin have been documented in the past. Indeed, considering their three-dimensional structures, leptin and its receptor belong to the class I cytokine receptor family (Zhang *et al.* 1996) and consequently will have an impact on the immune system. Several studies have clearly shown effects of leptin on the secretion of pro-inflammatory cytokines, such as the increase in pro-inflammatory cytokines production in murine peritoneal macrophages, such as IL-6 and TNF- α (Gainsford *et al.* 1996). Increases in TNF- α and IL-6 production were also seen after human monocyte activation by serum leptin (Santos-Alvarez *et al.* 1999). Studies conducted by Fraser *et al.* (1999), Martin-Romero *et al.* (1999) and more recently by Lord (2006) highlighted the potential for leptin to up-regulate activation markers such as CD25 and CD69 when present in very high concentration in the medium. In the present study, T-cell activation was assessed by the proportion of T-cells expressing CD69 which is the earliest inducible cell surface glycoprotein acquired during lymphoid activation. This Ca²⁺ dependent c-type lectin is expressed on B and T-cells, following a direct stimulation by exposition to an antigen or a mitogen (e.g. phorbol myristate acetate) (Chen *et al.* 1997). In a recent study Sancho

et al. (2005) has proposed a pro-inflammatory role of CD69, by showing that, in presence of a mitogen, the expression of CD69 enhances T-cell proliferation, tumour necrosis factor-alpha (TNF- α) through the production of IL-2. However, in vivo studies on CD69 knock-out mice didn't show any decreases in T-cell activation, challenging the hypothesis proposing CD69 to be a regulator of the immune response (Lauzurica *et al.* 2000). Consequently, although the exact role of CD69 is still unclear, its involvement in lymphocyte proliferation and signal-transmitting receptor in immune cells establishes it as a good marker of T-cells early activation (Diaz-Augustin *et al.* 1999).

As stated above, leptin alone did not activate the isolated T-cells, independently of the concentration used. However, when T-cells were stimulated with a mitogen (PMA), high concentrations of leptin (25 ng.mL⁻¹ and 50 ng.mL⁻¹) enhanced CD4+ T-cell activation and medium and high concentrations of leptin (5 ng.mL⁻¹, 25 ng.mL⁻¹, 50 ng.mL⁻¹) enhanced CD8+ T-cell activation. Interestingly, lower physiological concentrations of leptin did not enhance the PMA-induced T-cell activation. Although CD8+ T-cells appear to be more responsive to lower doses of leptin than CD4+ T-cells, the lowest concentrations of leptin used (0.5 ng.mL⁻¹ and 1.5 ng.mL⁻¹) correspond to the physiological serum leptin concentrations found in lean subjects (Prolo *et al.* 1998). The lack of activation of CD4+ and CD8+ T-cells by increasing concentrations of leptin alone might suggest that the leptin signaling pathway is not sufficient to generate T-cells activation and proliferation on its own, however it will enhance the activation in presence of a mitogenic or antigenic stimuli.

Another potential explanation lies in the low expression of the active form of the leptin receptor (OB-Rb) on the resting T-cell surface. Ob-Rb is expressed in the arcuate and ventromedial hypothalamic nuclei where it mediates the central effects of leptin (Glaum *et al.* 1996; Mercer *et al.* 1996; Tartaglia 1997), and has also been shown to be present

on the surface of all cell types of innate and adaptive immunity (Caldefie-Chezet *et al.* 2001; Zarkesh-Esfahani *et al.* 2001; Zhao *et al.* 2003; Lord *et al.* 1998). However the data presented in this study show that isolated resting CD3+, CD4+ and CD8+ T-cells lack cell-surface OB-Rb expression at the protein level, only a non-significant proportion of resting T-cells (less than 3%) expressed the long metabolically active form of the leptin receptor.

The lack of OB-Rb expression on resting T-cells has been previously observed (Zarkesh-Esfahani *et al.* 2001), and provides a potential explanation on the inability of leptin alone to activate isolated T-cells. Furthermore, it suggests that PMA stimulation up-regulates OB-Rb expression on T-cells, thus enhancing T-cells activation.

Although resting T-cells lacked expression of the cell surface OB-Rb, it was important to establish the presence of OB-Rb mRNA in those cells. Indeed because of the short exposure to the different stimulation conditions (4 hours), OB-Rb mRNA had to be already expressed in resting T-cells to potentially be translated into OB-Rb protein, and be stimulated by exogenous leptin. The results presented in this chapter confirmed the presence of OB-Rb mRNA in resting T-cells, suggesting even further that, although leptin alone did not activate T-cells, prior stimulation with a mitogen such as PMA is required to induce OB-Rb mRNA translation and expression on the cell surface, allowing serum leptin to exert its activating effect on the T-cells. This was confirmed by the RT-PCR' analysis of OB-Rb mRNA in stimulated T-cells. It was shown that high physiological doses of leptin (25 ng.mL^{-1}) without simultaneous PMA stimulation did not affect the expression of OB-Rb. However, OB-Rb mRNA was up-regulated (1.5 fold compared to the level of GAPDH housekeeping gene) when T-cells were stimulated with PMA alone, and with PMA and low concentrations of leptin (5 ng.mL^{-1}). Interestingly, cells incubated with PMA and higher concentrations of leptin (25

ng.mL⁻¹ and 50 ng.mL⁻¹) as observed in obese patients expressed significantly more OB-Rb mRNA than cells stimulated with PMA alone and PMA + 5 ng.mL⁻¹ of leptin. This novel finding potentially explains why high physiological concentrations of serum leptin enhance the PMA-induced T-cell activation. Extending the tunable activation threshold (TAT) hypothesis proposed by Gossman *et al.* (Grossman & Paul 1992; Grossman & Singer 1996; Grossman & Paul 2001), stating that T-cells could dynamically tune their activation threshold in response to TCR stimulation to allow a degree of controlled autoreactivity, to the up-regulation of OB-Rb could explain this effect of high concentrations of leptin on OB-Rb mRNA. Indeed, presence in the cellular microenvironment of high doses of leptin, may trigger a cascade reaction leading to the up-regulation of OB-Rb, to present increased number of available binding site. Following this hypothesis, lower leptin concentrations would not be sufficient to achieve the T-cell threshold of activation and would not lead to the up-regulation of OB-Rb mRNA.

Although no difference in OB-Rb mRNA expression was seen between T-cells stimulated with PMA alone and T-cells stimulated with PMA and 5 ng.mL⁻¹ of leptin, it is interesting to observe that more CD8⁺ T-cells expressed the activation marker CD69 when co-stimulated with PMA and leptin than PMA alone. This can be explained by the use of a mixed T-cell population to analyse OB-Rb mRNA expression. T-cells enrichments did not allow the extraction of sufficient amount of genetic material to perform the different mRNA analysis, and RNA was isolated from isolated PBMCs instead of pure T-cell subsets. To limit the impact of this limitation, RNA was only extracted from PBMCs samples containing a minimum of 80% of T-cells and samples containing an excessive amount of other cell types, such as NK cells or B cells were discarded.

Certain limitations exist in this study. Only lean subjects were recruited to take part in this study, in order to control for potential leptin resistance as observed in obese patients (Tschop *et al.* 2007). Exposure to high physiological doses of serum leptin over a prolonged period of time appears to induce alterations in leptin transport across the blood brain barrier (Banks & Farrell 2003), and may impair OB-Rb intracellular signaling (Munzberg & Myers 2005). Consequently the use of T-cells isolated from lean subjects controlled for such impairments. However, studies have shown an inverse correlation between body weight and serum OB-Re, the soluble form of the leptin receptor (Chan *et al.* 2002; Ogier *et al.* 2002; Shimizu *et al.* 2002). As a consequence of its high affinity for leptin, the complex OB-Re-leptin is formed when OB-Re is present in high concentrations, such as those observed with lean patients. This complex prevents leptin from binding and being internalized by OB-Rb (Yang *et al.* 2004; Tu *et al.* 2008), and thus preventing its action. The observed effects may consequently be due to the unusual exposure of T-cells to high concentrations of unbound leptin, which may have amplified the *in vivo* leptin effect on T-cells in obese individuals. Consequently, further research should study the effect of high leptin concentrations on T-cells isolated from obese individuals. Furthermore, future studies should also assess the effect of high physiological concentrations of leptin on T-cell proliferation.

An association between leptin concentration and late differentiated or senescent T-cells was previously shown in *Chapter 5*. The results presented in this study highlight a potential mechanistic effect of high physiological concentrations of serum leptin on human T-cells. Indeed, when T-cells are activated, as seen during latent viral reactivation or bacterial infection, they may become susceptible to further activation by endogenous leptin. This could further drive T-cell differentiation toward a senescent phenotype. In addition, leptin has been shown to increase anti-apoptotic proteins such as

Bcl-2, Bcl-X_L (Fujita *et al.* 2002) and T-bet (Lord *et al.* 2002), protecting thymocytes and mature T-cells from undergoing apoptosis. Suggesting further that prolonged exposure to high physiological concentrations of leptin could promote the accumulation of senescent T-cells, by enhancing their differentiation and preventing clearance of senescent T-cells via apoptosis. The leptin signaling pathway is known to be similar to cytokine activation via the recruitment of Janus kinases (JAK) and phosphorylation of the signal transducers and activators of transcription (STAT) (Baumann *et al.* 1996; Sanchez-Margalet & Martin-Romero 2001; Cheng *et al.* 2002). It has been suggested that the stimulation effect of normal concentrations of leptin on the immune system can be beneficial to the host, by enhancing neutrophil bactericidal capacities (Caldefie-Chezet *et al.* 2001; Caldefie-Chezet *et al.* 2003), potentially inducing lymphopoiesis (Bennett *et al.* 1996) and regulating thymocyte maturation (Howard *et al.* 1999) in mice. However similarly to very low concentrations of leptin, as seen during starvation, inducing lymphoid atrophy and increased sensitivity to endotoxic shock (Faggioni *et al.* 1999; Howard *et al.* 1999; Faggioni *et al.* 2000; Faggioni *et al.* 2001), sustained exposure to high physiological doses of leptin as seen in overweight and obese patients, can lead to enhanced T-cell activation, and possibly lead to premature replicative senescence.

In conclusion, the study presented in this chapter provides novel findings on the mechanistic effect of high physiological concentrations of leptin on T-cell activation. These findings support the data presented in *Chapter 5* that highlighted an association between high physiological leptin concentrations in obese individual and the increased proportions of late differentiated T-cells along with the reduction in early differentiated and naïve T-cell pool, independently of chronological ageing. In addition to the effect of physical activity on the proportions of the different T-cell subsets presented in *Chapter*

4, increased body weight and the consequent increase in circulating serum leptin concentration, is further demonstrating the impact of lifestyle factors on immunosenescence.

CHAPTER 7

THE IMPACT OF LATENT HERPESVIRUS INFECTIONS ON THE MOBILISATION OF VIRAL-SPECIFIC AND SENESCENT T-CELLS IN RESPONSE TO ACUTE AEROBIC EXERCISE IN MAN

7.1. Introduction

The impact of exercise on the immune system has been extensively investigated over the past century (Larrabee 1902; Nieman *et al.* 1990a; Nieman *et al.* 1990b). Studies have been distinguishing moderate exercise from acute bouts of strenuous exercise due to their observed different effects on the immune system. Indeed, while regular moderate exercise appears to improve immune function (Kohut *et al.* 2001; Drela *et al.* 2004; Gleeson 2007; Spielmann *et al.* 2011), acute bouts of strenuous or exhaustive exercise have immunosuppressive effects as demonstrated by the suppressed humoral immunity (Verde *et al.* 1992) and the increased upper respiratory tract infection (URTI) prevalence in training and competing athletes (Nieman *et al.* 1990a; Nieman *et al.* 1990b; Nieman 1997; Murphy *et al.* 2008). On a cellular level, acute exercise induces important changes in the T-cell subsets such as decreased naïve (CD45RA+) / memory (CD45RO+) and CD4/CD8 ratios, primarily due to a reduction in the naïve CD4+ T-cell pool (Baum *et al.* 1994; Weiss *et al.* 1995; Rebelo *et al.* 1998; Buyukyazi *et al.* 2004), and a significant reduction in T-cell activation and proliferative responses (Nieman *et al.* 1994; Nieman *et al.* 1995; Bury *et al.* 1996; Vider *et al.* 2001a; Gleeson 2007). Furthermore, acute bouts of exercise elicit a preferential mobilization and extravasation of effector-memory T-cells that exhibit a highly differentiated cell surface phenotype (TEMRA) (Campbell *et al.* 2009; Turner *et al.* 2010) or T-cells with shortened telomeres exhibiting a terminally differentiated cell surface phenotype (Simpson *et al.* 2007a; Simpson *et al.* 2008) in the peripheral blood compartment along with Natural Killer cells and $\gamma\delta$ T-cells (Anane *et al.* 2009). Indeed, acute exercise activates the hypothalamic pituitary adrenal axis (HPA), leading to the release of cortisol and catecholamines in the peripheral blood compartment, that will in turn regulate the

immune response by binding to surface β_2 -adrenergic-receptors expressed by lymphocytes (Sanders 2011). As a result of histone and DNA methylation-induced epigenetic regulations, effector-memory, TEMRA and terminally differentiated CD8+ T-cells along with NK cells exhibit very high levels of β_2 -adrenergic-receptors, potentially explaining the preferentially elevated response of these cell subsets to acute exercise (Kruger *et al.* 2008). Following this transient exercise-induced lymphocytosis, increased lymphocyte trafficking (Simpson *et al.* 2007b) combined to a lesser extent with moderated levels of cellular apoptosis (Mooren *et al.* 2002; Mooren *et al.* 2004; Kruger *et al.* 2008) lead to a lymphocytopenia.

Acute exercise has been shown to preferentially mobilize a population of CD8+ T-cells that express CD57 and KLRG1, but lack expression of the co-stimulatory molecule CD28 (Simpson *et al.* 2007a; Simpson *et al.* 2008; Simpson *et al.* 2010a). This surface phenotype is indicative of an exhausted cell population that may be senescent (Ibegbu *et al.* 2005) and present reduced proliferative capacities coupled with a potential resistance to apoptosis while still exhibiting effector cells properties (Spaulding *et al.* 1999). As a consequence of their reduced sensitivity to apoptosis, those stress-sensitive terminally differentiated cells have been shown to accumulate with ageing and with repeated reactivation of the latent herpesviruses Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (Wikby *et al.* 1998; Spaulding *et al.* 1999; Wikby A. 2003). Indeed, it was demonstrated in *Chapter 5* of this thesis that CMV and EBV infection were associated with increased proportions of senescent T-cells and a concomitant reduction in naïve T-cells, at a very young age. The accumulation of exhausted terminally differentiated T-cells eventually lead to the congestion of the immunological space, limiting the

homeostatic production of functional naïve T-cells, and ultimately contributing to poor vaccine efficacy and immunodepression.

As total eradication of an herpesvirus infection is never achieved by the host, the probability of a seronegative subject to be primarily exposed to CMV and/or EBV increases continuously with age, rationalizing the 50% (CMV) (Bate *et al.* 2010) to 80% (EBV) (Markin 1994) prevalence of single infection observed in the Western adult population. CMV and EBV reactivation causes naïve T-cells but also CMV-specific and EBV-specific memory T-cells to undergo clonal expansion, increasing the frequency of highly and terminally differentiated viral-specific T-cells relative to naïve T-cells within the host. In turn, the increased proportions of highly differentiated T-cells in seropositive subjects at rest should lead to an accentuated preferential exercise-induced mobilisation and extravasation of those cell subsets.

The data presented in *Chapter 4* highlighted clear associations between physical fitness and regular physical activity and lower proportions of senescent T-cells along with a protected naïve T-cell repertoire in middle-aged men. These results suggest that regular physical activity can prevent or reduce the age-associated accumulation in senescent T-cells, implying that the beneficial impact of regular physical activity on the immune system could be due to the periodic repetition of acute bouts of exercise performed over an extended period of time (Simpson 2011). Indeed it is postulated in the study presented in this chapter that a frequent mobilization of these cells with regular exercise may lead to their subsequent deletion via apoptosis, freeing up “immunological space” for naïve T-cells to reside. A recent study showed that CMV infection status largely influenced the mobilization of highly differentiated effector-memory T-cells (CD28-, CD27-/+, CD45RA-/+) in the peripheral blood compartment following acute bouts of

exercise (Turner *et al.* 2010). This study failed however to identify the impact of EBV infection and the potential interaction between CMV and EBV in cases of co-infection on the exercise-induced preferential lymphocytosis of the terminally differentiated T-cell subset. In addition to CMV- and EBV-specific CD8⁺ T-cells being preferentially mobilised in the blood after an acute psychological stress (Atanackovic *et al.* 2006). No study to our knowledge has considered the impact of latent herpesvirus on the exercise-induced mobilization of dysfunctional terminally differentiated T-cells.

The aim of the study presented in this chapter is to determine the relative contribution of CMV- and EBV-specific cells to the preferential mobilization and subsequent egress of highly-differentiated and senescent CD8⁺ T-cell subsets in response to exercise in subjects carrying a latent infection. It is hypothesized that CD8⁺ T-cells specific to epitopes of CMV or EBV will be mobilized by exercise and will largely account for the amplified exercise-induced mobilization and egress of differentiated CD8⁺ T-cells in people with CMV.

7.2. Methods

7.2.1. Subjects

Otherwise healthy adult males (n = 19) aged 22-35 years (mean \pm SD age: 28.4 \pm 5.4 years; mass: 84.0 \pm 14.5 kg; $\dot{V}O_{2max}$: 38.1 \pm 9.2 ml·kg⁻¹·min⁻¹) participated in this study. All participants were non-smokers, not taking medication affecting the immune system or supplements and were free from any infectious illness for 6-weeks prior to

testing. Subjects were asked to refrain from exercise 24 hours prior the exercise protocol. After receiving oral and written information pertaining to the risks and demands of the study, each subject signed an informed consent document. The Committee for the Protection of Human Subjects (CPHS) at the University of Houston provided ethical approval for the study prior to the beginning of the study. The physical characteristics of the participants are presented in Table 7.2.

7.2.2. Blood Sampling and PBMC isolation

Blood samples were drawn at rest before the start of the exercise protocol, immediately after and 1 hour following the end of the exercise protocol and Peripheral Blood Mononuclear Cells (PBMC) were isolated as described in the general materials and methods *Chapter 3.3*.

7.2.3. Serological Testing for CMV and EBV IgG Antibodies

Anti-CMV and anti-EBV antibody titers were determined as described in the general materials and methods *Chapter 3.4*.

7.2.4. Submaximal Exercise Test to Estimate Maximal Oxygen Uptake and Maximum Cycling Power and Cycling exercise protocol

Submaximal estimation of the subject's maximal oxygen uptake and subsequent exercise protocol were conducted as described in the general materials and methods

Chapter 3.2.3. The mean $\dot{V}O_{2\max}$ and maximum power for the subjects was 39.8 ± 10.0 mL.kg⁻¹.min⁻¹ and 244.3 ± 58.7 W respectively.

7.2.5. Antigen-specific T-cell staining and monoclonal antibodies labeling

PBMC's were divided into 1.0×10^6 aliquots and labelled with 5 μ l of undiluted CMV-specific Major Histocompatibility Complex (MHC) class I Pentamer or EBV-specific MHC class I Pentamer conjugated to APC (A*201 *NLVPMVATV* and A*201 *GLCTVAML* respectively, Proimmune, Sarasota, FL, USA) and incubated at room temperature in the dark for 15 mins. Following incubation, cells were labelled with a combination of directly conjugated monoclonal antibodies (mAbs) against the cell surface antigens KLRG1, CD57, CD28, CD45RA, and CCR7. The monoclonal antibodies were freshly diluted in PBS-BSA to their optimal concentration as determined by antibody titration before a volume of 50 μ L was added to the cell suspension. The monoclonal antibody panel used and the phenotypes of interest are presented in Table 3.2 and Table 7.1.

Table 7.1. Phenotypic identification of the blood T-cell subsets

Phenotypes	T-cell subsets	References
KLRG1-/CD28+ KLRG1-/CD57- CD45RA+/CCR7+	Naïve T-cells	(Koch <i>et al.</i> 2008) (Geginat <i>et al.</i> 2003)
KLRG1+/CD28- KLRG1+/CD57+	Senescent T-cells	(Voehringer <i>et al.</i> 2002; Ibegbu <i>et al.</i> 2005)
KLRG1+/CD28+ KLRG1+/CD57-	Pan-memory T-cells	(Ibegbu <i>et al.</i> 2005)
CD45RA-/CCR7+	Central-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)
CD45RA-/CCR7-	Effector-memory T-cells	(Sallusto <i>et al.</i> 1999; Sallusto <i>et al.</i> 2004)
CD45RA+/CCR7-	Highly differentiated TEMRA cells	(Geginat <i>et al.</i> 2003)

7.2.6. Flow cytometric analysis

PBMC phenotypes were assessed on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) as described in the general materials and methods *Chapter 3.5*.

7.2.7. Statistical Analysis

All data were assessed for assumptions of normality using the Shapiro-Wilk test and constant error variance prior to formal statistical testing. Skewed cell-surface phenotypic data were then normalised by logarithmic transformation prior to statistical analysis when possible, and non-parametric tests were used when phenotypic data could not be normalised. No statistical outliers were found. Independent t-tests were performed to assess the impact of CMV or EBV infection status on the resting T-cell

repertoire while the impact of CMV and EBV co-infection on the resting T-cells was assessed by conducting factorial ANOVA. The main effect of infection status and exercise along with their interactions was detected among the subjects using linear mixed models. The effects of infection status on cell mobilization and egress was determined using linear mixed models and independent t-test when interactions were found. The effect of exercise on the viral-specific T-cells was assessed using Kruskal-Wallis non parametric test. Statistical significance was set at $P < 0.05$. All values are presented as the mean \pm Standard Error of the mean (SEM), unless stated otherwise. All statistical analyses were performed using “Statistical Package for the Social Sciences” (SPSS v17.0, Chicago, IL, USA).

7.3. Results

All subjects successfully completed the exercise protocols. The physical characteristics and exercise performance measures of all subjects are presented in Table 7.2. No statistical differences were found between CMV or EBV seropositive and seronegative subjects for any of the physical characteristics or exercise performance measures ($p > 0.05$). More CMV seropositive subjects were also infected with EBV than CMV seronegative subjects (Chi Square; $p < 0.05$).

Table 7.2. Physical characteristics and exercise performance measures of the participants in relation to their viral infection status (mean \pm SD). No statistical differences in both the physical characteristics and exercise measures amongst the groups were observed. # indicates a significant difference due to CMV serostatus ($p < 0.05$).

Physical Characteristics	All participants (n = 19)	CMV- (n = 11)	CMV + (n = 8)
Age (years)	28.4 \pm 5.4	28.2 \pm 5.6	28.8 \pm 5.4
Body mass (kg.)	84.0 \pm 14.5	83.2 \pm 14.0	85.1 \pm 16.0
BMI (kg.m⁻²)	26.6 \pm 4.1	26.0 \pm 3.4	27.6 \pm 5.1
Body fat (%)	20.0 \pm 8.2	18.2 \pm 7.7	22.3 \pm 8.9
Physical Activity level (1-7)	4.6 \pm 2.0	4.3 \pm 1.8	5.0 \pm 2.3
Maximum Power (w.)	238.9 \pm 60.6	240.1 \pm 62.5	237.1 \pm 61.7
$\dot{V}O_{2max}$ (mL.kg⁻¹.min⁻¹)	38.1 \pm 9.2	39.6 \pm 11.5	35.7 \pm 3.1
EBV seropositivity (%)	73.7	63.7	87.5 [#]
Exercise performance measures			
Mean Power (w.)	203.4 \pm 60.0	204.3 \pm 57.8	202.1 \pm 67.2
Mean Power (% max)	85.2 \pm 13.6	85.5 \pm 13.5	84.9 \pm 14.7
Mean Heart Rate (beats. min⁻¹)	156.5 \pm 7.2	155.5 \pm 7.6	157.8 \pm 6.7
Mean Heart Rate (% max)	83.2 \pm 3.5	82.6 \pm 4.0	84.0 \pm 2.8

7.3.1. Impact of latent CMV and EBV infections on blood T-cell phenotypes in healthy young adult men.

While CMV and EBV infections are known to have a great impact on the proportions of resting naïve, memory and senescent T-cells in the elderly, little is known about their impact on younger adults. Consequently, the impact of latent virus infections was

initially assessed on the resting T-cell repertoire. The cohort could be divided in 4 groups according to their infection status: CMV+/EBV+ (n=7); CMV+/EBV- (n=1); CMV-/EBV+ (n=7) and CMV-/EBV- (n=4). As seen in Figure.7.1, subjects infected with CMV had higher proportions of CD4+ and CD8+ T-cells expressing CD57 ($p < 0.05$ and $p < 0.001$ respectively) and lower proportions of CD8+ T-cells expressing the co-stimulatory marker CD28 ($p < 0.01$) compared to CMV seronegative subjects. The frequencies of T-cells expressing those markers were not influenced by EBV infection. Naïve, memory and senescent T-cells frequencies in subjects with different infection status are presented in Figure.7.2, Figure.7.3 and Figure.7.4 respectively. The naïve CD8+ T-cells were affected by CMV infection as seronegative subjects had a greater proportion of naïve CD45RA+/CCR7+ ($p < 0.05$) but not by EBV infection (Figure.7.2). While non-significant, a similar trend was observed in the proportions of naïve KLRG1-/CD28+ CD8+ T-cells ($p = 0.090$) (Figure.7.2). Proportions of senescent KLRG1+/CD57+ CD4+ and CD8+ T-cells ($p < 0.05$ and $p < 0.001$ respectively) and KLRG1+/CD28- CD8+ T-cells ($p < 0.001$) were increased in CMV+ subjects in comparison to CMV- subjects (Figure.7.4). The proportions of highly differentiated effector-memory CD8+ T-cells re-expressing CD45RA (TEMRA) were greater in the CMV+ subjects than the CMV- subjects ($p < 0.001$) (Figure.7.4). It is interesting to observe that a similar effect of CMV infection was seen in the CD4+ TEMRA cells, but it was not significant ($p = 0.075$) (Figure.7.4). Finally, memory CD4+ and CD8+ T-cells were not significantly influenced by viral infection status (Figure.7.3).

No interaction effect of CMV and EBV co-infection was observed on the frequencies of naïve, memory and senescent T-cells as presented in Figure.7.5. However, the impact of CMV single infection (CMV+/EBV-) on the proportions of the different T-cell subsets was based on only one subject.

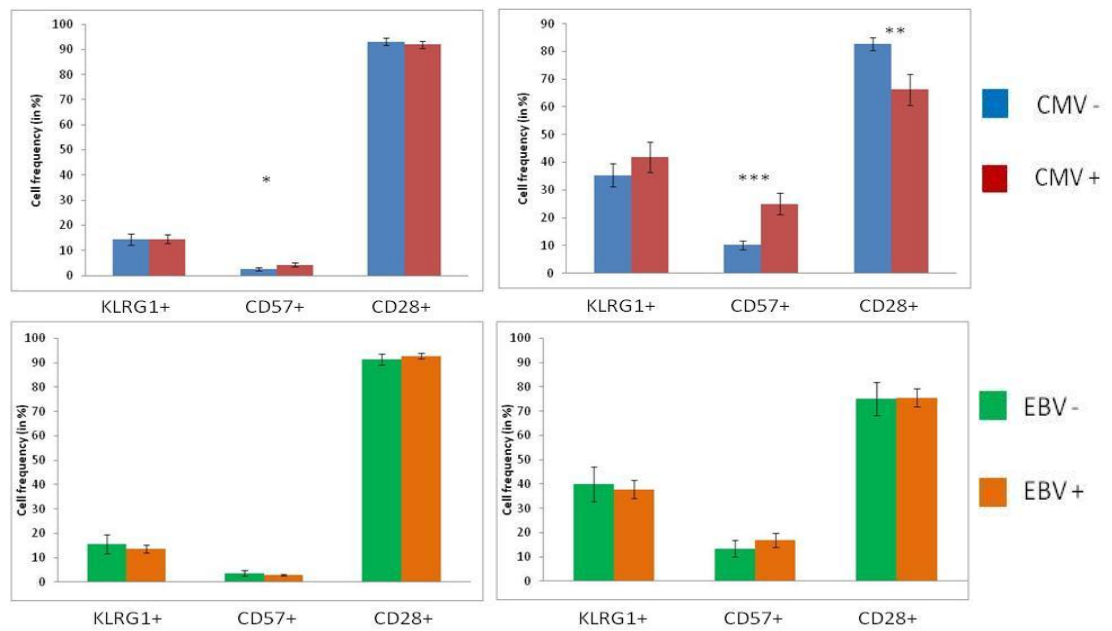


Figure 7.1. The impact of latent CMV or EBV serostatus on the proportions of (KLRG1+, CD57+, CD28+) cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences due to CMV infection is indicated by * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

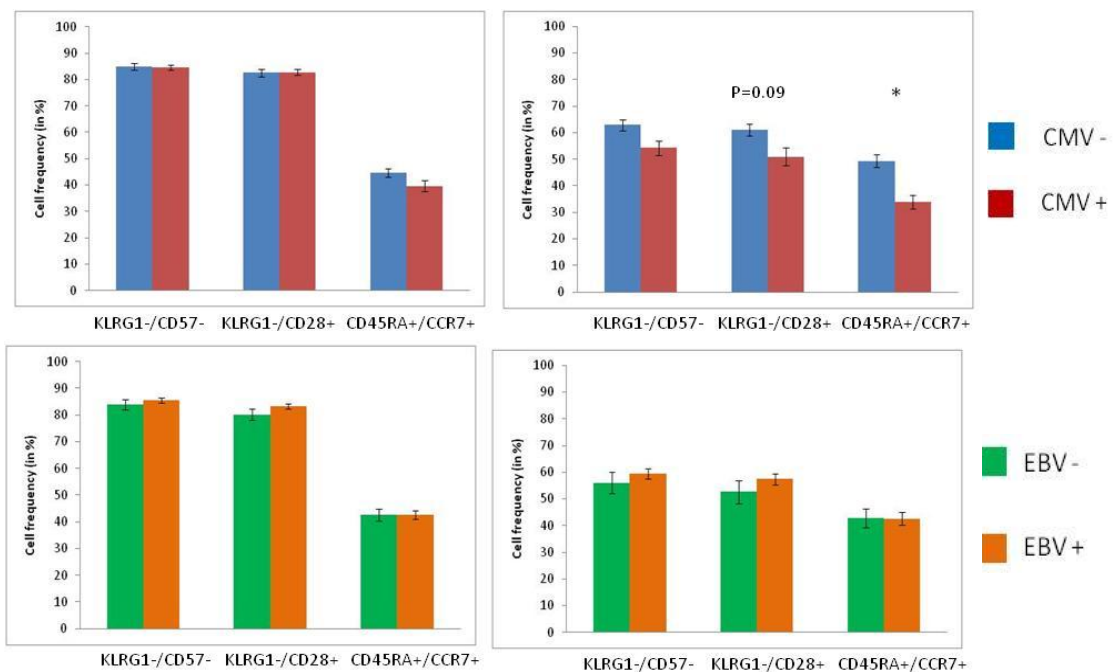


Figure 7.2. The impact of latent CMV or EBV serostatus on the proportions of naïve (KLRG1-/CD57-, KLRG1-/CD28-) and (CD45RA+/CCR7+) cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences due to CMV infection is indicated by * $p < 0.05$.

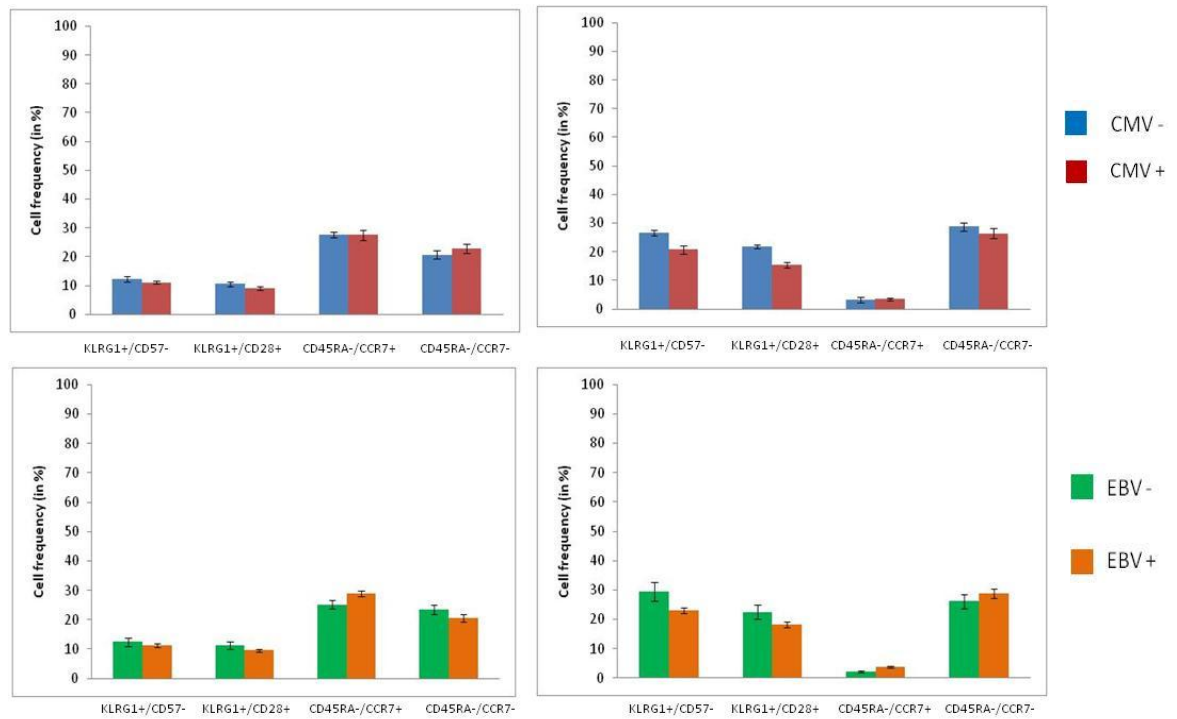


Figure 7.3. The impact of latent CMV or EBV serostatus on the proportions of memory (KLRG1+/CD57-, KLRG1+/CD28+), central-memory (CD45RA-/CCR7+) and effector-memory (CD45RA-/CCR7-) cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM.

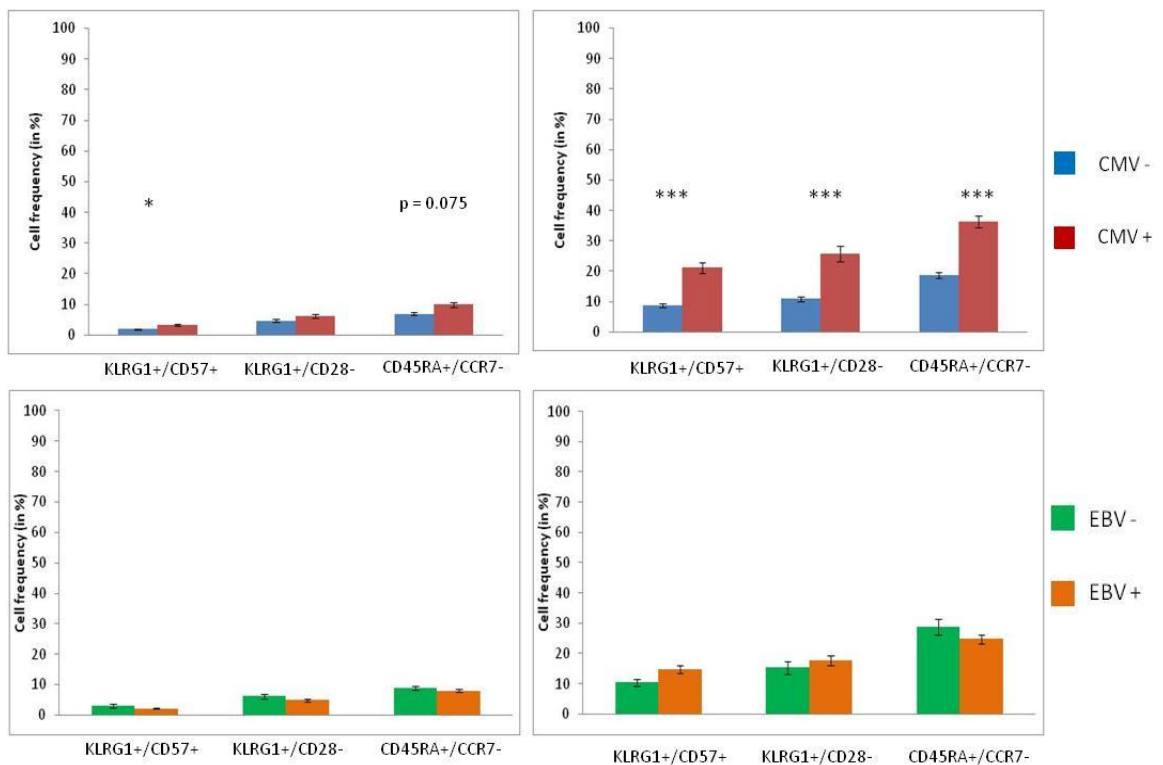


Figure 7.4. The impact of latent CMV or EBV serostatus on the proportions of senescent (KLRG1+/CD57+, KLRG1+/CD28-) and TEMRA (CD45RA+/CCR7-) cells in the CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences due to CMV infection is indicated by * p < 0.05; ** p < 0.01 and *** p < 0.001.

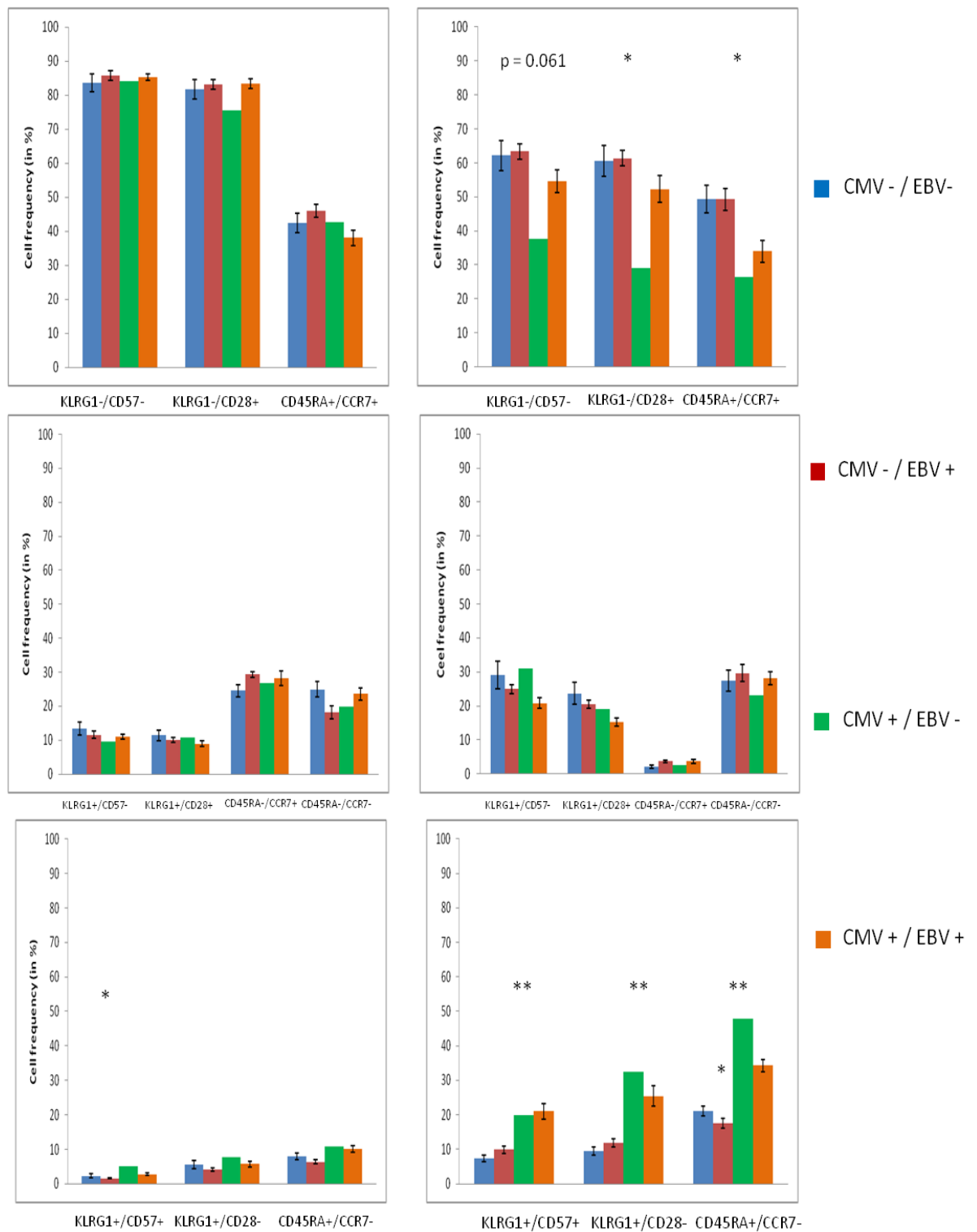


Figure 7.5. The impact of latent CMV and EBV co-infection on the proportions of the Naïve (KLRG1-/CD57-, KLRG1-/CD28+ and CD45RA+/CCR7+) (Top), Memory (KLRG1+/CD57-, KLRG1+/CD28+), Central-memory (CD45RA-/CCR7+), Effector-memory (CD45RA-/CCR7-) (Middle) and Senescent (KLRG1+/CD57+, KLRG1+/CD28- and CD45RA+/CCR7-) (Bottom) CD3+/CD4+ (Left) and CD3+/CD8+ (Right) T-cell subsets (in %). Values are mean \pm SEM. Statistically significant differences due to CMV single-infection (CMV+/EBV-) is indicated by * $p < 0.05$; ** $p < 0.01$ and statistically significant differences due to EBV single-infection (EBV+/CMV-) is indicated by # $p < 0.05$.

7.3.2. The impact of the latent CMV and EBV infection on the exercise-induced changes in T-cells concentration in peripheral blood.

Exercise-induced changes in the total number of T-cells, and the different CD4+ and CD8+ T-cell subsets are presented in Table 7.3. The acute bout of exercise performed by the subjects was associated with significant changes in the T-cell repertoire. A significant increase in total T-cell numbers was observed immediately after exercise ($p < 0.001$), followed by a significant reduction in T-cells numbers during the recovery phase (1H Post exercise) ($p < 0.001$). While both CD4+ and CD8+ T-cell numbers followed this pattern, only the exercise-induced lymphocytosis and lymphocytopenia of the CD8+ T-cell subset were statistically significant ($p < 0.001$) (Table 7.3.).

Table 7.3. Total numbers of peripheral blood lymphocytes and T-cell subsets in response to acute cycling exercise (cells.μL⁻¹) Values are mean ± SEM. Significant differences from pre-exercise (* p<0.05, ** p<0.01, * p<0.001) and post-exercise (# p<0.05, ## p<0.01, ### p<0.01) (n=24).**

Cell Subsets	Cell Phenotype	Before Exercise	After Exercise	1H after exercise
T-Lymphocytes	CD3+	1428 ± 78	2055 ± 104 ***	1358 ± 84 ###
Helper T-cells	CD3+/CD4+	583 ± 37	706 ± 53	580 ± 40
Cytotoxic T-cells	CD3+/CD8+	316 ± 20	439 ± 27 ***	301 ± 18 ###
Naïve CD4+ T-cells	CD4+/KLRG1-/CD28+	483 ± 35	549 ± 47	493 ± 36
Naïve CD8+ T-cells	CD8+/KLRG1-/CD28+	185 ± 20	209 ± 26	180 ± 18
Memory CD4+ T-cells	CD4+/KLRG1+/CD57-	68 ± 8	111 ± 12 **	66 ± 8 ##
Memory CD8+ T-cells	CD8+/KLRG1+/CD57-	75 ± 7	126 ± 11 ***	77 ± 8 ###
Effector-memory CD4+ T-cells	CD4+/CD45RA-/CCR7-	126 ± 15	172 ± 19	137 ± 16
Effector-memory CD8+ T-cells	CD8+/CD45RA-/CCR7-	87 ± 9	138 ± 12 ***	85 ± 10 ###
TEMRA CD4+ T-cells	CD4+/CD45RA+/CCR7-	48 ± 5	78 ± 10	42 ± 5
TEMRA CD8+ T-cells	CD8+/CD45RA+/CCR7-	82 ± 9	131 ± 14 **	70 ± 7 ###
Senescent CD4+ T-cells	CD4+/KLRG1+/CD57+	15 ± 3	29 ± 6 ^{p=0.059}	13 ± 2 ##
	CD4+/KLRG1+/CD28-	31 ± 5	62 ± 11 **	22 ± 4 ###
Senescent CD8+ T-cells	CD8+/KLRG1+/CD57+	40 ± 5	85 ± 11 ***	35 ± 5 ###
	CD8+/KLRG1+/CD28-	51 ± 7	108 ± 14 ***	41 ± 5 ###

Changes in the number of naïve (KLRG1-/CD28+), memory (KLRG1+/CD57-), effector-memory (CD45RA-/CCR7-), TEMRA (CD45RA+/CCR7-) and senescent (KLRG1+/CD57+; KLRG1+/CD28-) CD8+ and CD4+ T-cell subsets in response to exercise are presented in the Table 7.3. The number of naïve KLRG1-/CD28+ CD4+ and CD8+ T-cells did not statistically change with exercise, however exercise mobilized

the memory KLRG1+/CD57- CD4+ and CD8+ T-cells ($p < 0.01$ and $p < 0.001$ respectively) along with the effector-memory CD45RA-/CCR7-CD8+ T-cells ($p < 0.001$). After 1 hour, naïve and memory T-cell numbers were comparable to their initial levels ($p < 0.001$). While exercise did not elicit the mobilisation of naïve T-cells, it preferentially mobilised KLRG1+/CD57+ senescent CD4+ and CD8+ T-cells in the peripheral blood compartment ($p < 0.01$ and $p < 0.001$ respectively) along with senescent KLRG1+/CD28- and TEMRA CD45RA+/CCR7- CD8+ T-cells ($p < 0.001$ and $p < 0.01$) (Table 7.3).

The impacts of CMV latent infection on exercise-induced lymphocytosis and lymphocytopenia are presented on Figure.7.6. While CMV infection did not statistically impact the exercise-induced CD4+ T-cells mobilisation, it was associated with a lower CD4+ T-cell count in blood 1 hour following the exercise (Figure.7.6). CMV infection did not impact the exercise-induced CD8+ T-cells and total lymphocyte shift.

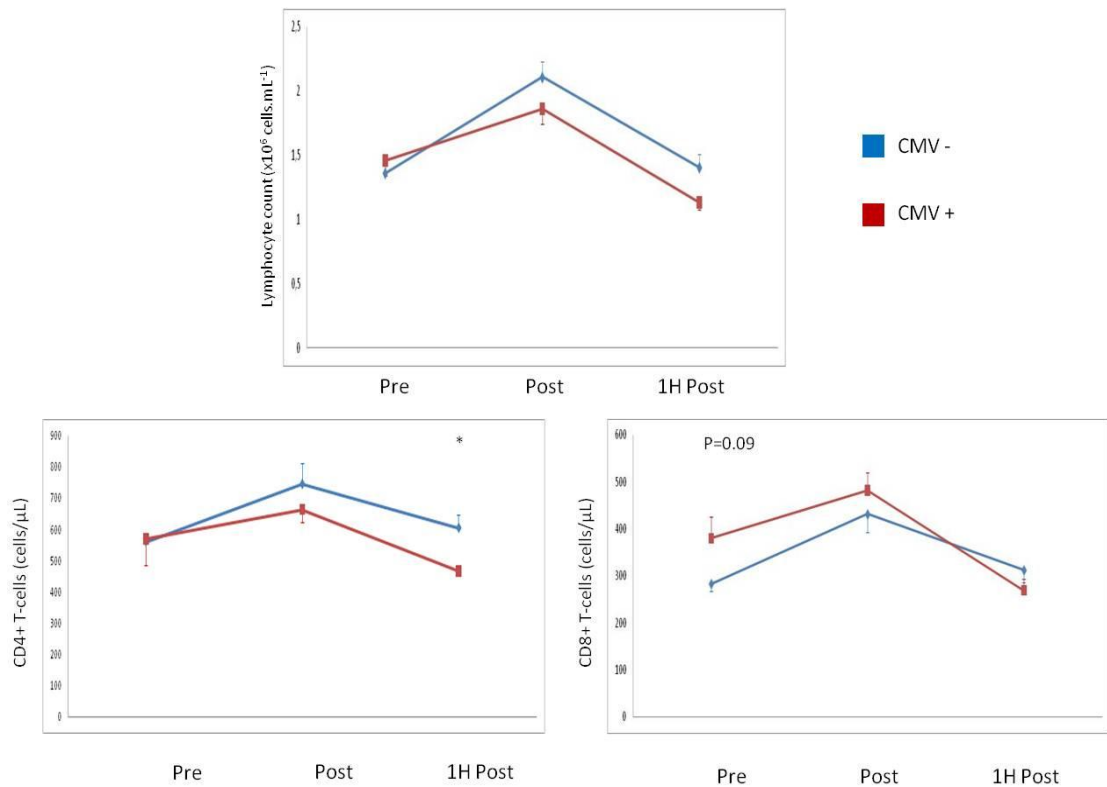


Figure 7.6. The impact of CMV latent infection on the total lymphocyte (top), CD3+/CD4+ (bottom left) and CD3+/CD8+ (bottom right) cell counts changes with exercise (cells. μL^{-1}). Values are mean \pm SEM. Statistically significant differences from the CMV positive group is indicated by * $p < 0.05$.

The effects of CMV latent infection on the naïve, memory and senescent T-cell subsets are presented on Figures 7.7., 7.8. and 7.9. respectively. The egress of naïve KLRG1-/CD57- CD4+ T-cells ($p < 0.05$) was greater in the CMV+ subjects than in the CMV- subjects (Figure.7.7). When other naïve CD4+ T-cells phenotypes were studied, a similar pattern was observed, and the egress of KLRG1-/CD28+ ($p = 0.077$) and CD45RA+/CCR7+ ($p = 0.101$) CD4+ T-cells was greater in CMV seropositive subjects, compared to the CMV seronegative subjects. Naïve CD45RA+/CCR7+ CD8+ T-cell egress was also greater in CMV+ subjects than in the non-infected subjects ($p < 0.05$).

While CMV seronegative subjects did not mobilize more memory KLRG1+/CD28+ CD4+ T-cells ($p > 0.05$) with exercise than the CMV seropositive subjects, they had more memory KLRG1+/CD28+ cells in the peripheral blood compartment 1 hour after

exercise ($p < 0.05$). A similar non-significant pattern was seen in the central-memory CD45RA-/CCR7+ CD4+ T-cells ($p = 0.083$) exercise-induced egress in CMV seronegative subjects. CMV serostatus did not have a clear effect on the memory KLRG1+/CD57-, central-memory CD45RA-/CCR7+ and CD45RA-/CCR7- CD8+ T-cells exercise-induced mobilisation or egress. However non-significant trend were seen in the association between CMV seropositivity and a lower memory KLRG1+/CD57- ($p = 0.100$) and memory KLRG1+/CD28+ CD8+ T-cells ($p = 0.079$) removal from the peripheral blood compartment during the recovery phase (Figure.7.8). The results presented in Figure. 7.9. show the impact of CMV latent infection on the exercise-induced senescent CD8+ T-cells shifts. A main effect for CMV infection was observed. In addition to having significantly larger proportions of the senescent KLRG1+/CD57+, KLRG1/CD28- and TEMRA CD45RA+/CCR7- CD8+ T-cells than CMV seronegative subjects ($p < 0.001$), CMV seropositive subjects also mobilise a greater number of those senescent cells in the blood with exercise ($p < 0.001$). The senescent cell counts remained higher in the hour following the exercise in the CMV positive subjects ($p < 0.001$) (Figure.7.9). No impact of CMV infection was seen on the CD4+ senescent T-cells ($p > 0.05$).

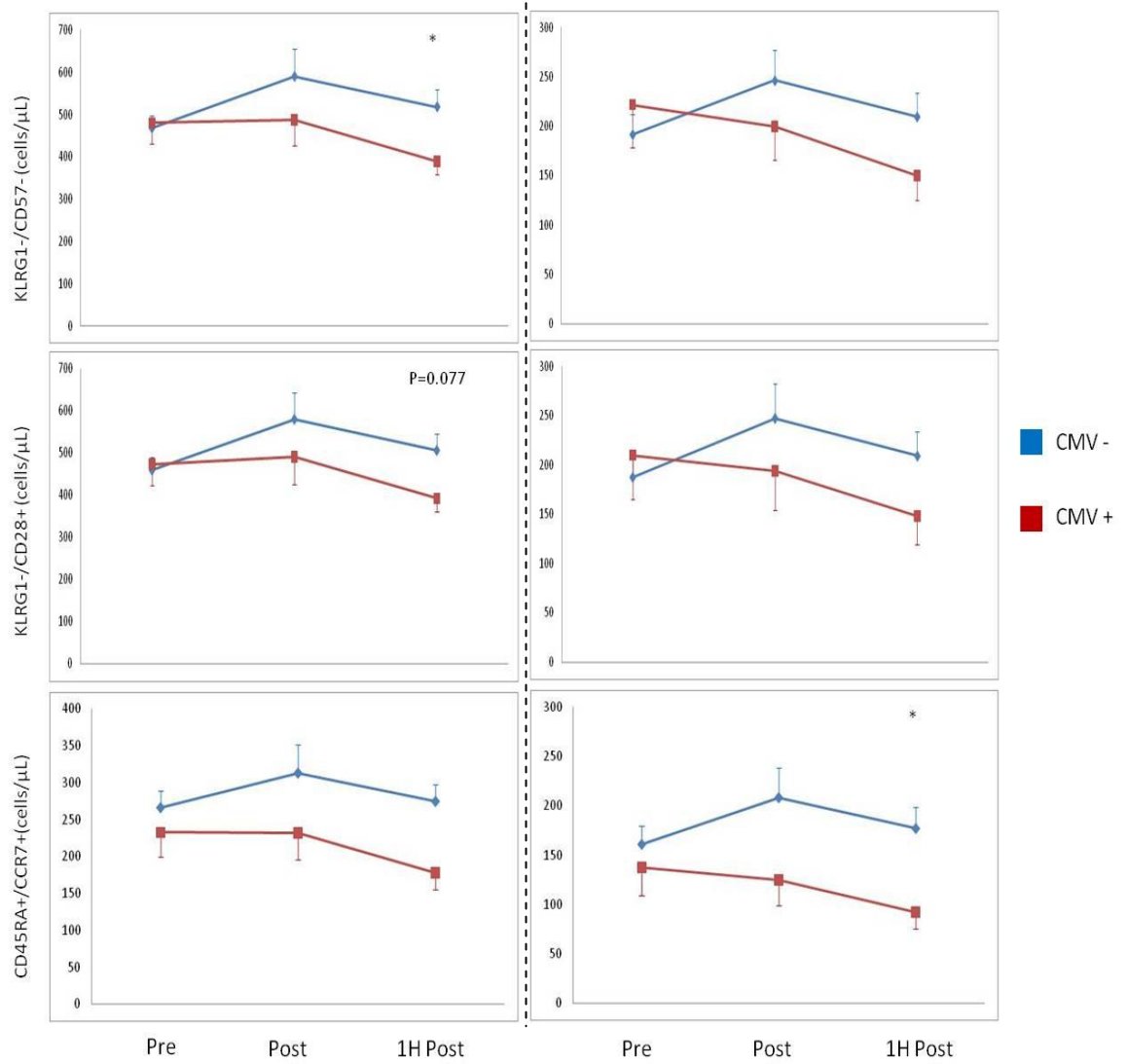


Figure 7.7. The impact of CMV latent infection and exercise on the number of CD3+/CD4+ (left) and CD3+/CD8+ (right) exhibiting a naïve phenotype (KLRG1-/CD57- (top), KLRG1-/CD28+ (middle) and CD45RA+/CCR7+ (bottom)) (cells.µL⁻¹). Values are mean ± SEM. Statistically significant differences from the CMV negative group is indicated by * p < 0.05.

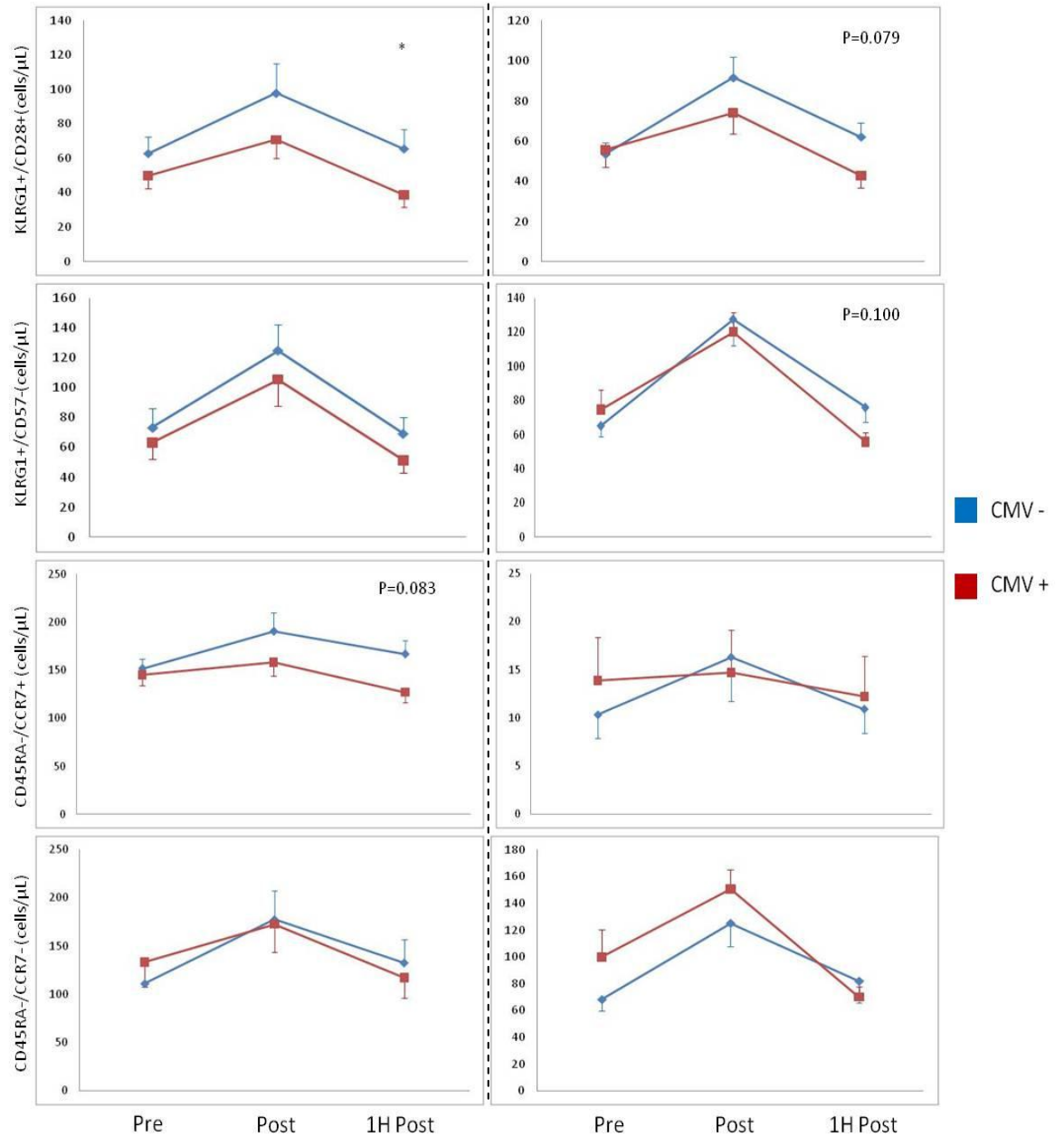


Figure 7.8. The impact of CMV latent infection and exercise on the number of CD3+/CD4+ (left) and CD3+/CD8+ (right) T-cells exhibiting a memory (KLRG1+/CD28+ and KLRG1+/CD57-), central-memory (CD45RA-/CCR7+) and effector-memory (CD45RA-/CCR7-) phenotype (cells.μL⁻¹). Values are mean ± SEM. Statistically significant differences from the CMV negative group is indicated by * p < 0.05.

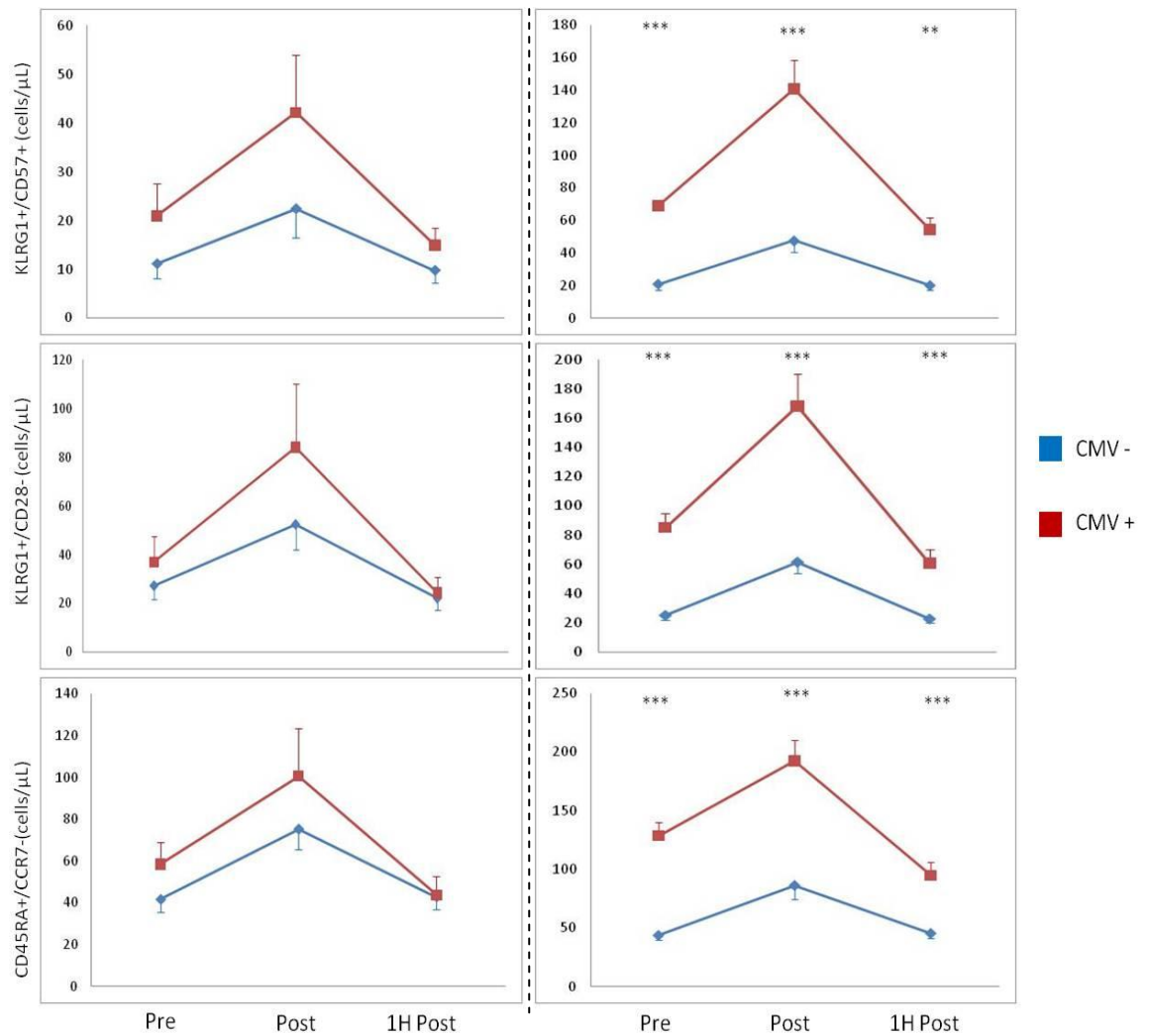


Figure 7.9. The impact of CMV latent infection and exercise on the number of CD3+/CD4+ (left) and CD3+/CD8+ (right) exhibiting a senescent phenotype (KLRG1+/CD57+ (top), KLRG1+/CD28- (middle)) and highly differentiated memory phenotype (CD45RA+/CCR7- (bottom))(cells.µL⁻¹). Values are mean ± SEM. Statistically significant differences from the CMV negative group is indicated by * p < 0.05, ** p < 0.01 and *** p < 0.001.

The impacts of EBV latent infection on exercise-induced lymphocytosis and lymphocyte efflux are presented on Figure.7.10. A main effect for EBV infection was observed on the proportions of CD8+ T-cells. Both resting CD8+ T-cell count and the exercise-induced CD8+ T-cells mobilization were statistically greater in EBV seropositive subjects, when compared to the seronegative group (p < 0.05). However, EBV infection did not statistically impact the exercise-induced total lymphocytes and CD4+ T-cells (Figure.7.10).

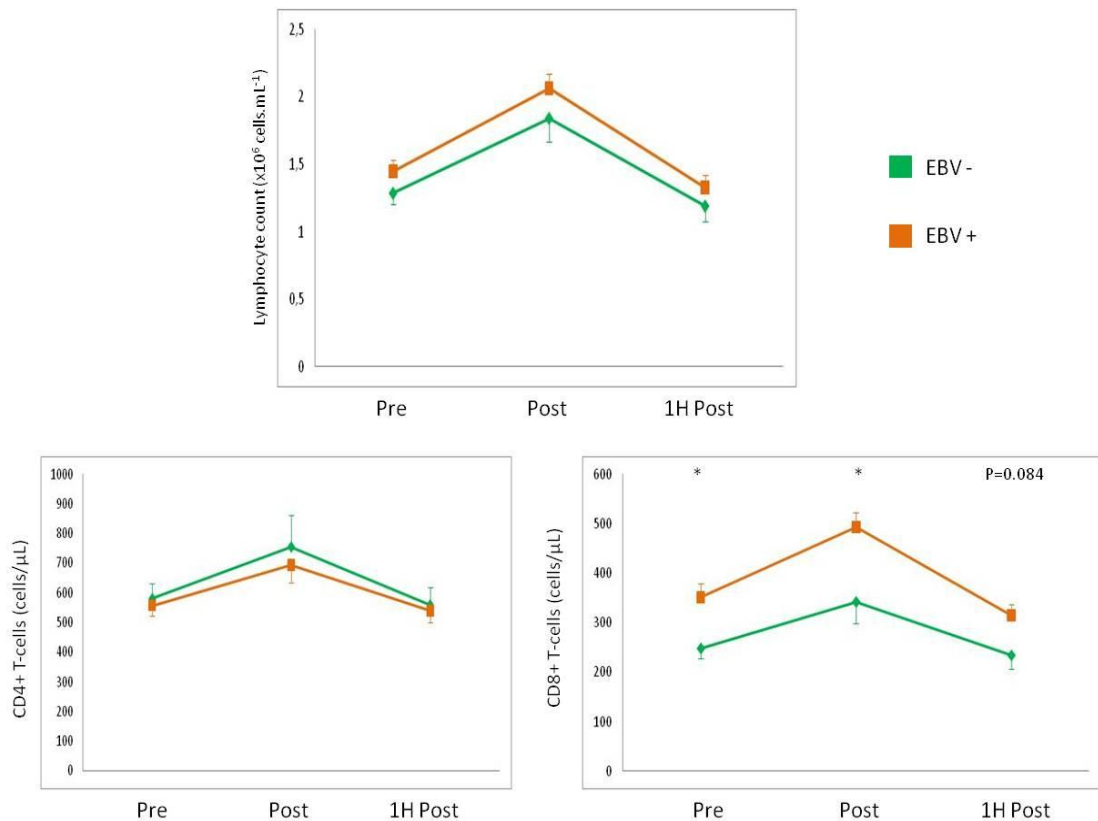


Figure 7.10. The impact of EBV latent infection on the total lymphocyte (top), CD3+/CD4+ (bottom left) and CD3+/CD8+ (bottom right) cell counts changes with exercise (cells.μL⁻¹). Values are mean ± SEM. Statistically significant differences from the EBV negative group is indicated by * p < 0.001.**

The effects of EBV latent infection on the naïve and senescent T-cell subsets are presented on Figures 7.11. and 7.12. respectively. A main effect for EBV infection was observed on the proportions of CD8+ T-cells. Latent EBV infection was not associated with any changes in the number of naïve KLRG1-/CD57- and KLRG1-/CD28+ CD8+ T-cells immediately after or 1 hour after completion of the cycling protocol (p > 0.05) (Figure. 7.11). Latent EBV infection had an impact on the central-memory CD45RA-/CCR7+ and effector-memory CD45RA-/CCR7- CD8+ T-cells initial concentration in the blood (p < 0.05), but was also associated with a greater mobilisation and egress (p < 0.01 and p < 0.05 respectively) of those cells in response to exercise (Figure. 7.12). Exercise-induced changes in memory CD4+ T-cell concentrations were not affected by

EBV infection ($p > 0.05$). EBV infection was associated with a greater mobilisation of senescent KLRG1+/CD28- CD8+ T-cells after exercise when compared to uninfected subjects ($p < 0.05$). This effect of EBV infection on the senescent CD8+ T-cell exercise-induced mobilisation was also seen on the senescent KLRG1+/CD57+ CD8+ T-cells as a non-significant trend ($p = 0.100$). No significant interaction effect was observed between EBV infection and the senescent KLRG1+/CD57+, KLRG1+/CD28- and TEMRA CD45RA+/CCR7- CD4+ T-cells concentrations at the different time points (Figure. 7.13).

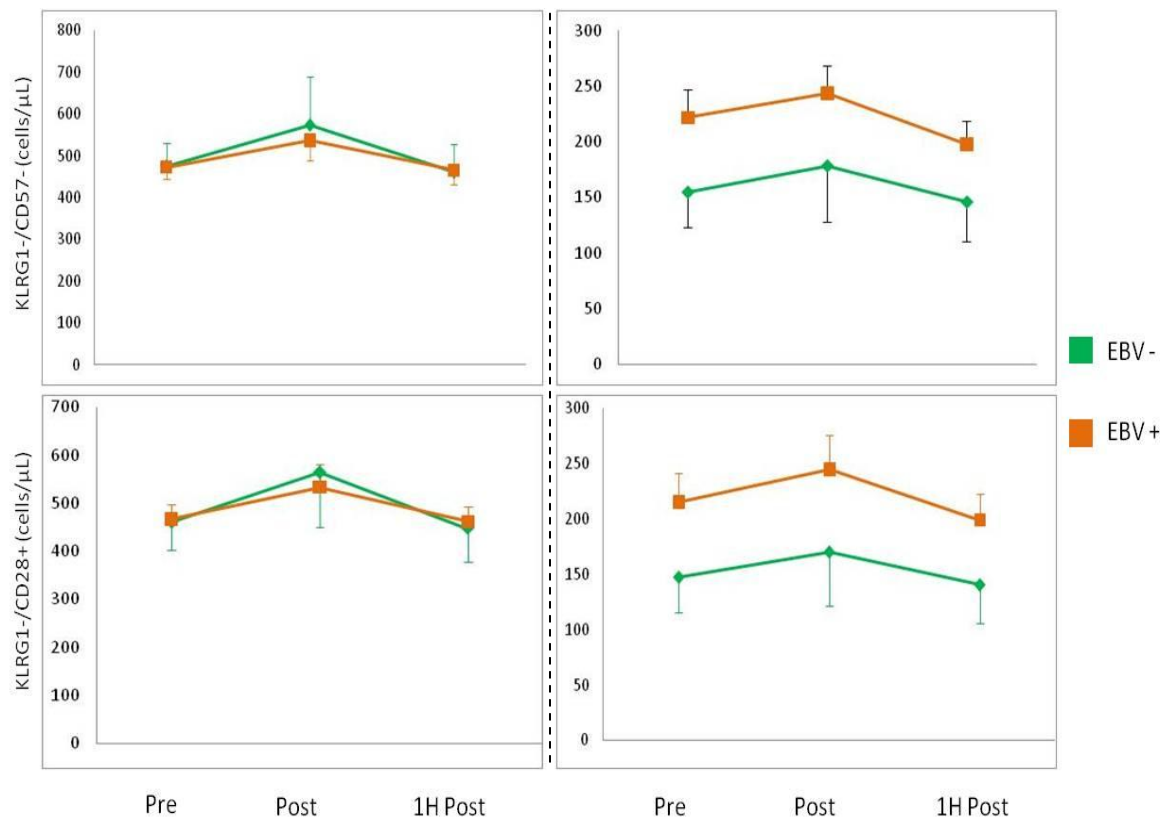


Figure 7.11. The impact of EBV latent infection and exercise on the number of CD3+/CD4+ (left) and CD3+/CD8+ (right) T-cells exhibiting a naïve phenotype (KLRG1-/CD57- (top) and KLRG1-/CD28+ (bottom)) (cells.µL⁻¹). Values are mean ± SEM. Statistically significant differences from the EBV negative group is indicated by ** $p < 0.01$.

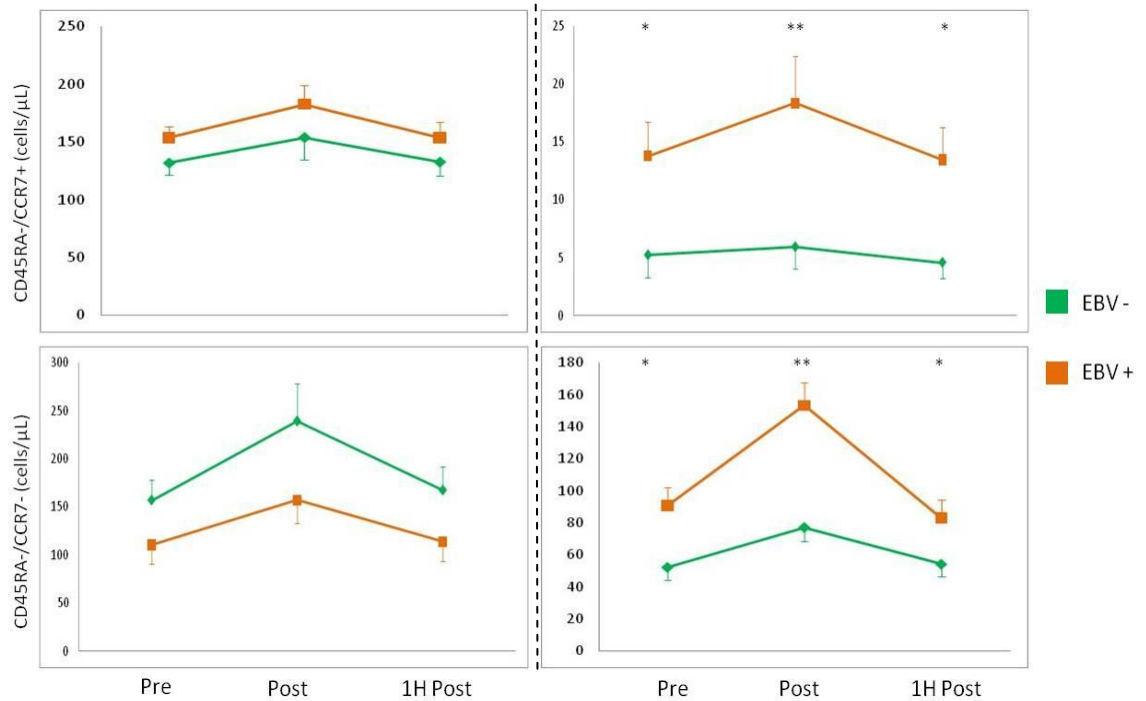


Figure 7.12. The impact of EBV latent infection on the central-memory CD45RA-/CCR7+ (top) and effector-memory CD45RA-/CCR7- (bottom) CD3+/CD4+ (left) and CD3+/CD8+ (right) cell counts changes with exercise (cells.μL⁻¹). Values are mean ± SEM. Statistically significant differences from the EBV negative group is indicated by * p < 0.05, ** p < 0.01.

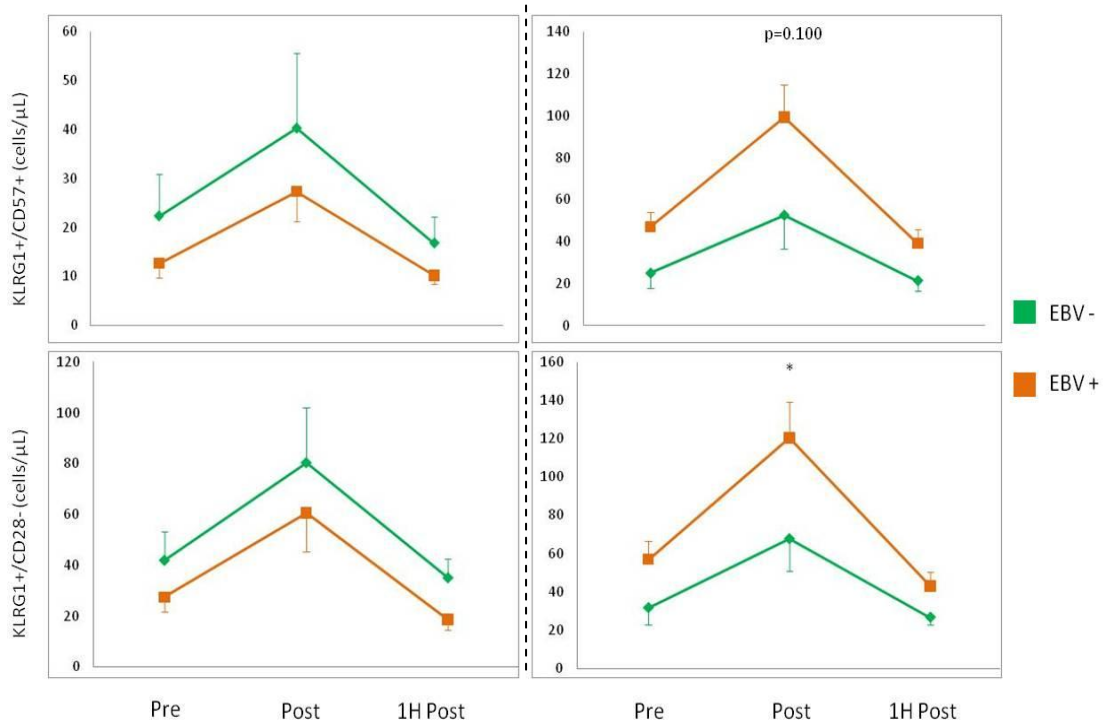


Figure 7.13. The impact of EBV latent infection and exercise on the number of CD3+/CD4+ (left) and CD3+/CD8+ (right) cell exhibiting a senescent phenotype (KLRG1+/CD57+ (top) and KLRG1+/CD28- (bottom)) (cells.μL⁻¹). Values are mean ± SEM. Statistically significant differences from the EBV negative group is indicated by ** p < 0.01.

In order to accurately represent the cellular shifts occurring between peripheral tissues and the blood compartment, cell mobilization and egress are represented in the Figures 7.14., 7.15., 7.16. and 7.17. Cellular influx corresponds to the number of cells entering the blood compartment during the acute bout of exercise and was obtained by subtracting the cell numbers present in the blood before the exercise (Pre) to the amount of cells present immediately after the exercise (Post). Cellular efflux corresponds to the number of cells leaving the blood compartment during the recovery phase and was obtain by subtracting the cell numbers present in the blood immediately after exercise (Post) to the amount of cells present in the blood after the recovery phase (1HPost).

$$\text{Cellular Influx} = \text{cell number}_{\text{post}} - \text{cell number}_{\text{pre}}$$

$$\text{Cellular Efflux} = \text{cell number}_{\text{1Hpost}} - \text{cell number}_{\text{post}}$$

CMV infected subjects presented a lower naïve CD45RA+/CCR7+ and KLRG1-/CD28+ CD8+ T-cell mobilization ($p < 0.05$) than their infection free counterparts. While the cycling protocol induced a net increase in naïve CD45RA+/CCR7+ CD8+ T-cells in the peripheral blood of CMV seronegative subjects, it promoted a reduction in those naïve cells in CMV seropositive subjects ($p < 0.05$). Although not significant, a similar trend was observed in the reduced exercise-induced mobilisation of the naïve KLRG1-/CD28+ ($p = 0.094$) and CD45RA+/CCR7+ CD4+ T-cells ($p = 0.114$) in CMV seropositive subjects. EBV infection did not have a significant effect on the different naïve CD4+ and CD8+ T-cell subsets mobilisation and egress. (Figure. 7.14).

CMV and EBV latent infection also had an effect on effector-memory CD45RA-/CCR7-. While CMV and EBV seropositive subjects statistically mobilised a similar amount of effector-memory CD8+ T-cells than infection-free subjects, they presented a greater extravastion of CD45RA-/CCR7- CD8+ T-cells during the recovery period

when compared to CMV ($p < 0.05$) and EBV ($p < 0.01$) seronegative subjects. No effect of CMV or EBV latent infections on the central-memory CD45RA⁻/CCR7⁺ CD4⁺ and CD8⁺ T-cells exercise-induced changes were observed ($p > 0.05$) (Figure. 7.15.). The senescent KLRG1⁺/CD57⁺ ($p < 0.05$) and KLRG1⁺/CD28⁻ ($p < 0.05$) mobilisation after exercise and egress ($p < 0.01$) during recovery phase was more prominent in CMV seropositive subjects, however EBV infection did not have an effect on the senescent CD4⁺ and CD8⁺ T-cells exercise-induced changes (Figure. 7.16.). While CMV infection did not have an impact on the TEMRA CD45RA⁺/CCR7⁻ CD8⁺ T-cells mobilization, it was associated with a greater extravasation of those cells during the recovery phase ($p < 0.01$) (Figure. 7.17).

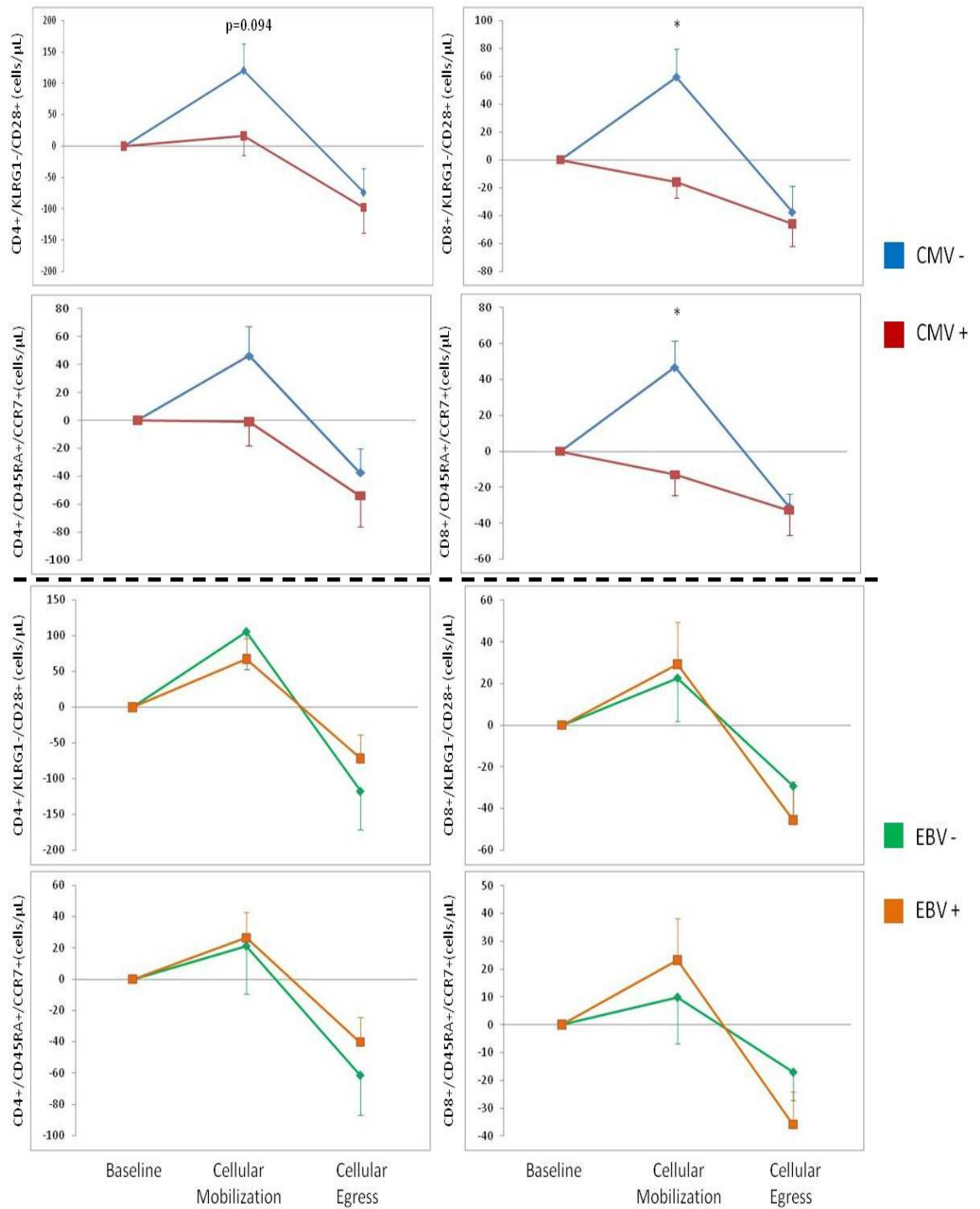


Figure 7.14. The impact of latent CMV (top half) and EBV (bottom half) infection on cellular mobilization and egress of naive KLRG1-/CD28+ (top) and CD45RA+/CCR7+ (bottom) CD3+/CD4+ (left) and CD3+/CD8+ (right) cell counts changes with exercise (cells.µL⁻¹). Values are mean ± SEM. Statistically significant differences from the CMV positive group is indicated by * p < 0.05

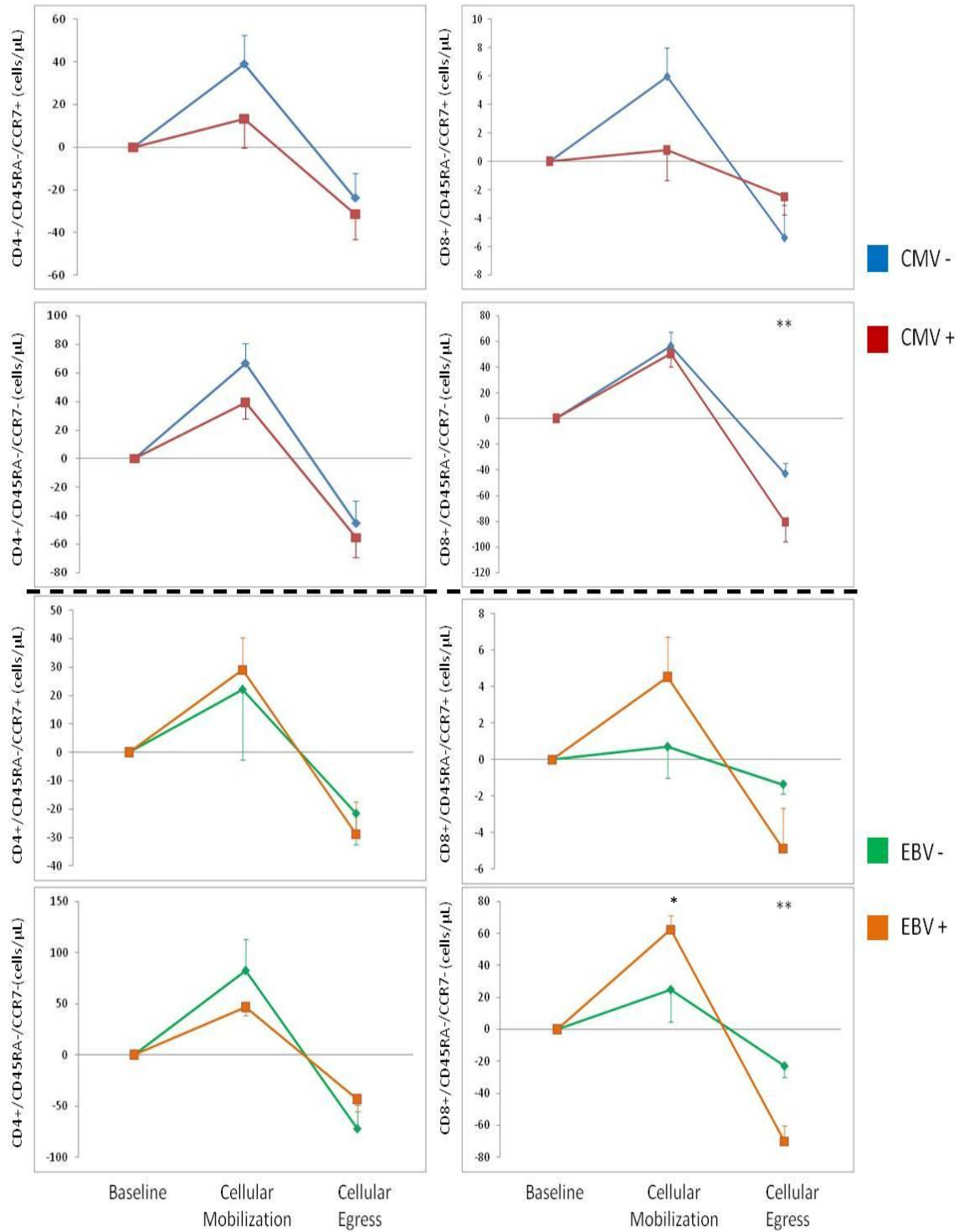


Figure 7.15. The impact of latent CMV (top half) and EBV (bottom half) infection on cellular mobilization and egress of central-memory CD45RA-/CCR7+ (top) and effector-memory CD45RA-/CCR7- (bottom) CD3+/CD4+ (left) and CD3+/CD8+ (right) cell counts changes with exercise (cells.μL⁻¹). Values are mean ± SEM.

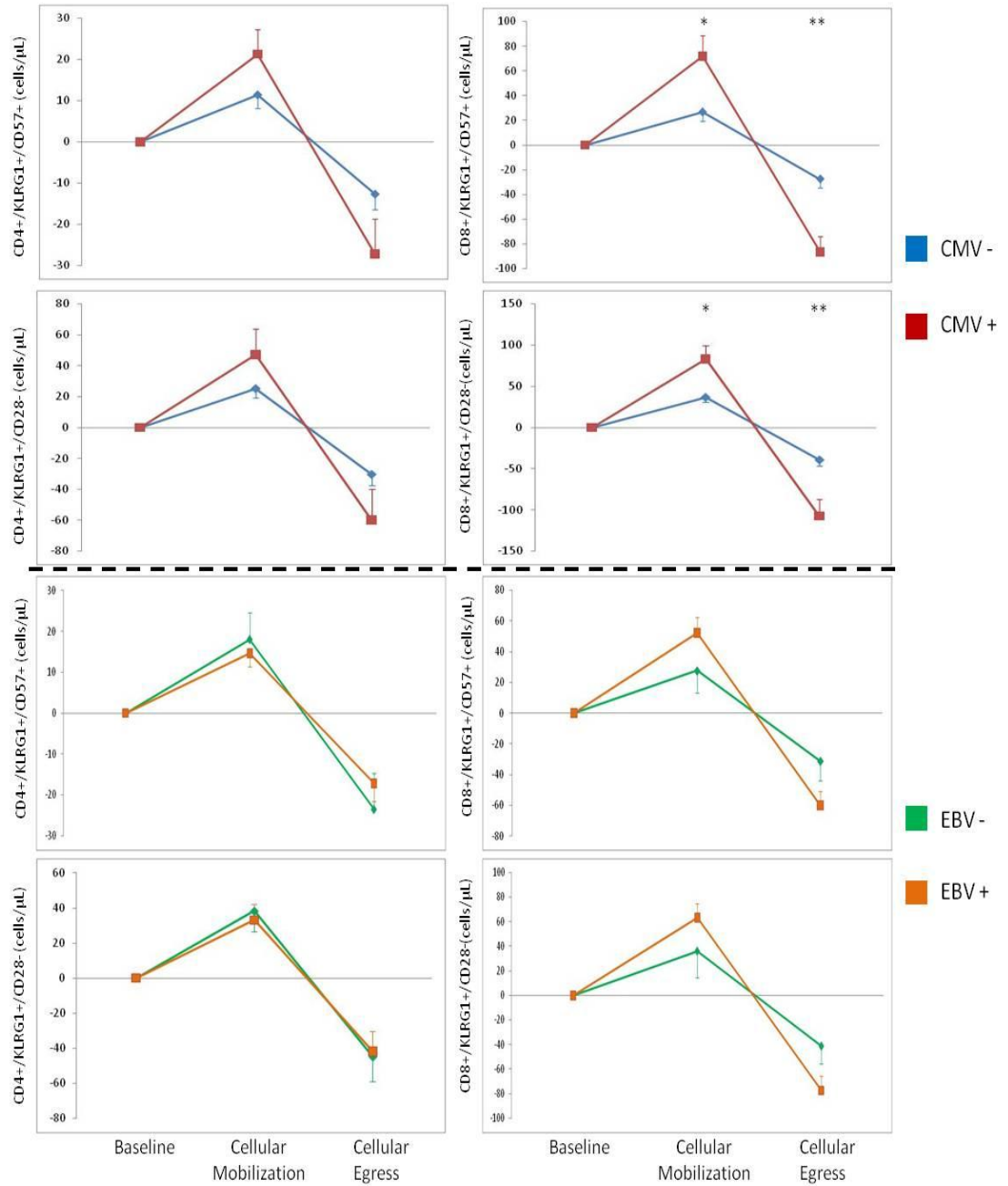


Figure 7.16. The impact of latent CMV (top half) and EBV (bottom half) infection on cellular mobilization and egress of senescent KLRG1+/CD57+ (top) and KLRG1+/CD28- (bottom) CD3+/CD4+ (left) and CD3+/CD8+ (right) cell counts changes with exercise (cells.μL⁻¹). Values are mean ± SEM. Statistically significant differences from the CMV positive group is indicated by * p < 0.05

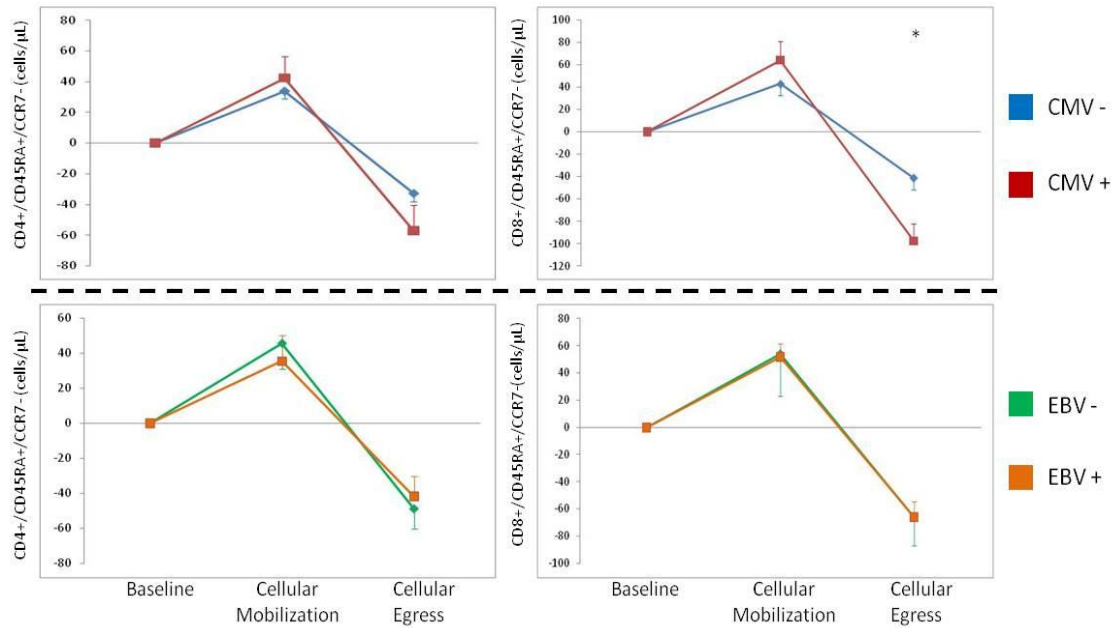


Figure 7.17. The impact of latent CMV (top) and EBV (bottom) infection on cellular mobilization and egress of TEMRA CD45RA+/CCR7- CD3+/CD4+ (left) and CD3+/CD8+ (right) cell counts changes with exercise (cells. μL^{-1}). Values are mean \pm SEM. Statistically significant differences from the CMV positive group is indicated by * $p < 0.05$

7.3.3. The contribution of viral-specific T-cells to the mobilization of memory and senescent T-cells with exercise.

The impact of exercise on the total CMV pp65-specific (n=6) and EBV Imp2-specific (n=7) CD8+T-cells are presented on Figure 7.18 and 7.19 respectively. The impact of exercise on the total CMV pp65-specific and EBV Imp2-specific CD8+T-cells along with the different T-cell subsets are presented on Figure 7.20. The cycling protocol induced a significant mobilisation of the total CMV-specific and EBV-specific CD8+ T-cells ($p < 0.05$), followed during the recovery phase by the egress of those viral-specific cells to attain the initial level observed in the peripheral blood compartment in the Pre sample. As hypothesised, senescent KLRG1+/CD57+ CMV-specific CD8+ T-cells were preferentially mobilised by the exercise (Figure. 7.20.) ($p < 0.05$). A similar exercise-

induced mobilisation was observed on KLRG1-/CD57+ CMV-specific T-cells ($p < 0.05$) (Figure. 7.20.). Levels of “naïve” KLRG1-/CD57- and “memory” KLRG1+/CD57- CMV-specific CD8+ T-cells did not significantly change with exercise. The cycling protocol also elicited a significant mobilisation of the total EBV Imp2-specific CD8+ T-cells ($p < 0.05$). We observed that naïve (KLRG1-/CD57-), memory (KLRG1+/CD57-) and senescent (KLRG1+/CD57+) EBV-specific CD8+ T-cells were significantly mobilised into the circulation by exercise ($p < 0.05$). This was not the case for the KLRG1-/CD57+ EBV-specific CD8+ T-cells ($p > 0.05$) (Figure. 7.20.).

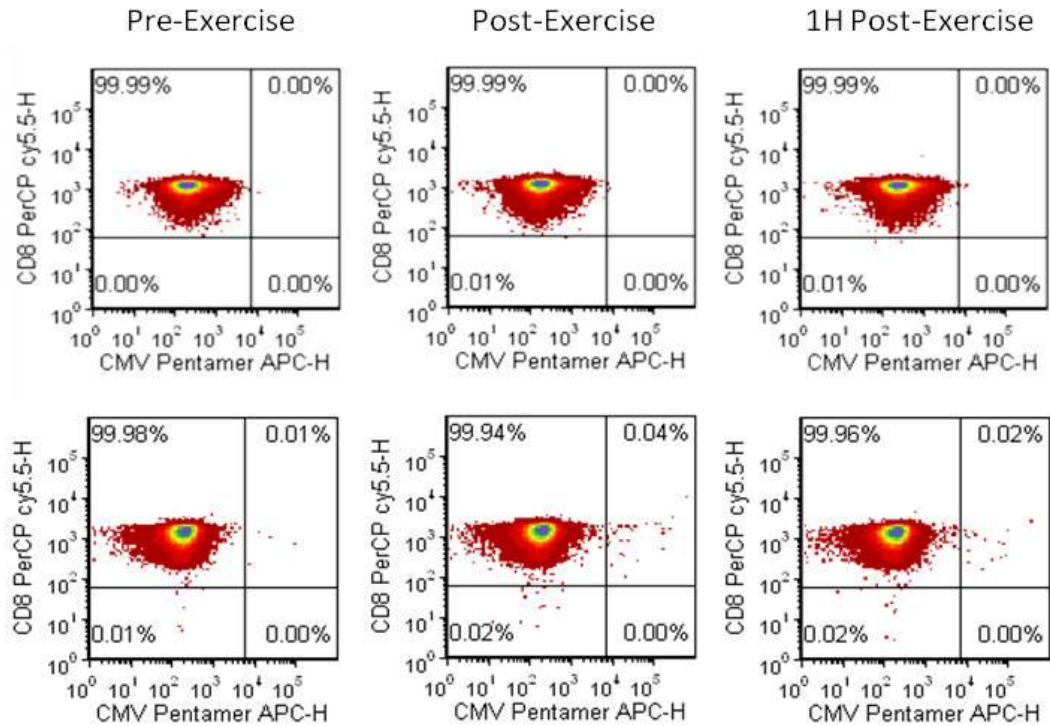


Figure 7.18. Representative flow cytometry dotplots highlighting the impact of exercise on the CMV-specific pp65+ CD8+ T-cells mobilization in a CMV seronegative subjects (top) and a CMV seropositive subject (bottom).

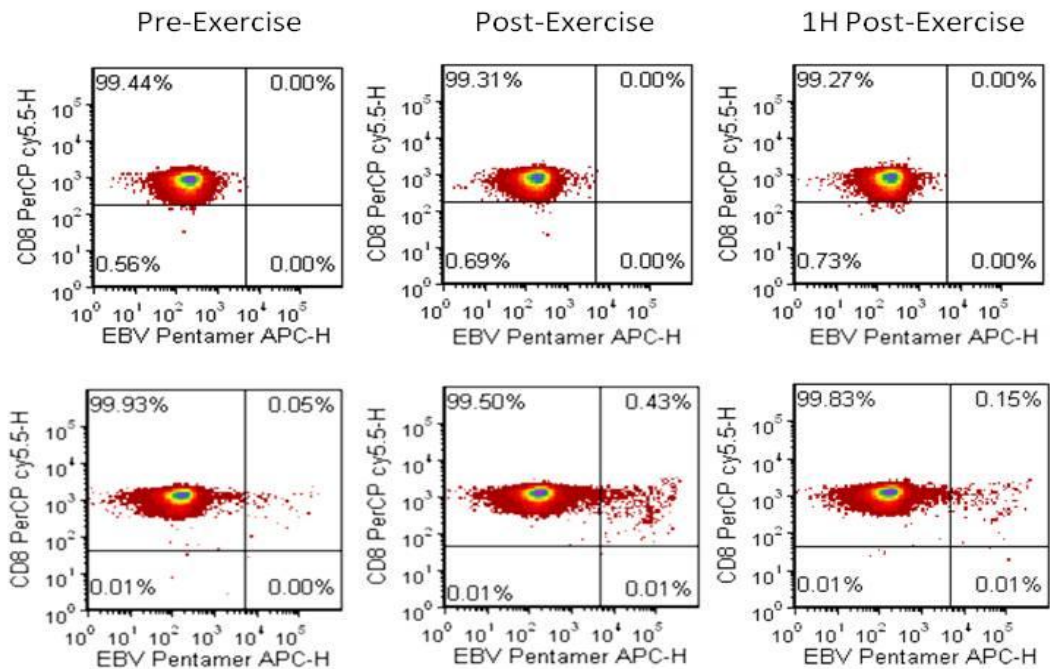


Figure 7.19. Representative flow cytometry dotplots highlighting the impact of exercise on the EBV-specific Imp-2+ CD8+ T-cells mobilization in a EBV seronegative subjects (top) and a EBV seropositive subject (bottom).

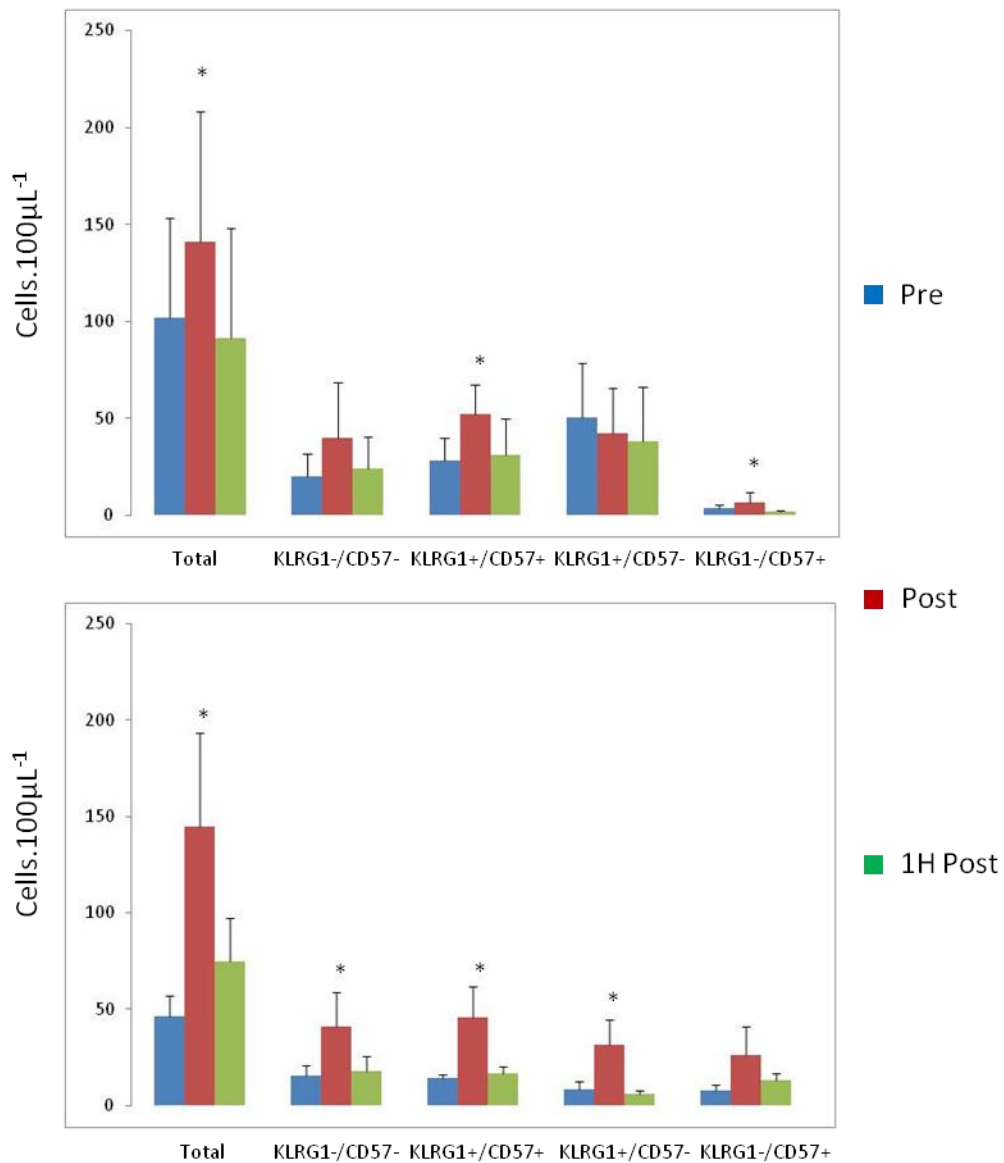


Figure 7.20. Changes in the total number of CMV pp65-specific CD8+ T-cells (top) and EBV Imp-2-specific CD8+ T-cells (bottom) and the different subsets in response to exercise in HLA/A2* subjects (cells. 100 μ L⁻¹). Values are mean \pm SEM. Significant differences from pre-exercise is indicated by * $p < 0.05$.

The mobilisation and egress of each viral-specific T-cell subtype is presented in the Figures 7.21. and 7.22. Exercise induced a preferential mobilisation of senescent KLRG1+/CD57+ pp65 CMV-specific and Imp2 EBV-specific CD8+ T-cells. While memory KLRG1+/CD57- Imp-2 EBV-specific CD8+ T-cells were also mobilised by the acute bout of exercise, the memory KLRG1+/CD57- pp65 CMV-specific CD8+ T-cells had exited the blood stream immediately following exercise (Figure. 7.21). A

preferential egress of senescent KLRG1+/CD57+ pp65 CMV-specific and Imp2 EBV-specific CD8+ T-cells was also observed during the recovery phase. The memory KLRG1+/CD57- CD8+ Imp2 EBV-specific CD8+ T-cells were also seen to be importantly removed from the peripheral blood compartment during the recovery phase. While some antigen-specific KLRG1-/CD57- cells were identified they are not considered as naive cells, but very early differentiated cells that are yet to undergo further differentiation (Figure. 7.22).

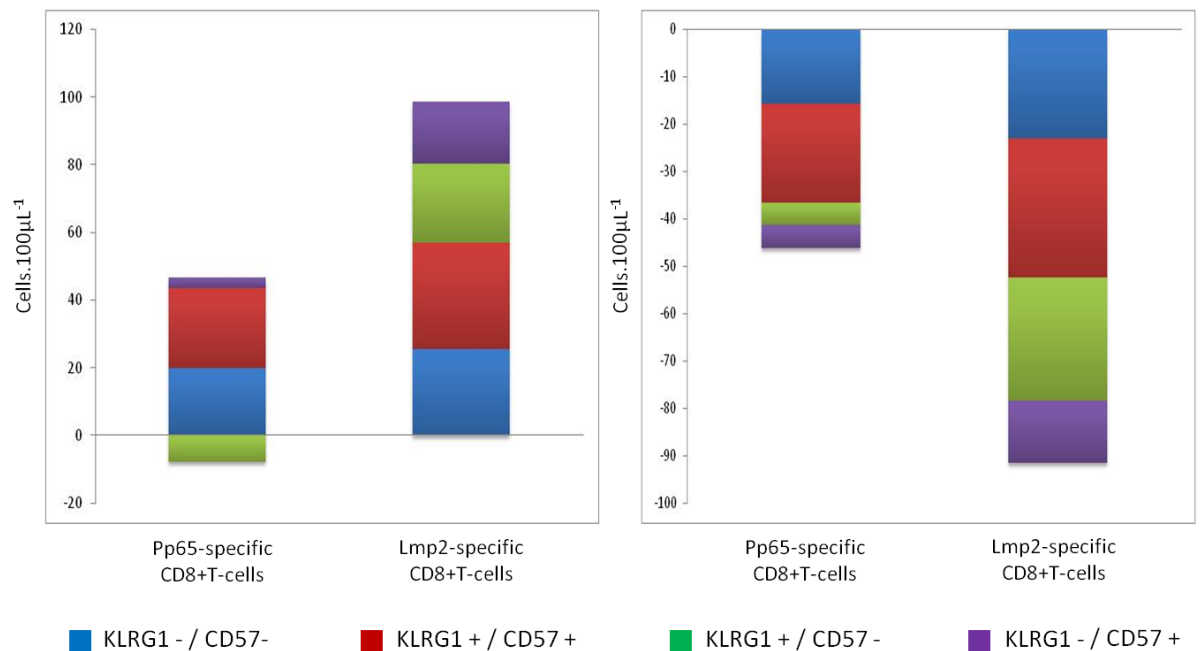


Figure 7.21. Mobilization (left) and egress (right) of the total number of CMV pp65-specific CD8+ T-cells and EBV Imp-2-specific CD8+ T-cells and the different subsets in response to exercise in HLA/A2* subjects (cells. 100µL⁻¹). Values are mean ± SEM.

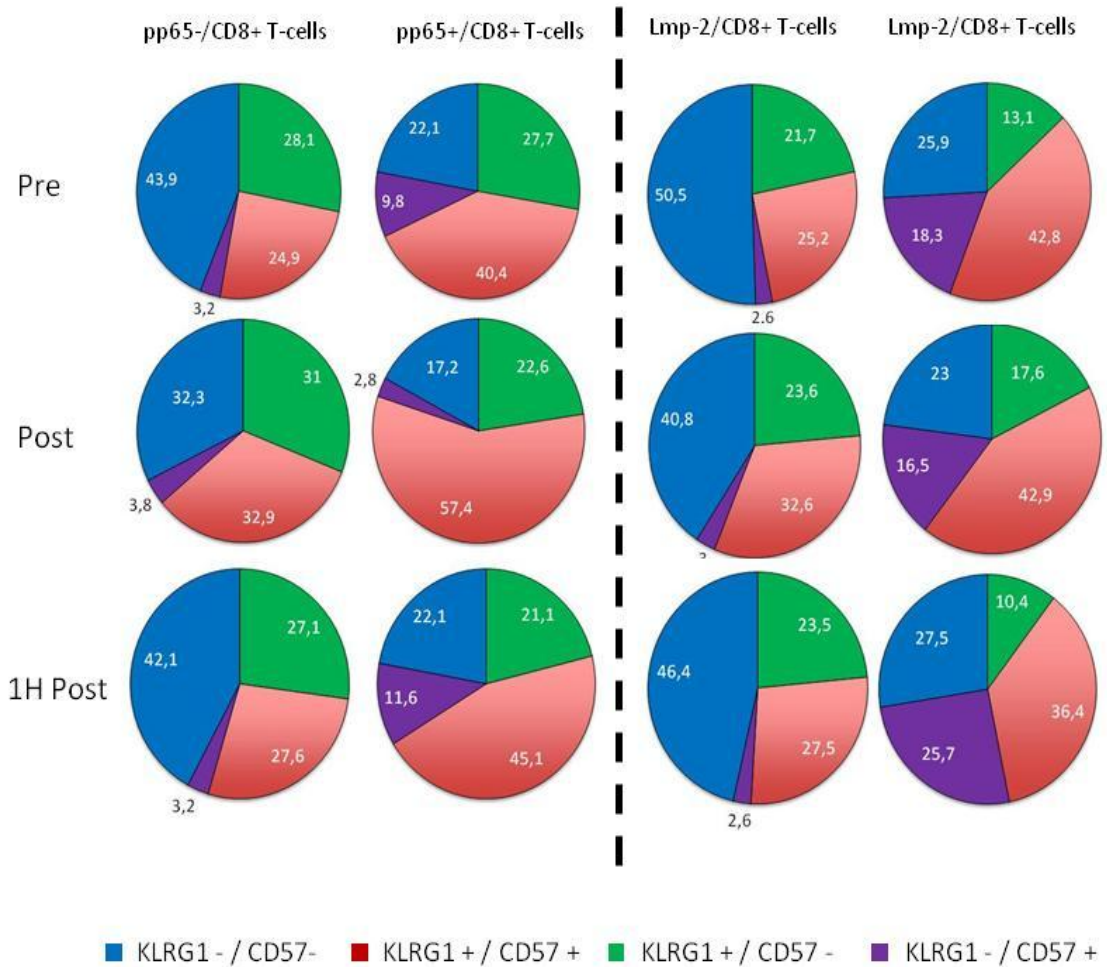


Figure 7.22. Changes in the proportions of total CMV pp65-specific (left) and EBV Imp-2-specific (right) CD8+ T-cells and their different cell subsets in response to exercise. The different cell subsets are characterised depending on KLRG1 and CD57 surface expression. Values are expressed as the percentage of all gated CD3+/CD8+ pentamer positive or pentamer negative T-cells (in %). Values are mean \pm SEM.

The effect of exercise on the mobilization and egress of total CD8+ T-cells and viral-specific CD8+ T-cells exhibiting a senescent phenotype (KLRG1+/CD57+) are presented on Figure 7.23. Both latent CMV and EBV infection were associated with a greater mobilization and egress of total senescent CD8+ T-cells than their seronegative counterpart (Figure. 7.23). However, the number of senescent pp65 CMV-specific CD8+ T-cells and Imp2 EBV-specific CD8+ T-cells entering and leaving the peripheral

blood compartment in response to exercise accounted for less than 1% of all mobilized cells (Figure. 7.23).

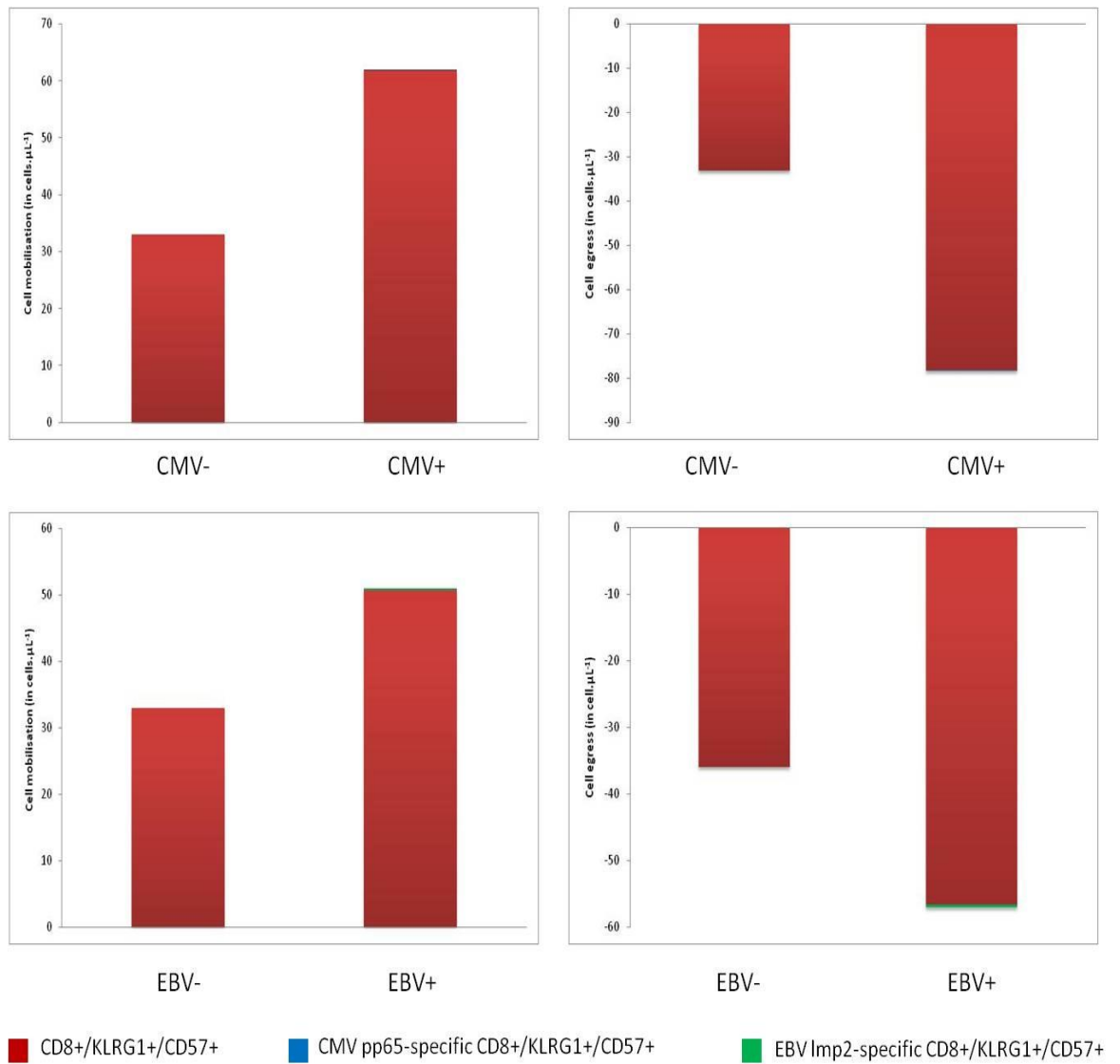


Figure 7.23. The impact of latent CMV (top) and EBV (bottom) infection on the mobilisation (left) and egress (right) of total and either pp65 CMV-specific or Imp2 EBV-specific CD8⁺ T-cells exhibiting a senescent phenotype (KLRG1⁺/CD57⁺).

7.4. Discussion

The study presented in this chapter sought to determine the effect of acute aerobic exercise on highly differentiated effector-memory and senescent CMV-specific or EBV-specific T-cells and more generally to characterise the impact of CMV and/or EBV latent infection on the exercise-induced immune changes in young adults. The major findings in this study were: (1) CMV infected subjects had more senescent T-cells than non-infected. Furthermore those senescent T-cells were mobilized and left the peripheral blood compartment in greater numbers in response to exercise in CMV infected subjects than non-infected. (2) EBV infection did not impact the mobilization or egress of senescent T-cells in response to exercise (3) CD8+ T-cells specific to epitopes of the CMV and EBV viruses were mobilized into the blood with exercise but unlike recent hypothesis (Turner *et al.* 2010), these contributed very little to the amplified mobilization of senescent T-cells seen in CMV and EBV infected people.

TEMRA and senescent T-cells are preferentially mobilised in the peripheral blood compartment following acute bouts of exercise, in young and older adults (Simpson *et al.* 2007a; Simpson *et al.* 2008; Turner *et al.* 2010). As latent herpesvirus infections have also been associated, although to a lesser extent, with increased frequencies of senescent T-cells in younger individuals; it was hypothesised in this study that young adults carrying a latent CMV and/or EBV infection would mobilize greater numbers of terminally differentiated T-cells following exercise than non-infected subjects. While a recent study had already highlighted the potentiating effects of latent CMV infection on the exercise-induced egress of effector-memory and TEMRA T-cells (Turner *et al.* 2010), this is the first study to show that CMV infection is associated with a greater

exercise-induced preferential mobilisation of senescent T-cells, in the detriment of the naïve T-cells mobilisation as seen in CMV seronegative subjects. Although EBV infection is often regarded as having less consequential impacts on the immune system than CMV infection, we discovered that EBV seropositive subjects were experiencing similar increased exercise-induced changes on the proportions of memory, senescent and TEMRA CD8+ T-cells. We did not however verify our third hypothesis stating that preferential mobilization and egress of highly differentiated effector-memory and senescent viral-specific T-cells in CMV and/or EBV infected subjects were the principal actors of the enhanced memory and senescent T-cell shifts seen in viral seropositive subjects following acute bouts of exercise.

7.4.1. Latent viral infections impact on the immune system of young men

Repeated immune challenges throughout a lifetime lead to the accumulation of highly-differentiated memory and senescent T-cells in blood (Koch *et al.* 2008; Simpson *et al.* 2008). While increased proportions of memory and dysfunctional senescent T-cells are a feature of normal chronological ageing in mammals, the rate by which they accumulate in the elderly has been shown to be drastically influenced by latent herpesvirus infections (Kern *et al.* 1999; Wikby *et al.* 2002; Ouyang *et al.* 2003a; Ouyang *et al.* 2003b; Thimme *et al.* 2005). Although the role played by latent herpesvirus infection on the precipitation of senescent T-cell accumulation has been well documented in the elderly, only very few studies have assessed the impact of herpesvirus infections alone by studying young populations (Akbar & Fletcher 2005; Vescovini *et al.* 2007; Turner *et al.* 2010). Indeed senescent T-cells accumulation in the blood with chronological ageing is assumed to follow a linear progression; however

herpesvirus infections could modify this dynamics into an inverse exponential progression due to initial infection followed by a progressive decline in reactivation frequencies. The differences in senescent T-cell proportions seen among elderly populations have great clinical implications and are partial predictors of morbidity and mortality when grouped under the IRP with other immune markers, including a CD4/CD8 ratio inferior to 1, reduced proportions of naïve T-cells and latent infections to the herpesvirus CMV and EBV (Wikby *et al.* 1998; Olsson *et al.* 2000; Wikby A. 2003). In the present study, EBV seropositivity alone was associated with a reduced CD4/CD8 ratio compared to infection-free subjects, probably linked to an increase in memory CD8⁺ T-cells; however, the CD4/CD8 ratio reduction was not substantial enough to be qualified as a feature of the IRP. This could be explained by the potential recent EBV contamination of the subjects tested in this study as a direct consequence of their young age (mean 28.5 ± 5 years old). Similarly to CMV single-infection, latent CMV and EBV co-infection did not have an impact on the CD4/CD8 ratio. It is interesting to observe that while some researchers have reported a greater prevalence of EBV seropositivity amongst CMV seropositive subjects (Alvarez-Lafuente *et al.* 2008; Wang *et al.* 2010), in young adults EBV infection appears to be unrelated to CMV infection ($p > 0.05$).

In order to analyse the impact of herpesvirus infection on the proportions of resting naïve, memory and senescent T-cells, different cell populations based on their expression of cell surface molecules (Table 7.1). Highly differentiated effector-memory T-cells (TEMRA) were isolated based on their expression of CD45RA concurrent with their down-regulation of CCR7. While the common perception is that CD45RA⁺ T-cells that do not express CCR7 identifies a population of terminally differentiated T-cells, this is contentious as CD45RA, in addition to being highly expressed on naïve T-

cells, is also re-expressed on T-cells that have not encountered their specific antigen in a certain time span, and is not related to their proliferative capacity (Dunne *et al.* 2002; Carrasco *et al.* 2006). Consequently a distinction was made between TEMRA and senescent T-cells. Classified as such, a correlation between CMV infection and reduced frequencies of naïve CD45RA+/CCR7+ CD8+ T-cells was found, and an increase in both TEMRA CD8+ T-cells and senescent CD4+ and CD8+ T-cells. While EBV infection did not have an impact on the naïve and senescent T-cells frequencies, it was associated with a non-significant reduction of TEMRA CD8+ T-cells, potentially due to the confounding effect of CMV co-infection, dampening the EBV-associated reduction in TEMRA CD8+ T-cells. In accordance with other studies (Pawelec *et al.* 2005), no effect of CMV or EBV infection on the different memory CD4+ and CD8+ T-cell populations was identified. Interestingly, contrarily to the hypothesis of the study presented in this chapter, no interaction or synergistic effects of CMV and EBV in co-infected subjects was observed on the resting T-cell repertoire. Although this could be explained by the lack of impact of EBV infection on the different T-cell subsets or the small amount of EBV+/CMV- subjects, it could also be due to the limited amount of time the subjects were infected by both viruses.

7.4.2. Latent viral infections impact exercise-induced immune changes in young men

The rapid mobilization of lymphocytes into the peripheral blood compartment following an acute bout of exercise is consistently observed throughout the literature. Senescent and highly-differentiated T-cells have been shown to be more sensitive to exercise-induced lymphocytosis by being mobilized in relatively greater number compared to naïve and early differentiated T-cells (Simpson *et al.* 2007a; Simpson *et al.* 2008;

Simpson *et al.* 2010b; Turner *et al.* 2010), however the potential confounding effect of herpesvirus infections is still to be determined.

In accordance with the current literature, a transient mobilization of senescent and memory T-cells was seen immediately after exercise, followed by the return of cell numbers to baseline restoration (Simpson *et al.* 2007a; Simpson *et al.* 2008; Turner *et al.* 2010). CMV infection did not have an impact on the concentration of CD8⁺ T-cells in peripheral blood compartment following exercise or during the recovery period. This could be explained by the preferential shift of certain cell subsets. Indeed, while naïve CD8⁺ T-cells (KLRG1⁻/CD28⁺ and CD45RA⁺/CCR7⁺) in CMV seronegative subjects exhibited a classical exercise-induced mobilization followed by a decrease in cell number during the recovery phase, naïve CD8⁺ T-cells in CMV seropositive subjects did not appear to respond to the exercise protocol. Contrarily, senescent (KLRG1⁺/CD57⁺ and KLRG1⁺/CD28⁻) and TEMRA (CD45RA⁺/CCR7⁻) CD8⁺ T-cells from CMV seropositive subjects were mobilized in greater number with exercise than the same cell types isolated from CMV seronegative subjects.

TEMRA and senescent T-cells have increased effector capacities (Ibegbu *et al.* 2005), consequently, their increased exercise-sensitivity could be related to a required enhanced immune-surveillance induced by stressors such as physical exercise or psychological stressors (Bosch *et al.* 2009). It is interesting to observe that different cell types responded to exercise in EBV seropositive subjects. Indeed while naïve CD4⁺ and CD8⁺ T-cells had a similar response regardless of EBV infection status, EBV seropositive subjects had a greater number of central-memory (CD45RA⁻/CCR7⁺) CD8⁺ T-cells prior, immediately after and 1 hour after exercising than EBV seronegative subjects who did not elicit a response in central-memory cell counts throughout the test. The absolute exercise-induced mobilization and egress of those cell

types were however not different in EBV seropositive and EBV seronegative. Effector-memory (CD45RA-/CCR7-) cells were also mobilized and egressed in greater number in EBV-infected subjects in response to exercise. Central-memory cells constitutively express the chemokine receptor CCR7, necessary for cell extravasation through high endothelial venules and migration to secondary lymphoid organs (Forster *et al.* 1999), making them more available for antigenic stimulation than effector-memory cells. These findings are consistent with the study hypothesis that exercise enhances immune cells recirculation in a context of immune vigilance in response to stressors. Furthermore, highly differentiated T-cells have been shown to express higher level of the catecholamine receptor β 2-adrenergic receptor (Karaszewski *et al.* 1991; Dimitrov *et al.* 2009; Dimitrov *et al.* 2010), and are consequently likely to respond specifically to the increased concentrations of catecholamine seen during acute bouts of exercise (Christensen & Galbo 1983; Mazzeo 1991). However no interaction effect between CMV and EBV co-infection was observed on the exercise-induced T-cell response.

Latent herpesvirus infection is associated with alteration in the resting T-cell repertoire, as described above. In order to assess accurately cell exercise-induced trafficking in function of infection status, cell mobilization and egress were analysed independently to the number of cells present in the blood prior to the exercise. Interestingly only a non-significant enhanced mobilization of CD4+ naïve T-cells (KLRG1-/CD28+; CD45RA+/CCR7+) ($p = 0.094$ and $p = 0.110$ respectively) in CMV seronegative subjects was observed when compared to the CMV seropositive subjects and a greater senescent and TEMRA CD8+ T-cell withdrawal in CMV seropositive subjects. A recent study has shown that effector-memory and highly-differentiated T-cells were more mobilized during acute bouts of exercise and exited the peripheral blood compartment to a greater extend in CMV seropositive subjects (Turner *et al.* 2010). This is in partial

concordance with the results presented in this chapter, where an impact of CMV on highly differentiated TEMRA and senescent CD8⁺ T-cells egress was seen, but there was no difference in mobilization of those cells between CMV positive and negative subjects. Besides the phenotypical differences used in the characterization of highly differentiated and senescent T-cells, the divergence of results is inferable to the longer and more intense exercise protocol presented in Turner *et al.* (2010). Indeed as the preferential mobilization of highly differentiated TEMRA and senescent T-cells in response to exercise has been shown to be varying according to the type, intensity and duration of the bout of exercise (Simpson *et al.* 2007a; Simpson *et al.* 2008; Campbell *et al.* 2009). EBV infection alone, or in combination with CMV, did not appear to influence the exercise-induced shifts in the numbers or proportions of naïve, memory or senescent CD4⁺ and CD8⁺ T-cell.

The results presented in this chapter show a greater egress of senescent T-cells during the recovery phase in CMV seropositive subjects, compared to CMV seronegative subjects. Some researchers have hypothesized that the reduction in highly differentiated and senescent T-cells observed after acute bouts of exercise was the result of cell clearance via immediate apoptosis in the peripheral blood compartment instead of cellular extravasation (Cioca *et al.* 2000; Mooren *et al.* 2002; Mooren *et al.* 2004), but recent literature tends to disagree (Peters *et al.* 2006; Simpson *et al.* 2007b; Navalta *et al.* 2010). It is indeed more likely that those highly cytotoxic T-cells are being re-directed towards peripheral tissues to either be eliminated via delayed apoptosis (up to 24 hours post-exercise) in peripheral organs such as the spleen, lungs or Peyer's patches (Kruger *et al.* 2009) or to ensure immune integrity of the host by enhanced potential antigen contact. Consequently, by periodically recreating acute aerobic exercise, this exercise-induced lymphocytosis and delayed cell apoptosis could be a potential

mechanism for creating “vacant immune space” and inducing homeostatic production of naïve T-cells (Simpson & Guy 2010). Although this could be a mechanistic explanation of the beneficial effect of regular physical activity on the naïve T-cell repertoire described in *Chapter 4* of this thesis; this hypothesis remains to be tested as no study to date has assessed the exact tissue destination and fate of those cells after their extravasation from the peripheral blood compartment.

7.4.3. Contribution of viral-specific T-cells to the exercise-induced preferential mobilization of memory and senescent T-cells.

The study presented in this chapter sought to determine if the greater numbers of senescent cells that are mobilized in CMV and EBV seropositive subjects were specific to these viruses. Indeed, as complete clearance of the virus is never achieved, CMV and EBV will be periodically reactivated in response to physical or psychological stress (Almanzar *et al.* 2005) throughout the lifetime, inducing the clonal expansion of virus-specific CD8⁺ T-cell clone and thus contributing to immune exhaustion (Koch *et al.* 2006). It was hypothesized that the CMV and EBV associated increase in TEMRA and senescent T-cells numbers in response to exercise was due to viral-specific T-cell preferential mobilization. Contrarily to this hypothesis, the results presented in this chapter showed that the number of viral-specific T-cells exhibiting a senescent phenotype mobilized and exiting the peripheral blood compartment in response to exercise only accounted for less than 1% of the difference in senescent T-cell mobilization between CMV seropositive and CMV seronegative subjects.

It was found that although the numbers of resting naïve, memory and senescent pp65 CMV-specific and Imp2 EBV-specific CD8⁺ T-cells appear to be similar, the proportions of senescent pp65 CMV-specific and Imp2 EBV-specific senescent T-cells are greater than the proportions of naïve viral-specific T-cells (CMV-specific: 40.4% vs 24.9%; EBV-specific: 42.8% vs 25.2%). Consequently, CMV infection and EBV infection are associated with increased proportions of memory and senescent viral-specific T-cells as hypothesized. Furthermore, pp65 CMV-specific senescent T-cells were preferentially mobilized with exercise along with memory (KLRG1⁻/CD57⁺) T-cells compared with “naïve” CMV-specific T-cells. Exercise also mobilized Imp2 EBV-specific “naïve” (or central-memory), memory (KLRG1⁺/CD57⁻) and senescent T-cells. Total CMV-specific T-cells mobilization is principally resulting from senescent CMV-specific T-cells and to a small extent memory viral-specific T-cells entry in the blood compartment, while total EBV-specific T-cells mobilization is principally resulting from “naïve”, memory and senescent EBV-specific T-cells mobilization. Similarly to total memory and highly differentiated effector-memory T-cell egress during the recovery phase in EBV seropositive subjects, EBV-specific memory T-cells are preferentially leaving the blood compartment during the recovery phase. However, as stated above, the very small number of viral-specific T-cells exhibiting a senescent phenotype mobilized and exiting the peripheral blood compartment in response to exercise could not explain the CMV and EBV-associated difference in senescent T-cell mobilization.

Consequently, a direct impact of CMV on T-cell proliferation via a classical adaptive immune response does not appear to be the main culprit for the increase of highly differentiated and senescent T-cells in seropositive subjects, other CMV-induced mechanisms should be considered. One of which could be T-cell bystander activation

during latent herpesvirus reactivation. Activated antigen-presenting cells produce interferon- α (IFN- α) and interferon- β (IFN- β) during herpesvirus reactivation, leading to STAT-1 activation and ultimately to IL-15 release (Tough *et al.* 1996; Tough *et al.* 1997). This production of IL-15 will induce the heterologous proliferation of cells expressing high level of the β chain of the IL-2 receptor, such as memory and highly differentiated CD8⁺ T-cells (Zhang *et al.* 1998), potentially leading to the accumulation of heterologous CD8⁺ T-cells, that are not specifically directed towards CMV or EBV. Interestingly, CD4⁺ T-cell bystander activation has also been proposed in activated CD4⁺ T-cells expressing the complete form of the IL-2 receptor (CD25 and CD122) in response to IL-2 produced by antigen-specific activated CD4⁺ T-cells (Boyman 2010; Di Genova *et al.* 2010). However, this hypothesis remains to be tested in context of CMV and EBV infections.

Regarding the limitations of this study, viral-specific T-cells could not be assessed in all CMV and EBV infected subjects due to the viral-specific pentamers used. Indeed the pentamere technology requires subjects to express a specific HLA allele (HLA*A201), and only 6 of the CMV positive subjects expressed this allele and 7 of the EBV positive subjects were HLA*A201 positive. While HLA*A201 is the most prevalent allele in the general population, only around 50% of the population are expressing HLA*A201 (Lee *et al.* 1990) and consequently viral-specific T-cells could not be isolated in CMV and EBV seopositive subjects that expressed another HLA allele, as their T-cell receptor would not recognize the HLA*A201-pentamere complex. Furthermore, another limitation in the characterization of viral-specific T-cells may have led to their under-estimation. Indeed many viral-proteins are recognized by T-cells, and although pp65 and Imp2 have been shown to be good target proteins to monitor CMV and EBV-specific T-cells respectively (Gibson *et al.* 2004; Gibson *et al.* 2007; Lalonde *et al.*

2007), 20 other peptides have been shown to be immunogenic for T-cells (Sylwester *et al.* 2005). While other techniques have been used in the past to characterize the proportions of viral-specific T-cells, such as ELISPOTs, the use of flow cytometry to assess the proportions of pentamer-bound viral-specific T-cells is considered to be the most accurate determination technique. However the single use of pp65-specific or Imp2-specific pentamers may not be representative of the entire population of CMV-specific and EBV-specific T-cells and the proportions of viral-specific T-cells observed may have been under-evaluated. Another limitation of this study is to be found in the protocol used to estimate individual subjects' workload. Subjects cycled for 30 minutes at a fixed-intensity corresponding to 85% of their maximum Wattage. However, this intensity was estimated based on a submaximal $\dot{V}O_{2max}$ test (Astrand 1960), which could have potentially led to inaccuracies and to overestimated workload in certain subjects. Finally both the sample size (n=19) and sex (exclusively male) can be seen as a limitation. While additional subjects could have been tested, it was decided to exclude women from the study, mostly because CMV effect on the immune system is more pronounced in men. Indeed T-cell functions are known to be influenced by progesterone and oestradiol (as reviewed by (Beagley & Gockel 2003)) in a menstrual cycle-dependent manner (White *et al.* 1997) and could have been a confounding factor in the analysis of CMV and EBV infection impact on the exercise-induced cell trafficking. Furthermore, the results presented in *Chapter 5* of this thesis suggest that women have fewer senescent T-cells than men during adolescent. Although no study in this thesis analysed the effect of gender on the accumulation of senescent T-cells at an older age, the potential delayed onset of immunosenescence could be protecting female subjects from enhanced immune-senescence triggered by herpesvirus infections. A future study should analyse gender-associated difference in immunosenescence across the lifespan.

In conclusion, latent CMV and EBV infections are associated with immune changes and following acute bouts of exercise. While the association between CMV infection and increased proportions of senescent T-cells in the elderly are well documented (Wikby *et al.* 2002; Wikby A. 2003; Pawelec & Gouttefangeas 2006; Pawelec *et al.* 2009), the results presented in this study showed a similar impact on younger adults. They also highlighted that senescent T-cells in CMV infected subjects are mobilized in great quantities in blood and preferentially exit the peripheral blood compartment. This could be interpreted as an increased stress-sensitivity of senescent cells in subjects infected with CMV, leading to an increased immune surveillance by the recirculation of cells with high killing capacities. Finally it was demonstrated that the increase of TEMRA and dysfunctional senescent T-cells observed in subjects infected by latent herpesviruses, is not related to viral-specific T-cells, and consequently cannot be linked to oligoclonal T-cell expansion.

The study presented in this chapter was conducted to assess the impact of herpesvirus infections on the proportions of resting naïve, memory and senescent T-cells along with their impact on exercise-induced immune changes in young adults. However chronological ageing has been reported to greatly impact the proportions of those specific cell-types (Wikby *et al.* 1998; Brzezinska 2005), consequently future studies should assess the effect of herpesvirus infections on the age-associated increase in senescent T-cells. Such a study could determine if the age-associated accumulation of TEMRA and senescent T-cells is indeed due to chronological ageing, or rather CMV infection and repeated viral reactivations.

CHAPTER 8
GENERAL DISCUSSION

The studies comprising this thesis investigated the effects of lifestyle factors such as physical activity or obesity, along with latent viral infections on T-cell differentiation indicative of immunosenescence. Data was gathered from human subjects of various age, physical activity, body composition and viral infection status and the analysis of cell surface phenotypes was conducted using four-colour flow cytometry. When mechanisms potentially affecting T-cell phenotypic shifts were identified, *in vitro* stimulation assays followed by cell surface phenotype and gene expression analysis on isolated T-cell subsets were performed. Where the effect of acute bouts of exercise on T-cells subsets was characterized, differences in peripheral blood composition based on cell surface phenotypes among lymphocytes in resting, exercised and recovery blood samples were assessed.

8.1. Main findings of the studies presented in this Thesis

The aim of this thesis, as stated in *Chapter 2*, was to address the dearth of information regarding the impact of different lifestyle factors and viral infection status on the accumulation of highly differentiated and senescent T-cells based on the cell surface expression of identified markers of senescence. Although chronological ageing has been considered as the main culprit of immunosenescence, the studies presented within this thesis were conducted to highlight the importance of behaviors and viral infection history on both the accumulation of senescent T-cells and the shrinkage of naïve T-cell repertoire, independently of ageing. Many novel findings have resulted from the different studies comprised within this thesis. In respect to the specific aims of those studies, described in *Chapter 2*, the main findings of the studies described within this thesis are:

Aim 1: Investigate the association of physical activity and aerobic fitness and the age-related increase of effector-memory and senescent T-cells in adult men (*Chapter 4*).

Main Finding: Aerobic fitness is associated with a lower proportion of senescent and a higher proportion of naïve T-cells, particularly within the CD8+ T-cell compartment in healthy adult men. The beneficial impact of aerobic fitness, and consequently of regular physical activity, on the ageing immune system is independent of age, latent viral infection status and body composition.

Aim 2: Investigate the relationship between excess body mass and the proportions of the different T-cell subsets in an age-controlled population of adolescents (*Chapter 5*).

Aim 3: Investigate the association between serum leptin concentrations and the proportions of effector-memory and senescent T-cells in an age-controlled population of adolescents (*Chapter 5*).

Main Findings: (1) Excess body mass was associated with reduced proportions of naïve T-cells in Mexican-American adolescents. Obese subjects and subjects at risk for overweight had greater frequencies of CD8+ effector-memory T-cells and CD4+ senescent T-cells than subjects with an appropriate weight.

(2) Elevated serum leptin concentrations were associated with reduced frequencies of naïve T-cells. Individuals with the highest serum leptin concentrations had greater proportions of effector-memory CD8⁺ T-cells than subjects with lower serum leptin concentrations. Elevated serum leptin concentrations were also associated with an accumulation of senescent CD4⁺ T-cells.

(3) After adjusting for age, latent viral infection, gender and quality of life, excess body mass was still associated with a reduction in naïve T-cell frequencies and an increase in effector-memory T-cells and senescent CD4⁺ T-cells. Latent viral infections were associated with reduced frequencies in naïve T-cells and in increased frequencies in memory and senescent T-cells. Unexpectedly, while quality of life did not have an impact on the T-cell repertoire, gender had a significant impact on the different T-cell subsets frequencies. Indeed, female individuals had greater frequencies of naïve T-cells and fewer effector-memory and senescent T-cells

Aim 4: Investigate the impact of high concentrations of serum leptin on T-cell activation state (*Chapter 6*).

Main Findings: (1) Serum leptin alone did not induce peripheral blood T-cell activation, however leptin enhanced the mitogen-induced T-cell activation.

(2) A limited proportion of resting T-cells expressed OB-Rb, however they constitutively expressed OB-Rb mRNA. Mitogen-induced T-cell activation led to the up-regulation of OB-Rb mRNA when cells were costimulated with high physiological doses of leptin typically seen in obese individuals, but not with the low level of leptin typically seen in lean subjects.

Aim 5: Investigate the impact of latent viral infections on blood T-cell phenotypes in healthy young adult men (*Chapter 7*).

Aim 6: Investigate the impact of the latent viral infections on the exercise-induced changes in T-cell subsets (*Chapter 7*).

Aim 7: Investigate the contribution of viral-specific T-cells to the mobilization of memory and senescent T-cells with exercise (*Chapter 7*).

Main Findings: (1) CMV infected individuals had reduced proportions of naïve CD8+ T-cells and greater proportions of highly differentiated effector-memory and senescent T-cells than non-infected individuals. EBV infection was not associated with changes in the T-cell repertoire.

(2) Senescent T-cells were mobilized and left the peripheral blood compartment in response to exercise in greater numbers in CMV

seropositive individuals, compared to non-infected individuals. A greater number of highly differentiated effector-memory T-cells were removed from the blood during the recovery phase in CMV seropositive individuals than in CMV seronegative individuals. EBV infection did not impact the mobilization or egress of naïve and senescent T-cells, however a greater number of effector-memory CD8+ T-cells were mobilized and left the blood in EBV seropositive individuals than in non-infected individuals.

(3) CD8+ T-cells specific to epitopes of the CMV and EBV viruses were mobilized into the blood compartment with exercise but unlike recent hypothesis (Turner *et al.* 2010), they did not contribute significantly to the amplified mobilization of senescent T-cells seen in CMV and EBV infected individuals.

8.2. Exercise

Immunosenescence has been linked with many chronic conditions and is believed to directly or indirectly contribute to mortality in later life (Cawthon *et al.* 2003; Valdes *et al.* 2005; Koch *et al.* 2007; Starr *et al.* 2007). One striking feature of immunosenescence and advancing age is the accumulation of highly differentiated memory and senescent T-cells and a concomitant reduction in the numbers and proportions of fully functional naïve T-cells within the periphery (Effros *et al.* 1994a; Pawelec *et al.* 1996; Effros 1997; Spaulding *et al.* 1997; Ouyang *et al.* 2004). In addition to chronological ageing, certain lifestyle factors such as reduced physical activity and excessively sedentarity

lifestyles have also been shown to precipitate leukocyte telomere shortening and up-regulation of $p16^{INK4a}$ mRNA expression (Cherkas *et al.* 2008; Ludlow *et al.* 2008; Mirabello *et al.* 2009; LaRocca *et al.* 2010; Song *et al.* 2010). However, while those measures are considered to be pertinent markers of biological ageing, the direct impact of regular physical activity on the T-cell repertoire composition was not known. Furthermore, because no physical determinations of fitness level were conducted in these studies, nor were used validated self-reported questionnaire for physical activity, no conclusion could be drawn on the specific impact of physical activity on T-cell senescence.

It has been demonstrated in this thesis that aerobic fitness has a moderating effect on the age-related accumulation of senescent T-cells (*Chapter 4*). It was established that above average scores of aerobic fitness were associated with lower proportions of senescent T-cells and higher proportions of naïve T-cells. The study presented in *Chapter 4* confirms suggestions made by other researchers who advocated that regular physical activity could have beneficial effects on the immune system (Valdes *et al.* 2005; LaRocca *et al.* 2010; Song *et al.* 2010). Contrarily to these studies the present experimental design allowed an accurate characterization of the impact of aerobic fitness on the proportions of the different T-cell subsets. Indeed exercise tests were performed on each participants to estimate their aerobic fitness levels, which is likely to yield more accurate estimates of aerobic fitness than the self-reported estimation of physical activity that have been used previously. Secondly, the cell surface phenotypic techniques used permitted the identification of biomarkers of ageing and the influence of chronological age and fitness status on blood lymphocyte subtypes (i.e. CD4⁺ and CD8⁺ T-cells), allowing for a more accurate quantification of the cell types involved. The impact of exercise on T-cell differentiation was examined using two methods, a self-reported physical activity level

score and an estimation of $\dot{V}O_{2\max}$ from a laboratory-based exercise test. While the questionnaire-based estimation technique is a simple, convenient way to quantify physical activity scores, it was not found to have as much of an impact on age-associated T-cell phenotypic changes compared to the $\dot{V}O_{2\max}$ scores obtained from the exercise test. As expected, the use of self-reported activity scores to determinate physical fitness levels presents inaccuracy and lacks sensitivity, possibly due to increased intra-group variations, when compared to a laboratory-based exercise test.

Using estimated $\dot{V}O_{2\max}$ measurements the results showed that a difference in estimated $\dot{V}O_{2\max}$ of $10\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ was associated with a 9.5% difference in the proportion of senescent CD8+ T-cells independently of age. Furthermore, the data presented in *Chapter 4* showed that this increase in aerobic fitness, corresponding to a transition from a $\dot{V}O_{2\max}$ below average to a $\dot{V}O_{2\max}$ above average, could offset the age-associated accumulation of CD8+ senescent T-cells, found to increase by 10% per decade. Senescent T-cells are known to accumulate in the immunological space to the detriment of naïve T-cells (Effros *et al.* 1994a; Cossarizza *et al.* 1996; Spaulding *et al.* 1997; Lazuardi *et al.* 2005) with chronological ageing, however, the data presented in *Chapter 4* are suggesting that regular physical activity may prevent the accumulation of senescent T-cells in middle-aged men, but also prevent the naïve T-cell repertoire shrinkage. Indeed the results showed that while every decade of age was associated with a reduction of 10% of naïve CD8+ T-cells, an increase in estimated $\dot{V}O_{2\max}$ of $10\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ was associated with an increase of the same proportion of naïve CD8+ T-cells independently of age. Furthermore, after accounting for fitness level, chronological age was no longer associated with senescent T-cell proportions. These data suggest that regular physical activity exerts direct protective effects on the ageing immune system, preventing the early transition to the IRP category in later life. This novel finding ties in

with the differences in immune state observed among the elderly population, providing another potential explanation on why some elderly are more competent in responding to newly evolving pathogens.

The mechanisms by which regular physical activity dampens the accumulation of senescent T-cells is still not known, however as proposed in *Chapter 7*, it is plausible to hypothesize that periodic repetition of acute bouts of exercise over an extended period of time may play an important role in this process. Indeed the study presented in *Chapter 7* investigated the effect of acute bouts of exercise on the preferential mobilization of senescent T-cells in the peripheral blood compartment. The data showed that TEMRA and senescent T-cells were preferentially mobilized in the blood following exercise, and preferentially exited the blood compartment during the recovery phase, confirming findings available in the current literature (Simpson *et al.* 2007a; Simpson *et al.* 2008). Furthermore, numerous studies have shown that acute bouts of exercise altered lymphocyte susceptibility to apoptosis. Indeed, acute bouts of exercise appear to up-regulate cell surface receptors involved in apoptosis, such as Fas receptor (Mooren *et al.* 2002; Mooren *et al.* 2004) and TNF α receptors 1 and 2 (Pedersen *et al.* 1999), but also increase the production of ROS contributing to oxidative stress (Vider *et al.* 2001b) and the secretion of salivary and plasma TNF- α (Rahman *et al.* 2010). Considering that senescent T-cells are sought to be more resistant to apoptosis than their less differentiated counterparts (Spaulding *et al.* 1999; Bryl *et al.* 2001a; Hsu *et al.* 2001; Effros 2004), their preferential mobilization combined with the pro-apoptotic signals in the environment and apoptosis-inducing alterations of the cell surface markers may enhance their susceptibility to be cleared via delayed apoptosis (up to 24 hours post-exercise) in peripheral organs such as the spleen, lungs or Peyer's patches (Kruger *et al.* 2009). While it is plausible to hypothesize that periodically recreating acute bouts of

exercise could lead to the creation of “vacant immune space” and the subsequent homeostatic production of naïve T-cells (Simpson 2011), providing a mechanistic explanation of the beneficial effect of regular physical exercise presented in *Chapter 4*, the exact fate of the mobilized T-cells following exercise remains to be investigated. Many of the currently available therapeutic interventions (i.e. gene, cytokine, hormone and monoclonal antibody therapy) that have been proposed to negate ageing immunity (Greenstein *et al.* 1987; Kendall *et al.* 1990; Phillips *et al.* 2004; Rosenberg *et al.* 2006) are risky and bear a large number of undesired side effects and ethical complications, consequently physical exercise could be considered a safer alternative strategy to combat age-related immunosenescence.

Although the impact of estimated $\dot{V}O_{2\max}$ on the T-cell subsets withstood adjustment for, BMI, percentage body fat and waist/hip ratio (*Chapter 4*), and body composition was not associated with the different T-cell subsets, the potential effect of an excess body mass and adiposity on the accumulation of senescent T-cell could not be refuted. Indeed the study presented in *Chapter 4* was conducted on a homogeneously lean population with less than 7% of obese subjects, in order to control for body mass and adiposity. It was consequently necessary to investigate the impact of excess body mass and high adiposity on the T-cell repertoire, using an age-controlled population with a wide range of body weight and adiposity (*Chapter 5*).

8.3. Obesity

Chapter 5 investigated the proportions of the different T-cell subsets, characterized by their expression of CD28, CD27, CD57, CD45RA and CCR7 in an age-controlled

population of Mexican-American adolescents of heterogeneous body mass and body composition. Reduced proportions of naïve or early differentiated CD4⁺ and CD8⁺ T-cells were observed in subjects at risk for overweight and in obese subjects when compared to the subjects with an appropriate weight. In addition, increased proportions of memory or intermediate CD4⁺ T-cell subsets were observed in obese subjects and greater proportions of effector-memory CD8⁺ T-cell subsets were observed in subjects at risk of obesity and in obese subjects when compared to the subjects with an appropriate weight. Similarly, greater proportions of senescent or late differentiated CD4⁺ T-cell subsets were found in obese and subjects at risk of obesity than in subjects with an appropriate weight. Potential confounding factors may affect the T-cell repertoire and accumulation of senescent T-cells, such as age (Spaulding *et al.* 1997), gender, quality of life or latent viral infection (*Chapter 7*), consequently generalized linear models were generated to analyze the data while controlling for all those factors. When controlled for all the potential factors significantly affecting the T-cell repertoire, age-adjusted body mass was still correlated with a reduction in naïve T-cell proportions, and a concomitant increase in effector-memory T-cells and senescent CD4⁺ T-cells. These data suggest an accumulation of differentiated T-cells along with senescent CD4⁺ T-cells in overweight and obese individuals, a new finding providing new insights on the state of immunosuppression associated with obesity. Furthermore, these interesting results were obtained from young subjects, suggesting that obesity could be associated with the premature onset of immunosenescence, independently of age. Studies have shown a primordial role of physical activity on weight regulation (Byers 1995; Booth *et al.* 2000), consequently the data also confirms the hypothesis presented in *Chapter 4* stating that lack of physical activity could lead to obesity and subsequently enhance the accumulation of senescent T-cells.

The accumulation of senescent T cells with obesity may play a role in the increased level of immunosuppression and cardiovascular diseases in obese individuals. However, the high level of diversity observed among the subjects may reflect the existence of a metabolically healthy but obese (MHO) group in the participants of this study. Individuals classified as MHO have previously been identified and shown to have a metabolically healthy profile including high levels of insulin sensitivity and a clinically normal lipid and inflammation profiles (Brochu *et al.* 2001; Karelis *et al.* 2005; Stefan *et al.* 2008). These individuals are not subject to the same immunosuppression and cardiovascular risk as obese individuals with metabolic disorder (Meigs *et al.* 2006) and could dampen the observed effect size.

Although the exact mechanisms by which obesity and excess body mass impact on the level of T-cell differentiation remain to be tested, two mechanisms are likely to play a role in these premature immune changes. Increased levels of oxidative stress associated with obesity (Epel *et al.* 2004; Valdes *et al.* 2005) may lead to an increased rate of telomere attrition (Makarov *et al.* 1997; von Zglinicki 2002; Epel *et al.* 2004; Kurz *et al.* 2004) and telomere-independent senescence (Dumont *et al.* 2000; Toussaint *et al.* 2000; Chen *et al.* 2001; Frippiat *et al.* 2001). Another direct consequence of obesity and excess adiposity is the increased concentration of circulating adipocyte-secreted proteins, such as leptin. Indeed supra-physiological concentrations of leptin have been shown to enhance T-cell activation *in vitro* (Lord *et al.* 1998; Martin-Romero *et al.* 2000; Lord *et al.* 2002) (*Chapter 6*), suggesting that high concentrations of circulating leptin could lead to an increased rate of telomere erosion and senescent T-cell accumulation. Indeed while plasma leptin concentrations have been positively correlated with telomere shortening (Valdes *et al.* 2005), the data presented in *Chapter 5* showed a clear positive association between serum leptin concentration and

intermediate differentiated or memory T-cells along with late differentiated or senescent T-cells. Furthermore, subjects with the highest concentrations of circulating leptin had fewer early differentiated or naïve T-cells than subjects with very low serum leptin concentrations.

Chapter 6 investigated the effect of physiological concentrations of human leptin on T-cell activation *in vitro*. Furthermore, the constitutional and inducible expression of the physiologically active form of the leptin receptor, both at the protein and mRNA level, was assessed in T-cells. Previous studies have shown that supra-physiological doses of leptin (160 ng.mL^{-1}) enhanced mitogen-induced T-cell activation (Martin-Romero *et al.* 2000), however it was not known if physiological doses of leptin also had an effect on T-cell activation. The data obtained in *Chapter 6* showed that lower physiological concentrations of leptin (25 ng.mL^{-1} and 50 ng.mL^{-1}), as seen in obese patients (Prolo *et al.* 1998), also induce the up-regulation of the early activation marker CD69 in mitogen-activated T-cells, suggesting a realistic *in vivo* mechanism leading to the accumulation of senescent T-cells in obese individuals. Indeed, during infections and the development of a normal immune response, the exposure of antigen-activated T-cells to high concentrations of circulating leptin may unnecessarily and uncontrollably enhance their activation state, possibly increasing T-cell differentiation and accelerating the rate at which telomere shortening occurs, which could eventually lead to premature accumulation of senescent T-cells. It was also shown that although OB-Rb mRNA appears to be expressed in resting T-cells, it is not expressed at the protein level on the surface of resting T-cells (*Chapter 6*). In addition, T-cell activation by a mitogen up-regulated OB-Rb mRNA, and when mitogen-activated T-cells were costimulated with high concentrations of leptin, OB-Rb mRNA was further up-regulated. This novel finding was only observed when concentrations of leptin corresponding to the one found

in obese individuals were used to stimulate the T-cells, but not when stimulated with leptin concentrations corresponding to the one found in lean individuals. The kinetic of the cell surface OB-Rb down-regulation has been investigated (Barr *et al.* 1999; Uotani *et al.* 1999) and OB-Rb is known to be internalized mediating leptin cellular uptake. It could however be hypothesized that leptin-induced OB-Rb up-regulation on activated T-cells during normal immune responses may have a period of latency during which T-cells would still express high amount OB-Rb after resolution of the immune challenge. This would lead to prolonged T-cell activation by circulating leptin, increasing the rate of telomere shortening, eventually leading to the accumulation of intermediate differentiated and senescent T-cells (*Chapter 5*).

8.4. Viral Infection status

Chapter 7 investigated the effect of the latent viral infections CMV and EBV on the preferential mobilization and subsequent egress of highly differentiated and senescent CD8⁺ T-cell subsets in response to exercise. Similarly to the data shown in *Chapter 5*, subjects infected with CMV had reduced proportions of naïve T-cells and greater proportions of highly differentiated effector-memory TEMRA and senescent T-cells when compared to their infection free counterparts. As previously shown by Turner *et al.*, CMV infection amplified the removal of TEMRA CD8⁺ T-cells from the peripheral blood compartment following exercise (Turner *et al.* 2010). The study presented in *Chapter 7* is however the first study to show a similar effect of CMV infection on the exercise-induced changes in senescent T-cell numbers. Senescent T-cells were indeed more responsive to the acute bout of exercise in CMV seropositive subjects, as they were mobilized and removed from the peripheral blood compartment in greater number

in response to exercise than non-infected subjects. Another novel finding of this study is the observed limited effect of EBV infection on the T-cell repertoire. Naïve and senescent T-cells proportions did not differ from EBV seropositive and seronegative subjects, however EBV infected individuals had increased proportions of central-memory T-cells and a greater non-significant elevated proportion of highly differentiated effector-memory T-cells in EBV infected subjects was noted. The major finding of the study presented in *Chapter 7* was that, although CMV-specific and EBV-specific CD8⁺ T-cells were mobilized into the peripheral blood compartment with exercise, their contribution in the amplified mobilization of senescent T-cells seen in CMV and EBV infected individuals did not appear to be substantial. While very interesting, this novel finding is contradicting the hypothesis made in the study. It is however necessary to highlight an important limitation in the characterization of viral-specific T-cells. Many viral-proteins are recognized by T-cells, and although pp65 and Imp2 have been shown to be good target proteins to monitor CMV and EBV-specific T-cells respectively (Gibson *et al.* 2004; Gibson *et al.* 2007; Lalonde *et al.* 2007), 20 other peptides have been shown to be immunogenic for T-cells (Sylwester *et al.* 2005). Consequently the characterization of viral-specific T-cells by single use of pp65-specific or Imp2-specific pentamers may not be representative of the entire population of CMV-specific and EBV-specific T-cells and the proportions of viral-specific T-cells observed may have been under-evaluated.

Latent viral infections to CMV and EBV induce exacerbated CD8⁺ T-cell clonal expansion, promoting immune exhaustion (Koch *et al.* 2006), by being periodically reactivated in response to physical or psychological stressors (Almanzar *et al.* 2005). The beneficial effect of regular physical activity on immune ageing presented in *Chapter 4* could be related to the repeated preferential mobilization of highly

differentiated and senescent T-cells in response to acute bouts of exercise and their subsequent deletion via apoptosis or AICD. Consequently, it was hypothesized that frequent highly differentiated and senescent CMV-specific and EBV-specific CD8⁺ T-cells preferential mobilization in the peripheral blood compartment in response to exercise, would lead to their subsequent deletion via apoptosis, restoring available immune space for homeostatic production of naïve T-cells. The data presented in *Chapter 7* are confirming the preferential mobilization of highly differentiated and senescent T-cells, while suggesting that the vast majority of these are not specific to CMV or EBV. Highlighting that senescent T-cells mobilized with acute exercise do not appear to be specific for CMV or EBV, corroborate the lack of association between the beneficial effects of aerobic fitness on immune ageing and latent viral infection status (*Chapter 4*).

As described above, CMV-specific and EBV-specific may not all recognize pp65 or Imp2 respectively, however current literature suggest that the majority of viral-specific T-cells should be specific for these antigens (Gibson *et al.* 2004; Gibson *et al.* 2007; Lalonde *et al.* 2007). Consequently these new data suggests that other viral-induced mechanisms may impact T-cell proliferation and the accumulation of senescent T-cells, such as T-cell bystander activation during latent viral reactivation. Herpesvirus reactivation have been shown to induce IFN- α and IFN- β production by APC, leading to subsequent production of IL-15 (Tough *et al.* 1996; Tough *et al.* 1997). The direct consequence of IL-15 secretion will be the heterologous proliferation of cells expressing high level of IL-2R β chain, such as memory and highly differentiated CD8⁺ T-cells (Zhang *et al.* 1998). No study to date has however assessed the impact of serum IL-15 on the accumulation of senescent T-cells in context of CMV and EBV infections.

8.5. Limitations of the studies presented in this Thesis

8.5.1. Lymphocytes phenotypic identification limitations

Different T-cell subsets can be identified according to their expression of particular cell surface glycoproteins. Four-color flow cytometry was used in the different studies comprised within this thesis to characterize the different T-cell subsets of interest. Some limitations are however present when using this technique, as accurate characterization of certain T-cell subsets require the identification of the expression (or lack of expression) of more than 4 glycoproteins simultaneously. Phenotypic identification of T-cell subsets to mark their stage of differentiation and functional status is still a contentious issue. However current literature tends to identify highly differentiated effector-memory T-cell, that encountered antigen and undergone multiple rounds of cell division without reaching terminal differentiation, as CD3+/CD45RA+/CCR7- and senescent T-cell, that has reached terminal differentiation, as CD3+/KLRG1+/CD57+ (Voehringer *et al.* 2001; Voehringer *et al.* 2002; Appay *et al.* 2008).

More discrepancies exist in the literature in the identification of memory T-cells based on their expression of KLRG1 and CD28. Cells with a KLRG1+/CD28+ phenotype are consistent with effector-memory cells in that they do not express CD45RA or CCR7, but may still express CD27 indicating that they are “early” stage differentiated in accordance with the classic CD27/CD28 phenotypic identification provided by Appay *et al.* (Appay *et al.* 2002). Supporting this phenotypic identification, Thimme *et al.* (2005) reported that KLRG1 expression can appear early in the differentiation process, although expressed at greater levels proportionally on late/terminal differentiated T-cells.

The different T-cell subsets analyzed in *Chapter 5* were identified without using the monoclonal antibody anti-KLRG1, due to technical limitations. Although the concomitant use of CD28 and CD27 to identify the different T-cell subsets have been well documented (Hendriks *et al.* 2000; Appay *et al.* 2008; Koch *et al.* 2008), the classification of T-cell subsets into early differentiated, intermediate differentiated and late differentiated were preferred to analyze the result accurately.

8.5.2. Experimental design limitations

In *Chapter 4* individuals' $\dot{V}O_{2\max}$ were estimated from a submaximal cycling protocol to limit the physical stress to be endured by the older volunteers that participated to the study. Although the protocol created by Astrand *et al.* (196) has been validated and used by the scientific community for the past 50 years, and although a validated non-exercise questionnaire (Jackson *et al.* 1990) was also used to compare the estimated $\dot{V}O_{2\max}$ value obtained by the cycling protocol (Pearson's $R = 0.88$), small inaccuracies could have occurred. The subjects that volunteered for the study presented in *Chapter 7* underwent the same $\dot{V}O_{2\max}$ estimation tests to ensure consistency throughout the thesis. Furthermore subjects cycled for 30 minutes at a fixed-intensity corresponding to 85% of their maximum Wattage. However, due to ethical institutional review board regulations, submaximal $\dot{V}O_{2\max}$ test (Astrand 1960) was used to estimate this intensity instead of more physically demanding maximal $\dot{V}O_{2\max}$ testing. Consequently, this could have potentially led to inaccuracies and to overestimated workload in certain subjects.

In *Chapter 7* viral-specific T-cells could not be assessed in all CMV and EBV infected subjects due to the viral-specific pentamers used. Indeed the pentamer technology requires subjects to express a specific HLA allele (HLA*A201), and only 6 of the CMV

positive subjects expressed this allele and 7 of the EBV positive subjects were HLA*A201 positive. While HLA*A201 is the most prevalent allele in the general population, only around 50% of the population are expressing HLA*A201 (Lee *et al.* 1990) and consequently viral-specific T-cells could not be isolated in CMV and EBV seropositive subjects that expressed another HLA allele, as their T-cell receptor would not recognize the HLA*A201-pentamer complex. Furthermore, as discussed previously, viral-specific T-cells were characterized by their specificity to the most dominant epitope of the most dominant antigen of either CMV (pp65) or EBV (Imp2). Consequently, viral-specific T-cells specific for other antigens were not detected using the pentamer technology, and other techniques should be used to characterize the total CMV-specific and EBV-specific T-cells, such as Enzyme-linked ImmunoSorbent Spot assays.

In *Chapter 4* and in *Chapter 7* it was decided to exclude women from the study, mostly because CMV effect on the immune system is more pronounced in men. Indeed T-cell functions are known to be influenced by progesterone and oestradiol (as reviewed by (Beagley & Gockel 2003) in a menstrual cycle-dependent manner (White *et al.* 1997) and could have been a confounding factor in the analysis of CMV and EBV infection impact on the exercise-induced cell trafficking. Furthermore, the results presented in *Chapter 5* of this thesis suggest that women have fewer senescent T-cells than men during adolescent. Although no study in this thesis or in the current literature analyzed the effect of gender on the accumulation of senescent T-cells at an older age, it is plausible to hypothesize that the differences in senescent T-cell proportions observed between males and females at an early age, will lead to a delayed onset of immunosenescence and consequently in lower rate of morbidity and mortality in later life.

Finally, the greatest limitation of the study presented in *Chapter 4* was the use of a cross-sectional design. Indeed, while only a longitudinal study could have shown clear effect of regular physical activity and aerobic fitness on the ageing immune system, such a study would have required to be conducted over an extended period of time and would have exceeded all resources available to complete the studies contained within this thesis.

8.6. Future Research

As demonstrated by the studies comprised within this thesis, immunosenescence and the rate by which alterations in the T-cell repertoire occur are not solely affected by chronological ageing. Regular physical activity is negatively associated with the accumulation of senescent T-cells (*Chapter 4*), and highly differentiated and senescent T-cells have an increased exercise-sensitivity when compared to less differentiated T-cells (*Chapter 7*). In respect with these observations, it is suggested that future studies address the following research questions:

- 1) Highly differentiated and senescent T-cells have been shown to exit the peripheral blood compartment following exercise, however their destination is still unknown. Furthermore, what is the fate of these specific cell types? Are they eliminated via apoptosis as hypothesized?
- 2) If they happen to be deleted by apoptosis after their extravasation, are they being replaced by fully functional naïve T-cells, and if so, where are these new T-cells coming from?

- 3) Could exercise be used as a clinical intervention to restore normal proportions of naïve T-cells, once an individual is already in the IRP?
- 4) Can regular physical exercise prevent the early onset of immunosenescence by other mechanisms, such as by enhancing telomerase activity?

The data presented in *Chapter 5* and in *Chapter 6* showed that excess body mass was associated with the accumulation of senescent T-cells and the reduction in the naïve T-cell pool at a very young age, potentially due to un-regulated T-cell activation by elevated concentrations of serum leptin. Further research should be conducted in this area, in order to address the following research questions:

- 1) Does leptin stimulation enhance T-cell proliferation when compared to mitogenic stimulation?
- 2) What is the mechanism by which leptin up-regulates OB-Rb expression on circulating T-cells?
- 3) Do other hormones associated with body mass, such as ghrelin, have similar effects on T-cells, or would it have an opposite effect on T-cell activation?
- 4) Would an exercise-based weight-loss intervention reduce the rate at which senescent T-cells accumulate? Furthermore could regular exercise lead to the elimination of highly differentiated TEMRA and senescent T-cells, helping to restore a biologically “young” immune system?

The data presented in *Chapter 7* showed that, although individuals infected with CMV and EBV had greater frequencies of highly differentiated and senescent T-cells than non-infected subjects, and that those T-cell subsets were more responsive to exercise

than less differentiated T-cells, pp65-specific and Imp2-specific T-cells were not accounting for the greater exercise-induced changes in highly differentiated and senescent T-cells numbers observed in infected individuals. Consequently, future research should be conducted in this area, in order to address the following research questions:

- 1) Although CMV and EBV latent infections have been associated with the IRP, many other viruses such as Herpes Simplex Virus 1 and 2 are also known for being periodically reactivated. Could the senescent T-cell pool be composed of heterogeneous viral-specific T-cells?
- 2) By studying the effect of CMV and EBV infection in older men, would the longer period of infection affect the observed exercise-induced T-cell changes?

Finally, the data obtained in *Chapter 5* highlighted a clear effect of gender on the proportions of naïve, memory and senescent T-cells. The effect of immunosenescence on female has not been studied extensively, due to the daily hormonal variations and the development of menopause in later life. However, future research should include gender as a factor in the characterization of immunosenescence.

The findings of this project demonstrate that T-cell senescence is not solely due to chronological ageing, but can also be affected by exercise, obesity and latent viral infections. Further research is required to fully understand the effects and mechanisms affecting immunosenescence. Indeed, with the constant extension of expected lifespan, understanding how regular physical activity and weight management could delay the onset of immunosenescence or restore a functional immune system, would have great

clinical applications in the prevention of infections, ultimately leading to the reduction in morbidity and mortality in the elderly.

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APPENDIX 1

SUBJECT CONSENT FORM

SUBJECT RIGHTS

1. I understand that informed consent is required of all persons participating in this project.
2. All procedures have been explained to me and all my questions answered to my satisfaction.
3. Any risks and/or discomforts have been explained to me.
4. Any benefits have been explained to me.
5. I understand that, if I have any questions about this study, I may contact Dr. Richard Simpson at (713) 743-9270 or rjsimpson@uh.edu.
6. I have been told that I may refuse to participate or to stop my participation in this project at any time before or during the project. I may also refuse to answer any question.
7. ANY QUESTIONS REGARDING MY RIGHTS AS A RESEARCH SUBJECT MAY BE ADDRESSED TO THE UNIVERSITY OF HOUSTON COMMITTEE FOR THE PROTECTION OF HUMAN SUBJECTS (713-743-9204). ALL RESEARCH PROJECTS THAT ARE CARRIED OUT BY INVESTIGATORS AT THE UNIVERSITY OF HOUSTON ARE GOVERNED BY REQUIREMENTS OF THE UNIVERSITY AND THE FEDERAL GOVERNMENT.
8. **All information that is obtained in connection with this project and that can be identified with me will remain confidential as far as possible within legal limits. Information gained from this study that can be identified with me may be released to no one other than Dr. Richard Simpson (the principal investigator). The results may be published in scientific journals, professional publications, or educational presentations without identifying me by name.**
9. **If you have an injury or emergency while you are in the lab as a subject in this study (i.e., bruise, muscle pain, dizziness, etc), you should inform a member of the study staff, who will provide you basic first aid care. This injury will be reported to the Human Subjects Committee. If an injury or emergency arises that is unrelated to this study (i.e., personal injury while not in the lab, injury at home or work, etc.), you should dial 911.**

CONFIDENTIALITY

Every effort will be made to maintain the confidentiality of your participation in this project. Each subject's name will be paired with a code number by the principal investigator. This code number will appear on all written materials. The list pairing the subject's name to the assigned code number will be kept separate from all research materials and will be available only to the principal investigator. The code list (linking subject names and ID numbers) will be destroyed once the project is complete and your self-report data has been verified (we anticipate that this will be within 1-month after your last visit to the laboratory). Confidentiality will be maintained within legal limits.

RISKS/DISCOMFORTS

1. **Blood Collection.** **The risks associated with blood sampling are similar to those experienced when donating blood. These include lightheadness, nausea, swelling, edema, and/or bruising. Collection of the blood sample while you are in a seated position by a qualified technician will minimize these risks.**
2. **Exercise Tests.** The exercise tests in this study should not place you under any significant cardiovascular risk; however, there is a remote chance of a serious cardiovascular event (e.g., heart attack, <0.01% in healthy individuals). Other possible risks include those normally associated with exercise: feeling of fatigue, shortness of breath, lightheadedness, sweating, and post-exercise muscle soreness. Persons trained in CPR, First Aid, and emergency response will be present during all testing. Proper warm-up and cool-down will be allowed prior to and after the test will minimize the risks/discomforts associated with the exercise test. If you feel uncomfortable for any reason at all during the study, a researcher will be on hand to assist you. If we cannot find an immediate solution to your discomfort, we will abort the exercise test.
3. In the event of injury, we will only provide immediate first aid. This general first aid would include band-aids for a cut, water, ice packs for muscle soreness, and CPR. If it is necessary for you to seek other medical advice (for example, your medical doctor or hospital services), you are responsible for paying for those services, not the university.

ALTERNATIVES

Participation in this study is voluntary and the only alternative is non-participation.

PUBLICATION STATEMENT

The results of this study may be published in professional and/or scientific journals. The results may also be used for educational purposes or for professional presentations. Data will be presented as group means and standard deviations, without mention of your name and personal information.

I HAVE READ (OR HAVE HAD READ TO ME) THE CONTENTS OF THIS CONSENT FORM AND HAVE BEEN ENCOURAGED TO ASK QUESTIONS. I HAVE RECEIVED ANSWERS TO MY QUESTIONS. I GIVE MY CONSENT TO PARTICIPATE IN THIS STUDY. I HAVE RECEIVED (OR WILL RECEIVE) A COPY OF THIS FORM FOR MY RECORDS AND FUTURE REFERENCE.

Study Subject (print name): _____

Signature of Study Subject: _____

Date: _____

I HAVE READ THIS FORM TO THE SUBJECT AND/OR THE SUBJECT HAS READ THIS FORM. AN EXPLANATION OF THE RESEARCH WAS GIVEN AND QUESTIONS FROM THE SUBJECT WERE SOLICITED AND ANSWERED TO THE SUBJECT'S SATISFACTION. IN MY JUDGMENT, THE SUBJECT HAS DEMONSTRATED COMPREHENSION OF THE INFORMATION.

Principal Investigator (print name and title): _____

Signature of Principal Investigator: _____

Date: _____

APPENDIX 2

PHYSICAL ACTIVITY RATING QUESTIONNAIRE

(PA-R)



48286

USE BLACK INK ONLY. DO NOT USE PENCIL.**3**TGR **TRAINING INTERVENTIONS AND GENETICS OF EXERCISE RESPONSE
PHYSICAL ACTIVITY HISTORY**Measurement: (1)-Baseline (2)-15 weeks (3)-30 weeksToday's date: / /
M M D D Y Y Y Y

Read the 'Activity Type' options from the first column in the table below, and from 'none', 'minimal', 'moderate' or 'vigorous', choose the group most applicable to you. From within that group, select the category from the 'Hours Spent in Activity' column that best describes your GENERAL LEVEL OF ACTIVITY WITHIN THE LAST MONTH, then circle the corresponding number (from 0 to 7) listed below in the 'Activity Rating' column.

Please circle the activity rating that best describes you

Activity Type	Hours Spent in Activity	Activity Rating
NONE - avoid walking or exertion (e.g., always use elevator, drive wherever possible instead of walking)	NONE	0
MINIMAL - walk for pleasure, routinely use stairs, occasionally exercise sufficiently to cause heavy breathing or perspiration	Infrequent activity	1
MODERATE - participate regularly in recreation or work requiring modest physical activity, such as golf, horseback riding, calisthenics, gymnastics, table tennis, bowling, weight lifting, yard work	10-60 minutes per week	2
	Over one hour per week	3
VIGOROUS - Participate regularly in heavy physical exercise such as running or jogging, swimming, cycling, rowing, skipping rope, running in place, or engaging in vigorous aerobic activity type exercise such as tennis, basketball, or handball	Run less than 1 mile per week or spend less than 30 minutes per week in comparable physical activity	4
	Run 1 to 5 miles per week or spend 30 to 60 minutes per week in comparable physical activity	5
	Run 5 to 10 miles per week or spend 1 to 3 hours per week in comparable physical activity	6
	Run over 10 miles per week or spend over 3 hours per week in comparable physical activity	7

07/21/2006

APPENDIX 3

**BIOTIN-CONJUGATED MONOCLONAL ANTIBODIES
COMPRISED IN THE T-CELL ENRICHMENT
COCKTAILS**

Enrichment kit	Monoclonal Antibodies	Clone
CD3 T-cells	Anti-human CD11b/Mac-1	ICRF44
	Anti-human CD16	3G8
	Anti-human CD19	H1B19
	Anti-human CD36	CB38 (NL07)
	Anti-human CD41a	HIP8
	Anti-human CD56	B159
	Anti-human CD235a	GA-R2 (HIR2)
CD4 T-cells	Anti- human CD8	SK1
	Anti- human CD11b/Mac-1	ICRF44
	Anti- human CD16	3G8
	Anti- human CD19	H1B19
	Anti- human CD36	CB38 (NL07)
	Anti- human CD56	B159
	Anti- human CD123	9F5
	Anti- human CD235a	GA-R2 (HIR2)
	Anti- human $\gamma\delta$ TCR	B1
CD8 T-cells	Anti-human CD4	L200
	Anti-human CD11b/Mac-1	ICRF44
	Anti-human CD16	3G8
	Anti-human CD19	H1B19
	Anti-human CD36	CB38 (NL07)
	Anti-human CD41a	HIP8
	Anti- human CD56	B159
	Anti- human CD123	9F5
	Anti- human CD235a	GA-R2 (HIR2)
	Anti- human $\gamma\delta$ TCR	B1

APPENDIX 4

**PUBLISHED PAPERS PRODUCED FROM THE STUDIES
PRESENTED WITHIN THIS THESIS**



Aerobic fitness is associated with lower proportions of senescent blood T-cells in man [☆]

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ABSTRACT

Senescent T-cells accumulate with age, lowering the naïve T-cell repertoire and increasing host infection risk. As this response is likely to be influenced by certain lifestyle factors, we examined the association between aerobic fitness ($\dot{V}O_{2max}$) and the age-related accumulation of senescent T-cells. Blood lymphocytes from 102 healthy males (18–61 yr) were analyzed for KLRG1, CD57, CD28, CD45RA, CD45RO surface expression on CD4⁺ and CD8⁺ T-cells by 4-color flow cytometry. Advancing age (yr) was positively associated with the proportion (%) of senescent (KLRG1⁺/CD57⁺; KLRG1⁺/CD28⁻) CD4⁺ ($B = 1.00$; 1.02) and CD8⁺ ($B = 0.429$; 1.02) T-cells and inversely associated with naïve (KLRG1⁻/CD28⁺) CD4⁺ ($B = -1.000$) and CD8⁺ ($B = -0.993$) T-cells. $\dot{V}O_{2max}$ was inversely associated with senescent CD4⁺ ($B = -0.97$) and CD8⁺ ($B = -0.240$). Strikingly, age was no longer associated with the proportions of senescent or naïve T-cells after adjusting for $\dot{V}O_{2max}$, while the association between $\dot{V}O_{2max}$ and these T-cell subsets withstood adjustment for age, BMI and percentage body fat. Ranking participants by age-adjusted $\dot{V}O_{2max}$ revealed that the highest tertile had 17% more naïve CD8⁺ T-cells and 57% and 37% less senescent CD4⁺ and CD8⁺ T-cells, respectively, compared to the lowest tertile. $\dot{V}O_{2max}$ was not associated with latent cytomegalovirus (CMV), Epstein-Barr virus (EBV) or herpes simplex virus-1 (HSV-1) infection, indicating that the moderating associations of $\dot{V}O_{2max}$ were not confounded by persistent viral infections. This is the first study to show that aerobic fitness is associated with a lower age-related accumulation of senescent T-cells, highlighting the beneficial effects of maintaining a physically active lifestyle on the aging immune system.

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1. Introduction

Human aging is associated with a progressive decline in the function of the immune system, which is commonly referred to as immunosenescence. This is characterized by poor vaccine efficacy, increased incidence of opportunistic infections, and high morbidity and mortality among the elderly (Koch et al., 2007). Immunosenescence is thought to be a consequence of an accumulation of multiple exposures to external pathogens and persistent viral infections throughout the lifespan, although certain lifestyle factors (i.e., smoking, inactivity and socio-economic status) have also been linked with biomarkers of immunosenescence such as leukocyte telomere shortening (Valdes et al., 2005; Ludlow et al., 2008). The adaptive arm of the immune system (i.e., T-cells, B-cells

and their products) appears to diminish most with increasing age (Pawelec, 2006; Pawelec et al., 2006), and age-related changes within the T-cell compartment (i.e., inverted CD4/CD8 ratio, low proliferative responses, memory cell inflation and low IL-2 synthesis) are hallmark features of the immune risk profile (IRP) – an amalgam of immune biomarkers that have been used to predict morbidity and mortality in seniors (Pawelec and Gouttefangeas, 2006).

T-cell clonal expansion in response to an antigenic stimulus is a fundamental process of adaptive immunity, allowing for the formation of antigen specific effector T-cells to combat invading pathogens. However, this clonal expansion is not unending, and after repeated and excessive rounds of cell division, T-cells undergo cell cycle arrest and become senescent (Spaulding et al., 1999). In this state, T-cells will no longer clonally expand upon further antigenic stimulation, but will still retain effector cell properties (i.e., recognizing and killing virally infected cells) and are still capable of producing large amounts of pro-inflammatory cytokines, such as TNF- α , IFN- γ and TNF- γ (Effros et al., 2003; Vescovini

[☆] Please see Brief Commentary by Suzi Hong found on page 1519 of this issue.

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et al., 2007). Senescent T-cells are reported to express a number of signature cell-surface proteins, such as the killer cell lectin-like receptor G1 (KLRG1) and/or CD57 (Voehringer et al., 2002; Brenchley et al., 2003) while also lacking surface expression of the co-stimulatory molecule CD28 (Brzezinska et al., 2004). However, whether or not these cells are truly senescent (i.e., in a state of permanent growth arrest) is a subject of debate, as KLRG1 blockade has been shown to restore the proliferative capabilities of KLRG1+/CD28- T-cells (Henson et al., 2009), suggesting that these may be “exhausted” cells that are still capable of proliferation under certain circumstances (Akbar and Henson, 2011). Memory T-cells that have not undergone terminal differentiation may also express KLRG1 but retain expression of CD28 and do not express CD57 (Ibegbu et al., 2005), and is a phenotype that may overlap both central memory and early effector-memory T-cells (Koch et al., 2008). With advancing age, there is a progressive reduction in the number and proportions of fully functional naïve T-cells and a concomitant increase in memory, effector-memory and senescent T-cells within the periphery. This occurs due to the age-associated atrophy of the thymus and a consequential reduced output of naïve T-cells, coupled with the homeostatic proliferation and differentiation of memory T-cells due to life-long exposure to various pathogens and persistent viral infections (Simpson, 2011). In this instance, the accumulated senescent T-cells, which have limited antigen specificity, occupy the “immune space” at the expense of naïve cells leading to a severely restricted T-cell repertoire. This, in turn, manifests as increased prevalence of infectious disease due to an impaired ability to recognize and respond to newly evolving pathogens (i.e., influenza, rhinovirus and respiratory syncytial virus) (Saurwein-Teissl et al., 2002).

There has been recent interest in the manipulation of certain lifestyle factors that could be used as effective countermeasures against immunosenescence within the aging population. In particular, the beneficial effects of regular exercise on immunity have been well-documented in older adults, although the mechanisms that underpin enhanced immunity with regular exercise are not well understood (Shinkai et al., 1995; Simpson and Guy, 2010). Nevertheless, many biomarkers associated with the IRP are positively displayed in physically active elderly compared to their sedentary counterparts (Simpson and Guy, 2010), which include longer leukocyte telomere lengths (Ludlow et al., 2008), enhanced *in vitro* T-cell responses to mitogens (Ferguson et al., 1995), elevated *in vivo* immune responses to vaccines and recall antigens (Targonski et al., 2007), and increased IL-2 synthesis and expression of the IL2 receptor (Flurkey et al., 1992; Ferguson et al., 1995).

The maximal oxygen uptake ($\dot{V}O_{2max}$) is a “gold standard” measure of aerobic capacity that is known to decline with age and is associated with risk of morbidity, mortality and quality of life (Jackson et al., 2009). Long-term follow-up studies have shown that individuals with a lower $\dot{V}O_{2max}$ are more likely to develop hypertension, diabetes and metabolic syndrome as well as higher mortality rates due to cardiovascular disease and cancer (Blair et al., 1996; Lynch et al., 1996; Evenson et al., 2003; Barlow

et al., 2006; Kodama et al., 2009). It is not known, however, if $\dot{V}O_{2max}$ is associated with the aging-related accumulation of memory and senescent T-cells, which are signature properties of an aging immune system.

The aim of this study was to examine the association of aerobic fitness, indicated by estimated $\dot{V}O_{2max}$, and the age-related increase of memory and senescent T-cells in a population of healthy adult men. It was hypothesized that $\dot{V}O_{2max}$ would be associated with a moderation of the increased proportions of memory and senescent T-cells that are seen with advancing age.

2. Experimental procedures

2.1. Participants

Participants were 102 healthy non-smoker males of various ethnicities (mean \pm SD: age = 39 \pm 6; BMI = 25.5 \pm 1.8) (Table 1). All subjects were recruited from the higher education-based community at Edinburgh Napier University, Scotland UK ($n=66$; mean \pm SD: age: 40 \pm 5.5; BMI: 25.0 \pm 1.7) or the University of Houston, Houston, TX, USA ($n=36$; mean \pm SD: age: 37 \pm 6.5; BMI: 26.5 \pm 1.8) and consisted of either students or academic members of staff. The percentage age distribution of the participants was: 18–31 yr: 29%; 32–41 yr: 20%; 42–51 yr: 31%; 52–61 yr: 20%. All subjects completed a health and lifestyle questionnaire prior to participating in this study. Subjects with excessive alcohol intake (>14 drinks/week), taking medication affecting the immune system, routinely using ibuprofen and/or aspirin, anti-depressants, medications designed to alter blood pressure or cardiovascular function and hormone replacement therapy were excluded from the subject pool. Subjects reporting major affective disorders, HIV infection, hepatitis, chronic/debilitating arthritis, central or peripheral nervous disorders, previous stroke or cardiac events, were bedridden in the past three months, suffer from known cardiovascular disease or autoimmune diseases were all excluded from the study. All volunteers gave written informed consent and ethical approval was obtained from the School of Life Sciences, Edinburgh Napier University and the Committee for Protection of Human Services at the University of Houston.

2.2. Procedures

All subjects made a single visit to the laboratory between 08:00 and 10:00 local time to provide a resting blood sample, complete a submaximal exercise test and a questionnaire to estimate current levels of physical activity (PA-R). Bioelectrical impedance analysis was also used to measure percentage body fat using a single-frequency (50 kHz) electric current produced by a tetrapolar body-stat 1500 (Bodystat, Douglas, UK) as described by Ghosh et al. (1997). Before their visit, subjects were instructed to refrain from any form of exercise considered as “vigorous” (Jackson et al., 1990) for 24 h prior to their arrival at the laboratory. Following a 5-min period of seated rest, 12 mL of blood was drawn from an antecubital vein in 6 mL collection tubes (BD vacutainer™, Franklin Lakes, NJ, USA) containing a serum gel or spray-coated with lithium heparin to prevent coagulation. Serum samples were removed from whole blood and stored at -80 °C until analysis for cytomegalovirus (CMV), Epstein-Barr (EBV) and herpes simplex virus-1 (HSV-1) IgG antibodies, which was performed on the Houston cohort only ($n=36$), by ELISA using a SpectromaxM2 plate reader (Molecular Devices, CA, USA). The procedures and serostatus (i.e., positive or negative) for each latent virus were determined in accordance with the manufacturer’s instructions (GenWay Biotech, CA, USA). Blood samples treated with the anticoagulant were

Table 1
Physical characteristics of the participants ($n=102$).

	Mean	Standard deviation	Range
Age	39	± 6	18–61
Body mass (kg)	81	± 6	53–110
BMI	25.5	± 1.8	20.1–35.1
% Body fat	19.9	± 3.2	6.6–38
Physical activity rating (PA-R)	5	± 2	1–7
$\dot{V}O_{2max}$ (mL.kg ⁻¹ .min ⁻¹)	41.7	± 3.7	27.8–58.5
Waist (cm)	87	± 5.8	64–117
Hips (cm)	92.8	± 4.6	77–111
Waist/hips ratio	0.94	± 0.04	0.80–1.09

immediately processed for mononuclear cell separation, direct immunofluorescence assays and flow cytometry analysis.

2.3. Estimation of maximal oxygen uptake ($\dot{V}O_{2max}$) and physical activity rating (PA-R)

The $\dot{V}O_{2max}$ of each subject was estimated using a submaximal cycling exercise protocol (Astrand, 1960). Subjects completed four 3-min heart-rate adjusted incremental stages on Trek road bike that was mounted to an indoor cycling ergometer (Computrainer, RacerMate, Seattle, WA). The subjects' heart rate (HR) was measured continuously during the exercise protocols by short-range telemetry (Suunto T6, Vantaa, Finland) and the average HR during the last minute of each stage was used to determine the age-adjusted resistance to apply in the following stage. The test was terminated when the desired heart rate was attained. The $\dot{V}O_{2max}$ of each subject was estimated from the test using the equations provided by Adams and Beam (1998). Prior to exercise, each participant also provided his physical activity score using a questionnaire, which assigns numerical value (physical activity rating; PA-R) for infrequent (0–1), moderate (2–3) and vigorous (4–7) exercisers (Jackson et al., 1990). The $\dot{V}O_{2max}$ was also estimated from the questionnaire (Jackson et al., 1990). The relationship between $\dot{V}O_{2max}$ estimated from the non-exercise questionnaire and the $\dot{V}O_{2max}$ estimated from the submaximal cycling test was $r = 0.88$ ($p < 0.001$).

2.4. Peripheral blood mononuclear cell separation

Intravenous blood samples were collected from an antecubital vein in 10 mL vacutainers[®] blood collection tubes (BD vacutainer[™], Franklin Lakes, NJ, USA) spray-coated with lithium heparin, using 3/4 in. BD Vacutainer Safety-Lok[™] (BD vacutainer[™], Franklin Lakes, NJ, USA) and into a 10 mL serum-separating tube SST[™] (BD vacutainer[™], Franklin Lakes, NJ, USA) coated with clotting agents and separated serum was stored frozen at -80°C . Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Lymphoprep[®], Axis-Shield, Dundee, UK, or Histopaque[®], Sigma-Aldrich, St Louis, MO, USA). Whole blood was diluted with an equal volume of 0.9% NaCl (Baxter[™], Deerfield, IL, USA), and 6 mL of the diluted blood was layered over 3 mL of density-gradient media. Samples were then centrifuged for 30 min at 800g. Following centrifugation, the distinct band formed by the PBMCs were carefully removed and washed twice in 0.9% NaCl for 10 min at 250g, then with phosphate buffered saline+1% bovine serum albumin (BSA) + 0.02% sodium azide.

2.5. Labeling of cell surface antigens

Aliquots of 1×10^6 isolated PBMCs were labeled with an APC-conjugated anti-CD3 (IgG2a, Clone MEM-57), an Alexa488-conjugated anti-KLRG1 (clones 13A2 and 13F12F2) (Voehringer et al., 2002; Marcolino et al., 2004) or an FITC-conjugated anti-CD45RA (IgG1, Clone F8-11-13), a PEcy-5-conjugated anti-CD4 (IgG1, Clone MEM-241) or anti-CD8 (IgG2a, Clone MEM-31), and either an anti-CD28 (IgG1 κ , Clone CD28.2), anti-CD57 (IgM, Clone NK-1) or anti-CD45RO (IgG2a κ , Clone UCHL1) PE conjugated monoclonal antibody (mAb) in a four-color direct immunofluorescence procedure. Cells were incubated with 100 μL of each pre-diluted mAb for 45 min at room temperature. The anti-CD45RO and the anti-CD28 mAb were purchased from BD Pharmingen (San Jose, CA, USA). The anti-CD45RA, anti-CD3, anti-CD4 and anti CD8 mAbs were purchased from Immunotools or Ebioscience (Immunotools, Friesoythe, Germany; Ebioscience, San Diego, CA, USA), and the anti-CD57 from Abcam (Cambridge, UK). Cells were also labeled with each mAb in a one-color immunofluorescence procedure to

account for spectral overlap and adjust compensation settings during flow cytometry analysis. Appropriately conjugated isotype controls were also used in each assay to account for background binding of Ig. The phenotypic identification of the T-cell subsets used in this study are shown in Table 2.

2.6. Flow cytometry

PBMC phenotypes were assessed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA USA) at Edinburgh Napier University equipped with a 15-mW argon ion laser emitting light at a fixed wavelength of 488 nm and a red diode laser emitting at a fixed wavelength of 635 nm; or on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) at the University of Houston equipped with a blue laser emitting light at a fixed wavelength of 488 nm and a red laser emitting light at a fixed wavelength of 640 nm. The cells were identified and electronically gated using the forward and side light-scatter mode using CELLQuest Pro (BD Biosciences, San Jose, CA, USA) or Accuri C6 (CFlow[®] v1) software. Side scatter against APC fluorescence was then used to identify and gate the CD3+ cells. The CD4+ or CD8+ populations (both mAb conjugated to PerCP Cy-5.5) were then identified in the CD3+ cell population. For each sample, 100,000 CD3+/CD4+ or CD3+/CD8+ events were collected for analysis. The expression of KLRG1-Alexa488/CD28-PE, KLRG1-Alexa488/CD57-PE, and CD45RA-FITC/CD45RO-PE were then assessed on the CD4+ and CD8+ T-cells by four color flow cytometry. Spectral overlap was corrected by electronic color compensation using the one-color samples prior to each four-color assay. Fluorescent signals were collected in logarithmic mode (4 or 6 decade logarithmic amplifier depending on the flow cytometer) and cell numbers per channel in linear mode. Following acquisition, FCS files were transferred to a third party software program (FCS Express v3.0, De Novo, Los Angeles, CA, USA) for analysis. The percentage of all CD3+/CD4+ or CD3+/CD8+ T-cell subsets expressing the markers of interest were tabulated for statistical analysis.

2.7. Statistical analysis¹

All data were assessed for assumptions of normality and constant error variance prior to formal statistical testing. Skewed cell-surface phenotypic data was then normalized by logarithmic transformation prior to statistical analysis. Initial analysis was performed by linear regression to examine the association between age and the proportion of T-cell subsets expressing a particular cell surface phenotype in accordance with Table 2. When significant associations were found, additional variables (i.e., age, $\dot{V}O_{2max}$, BMI and % body fat) were systematically added to subsequent blocks of the hierarchical multiple regression models to evaluate their impact on the proportion of T-cell subsets (Table 3). In all models, standardized regression coefficients (B) and R^2 values are reported as a measure of association (Table 4). Linear regression models were created by including the centered independent variables as suggested by Aiken and West (1991) to minimize multicollinearity. No significant interactions were found between the independent variables tested so interaction effects were excluded from the final models. Testing site (i.e., Edinburgh or Houston) was added to the model as a covariate but had no effect. A one-way ANOVA was also used to detect differences among age-adjusted $\dot{V}O_{2max}$ classifications (Whaley et al., 2006). When significant group effects were found, student *t*-tests with Bonferroni

¹ Testing site (Edinburgh or Houston) was added as a covariate in the regression analysis but was found to have no effect for any of the measured variables ($p > 0.05$). We also performed independent sample *t*-tests between all measures collected in Edinburgh and Houston but found no significant differences ($p > 0.05$).

Table 2
Phenotypic identification of blood T-cell subsets.

Surface phenotype	T-cell subset	Reference
KLRG1 ⁻ /CD28 ⁺ ^a	Naïve T-cells	Koch et al. (2008)
KLRG1 ⁺ /CD28 ⁻ ^b KLRG1 ⁺ /CD57 ⁺ ^b	Senescent T-cells	Ibegbu et al. (2005) and Voehringer et al. (2002)
KLRG1 ⁺ /CD28 ⁺ ^c	Memory T-cells	Ibegbu et al. (2005) and Koch et al. (2008)
CD45RA ⁺ / CD45RO ⁺ ^d	Transitional T-cells	Sandens et al. (1988) and Akbar et al. (1988)

^a Approximately 3–7% of all CD8⁺ T-cells displaying this phenotype may be central-memory T-cells (Koch et al., 2008).

^b The term “senescent” has been used to describe cells with this phenotype, however, some KLRG1⁺/CD28⁻ cells may be in a state of “exhaustion” and their proliferative capabilities may be restored under certain circumstances (Akbar and Henson, 2011). The relationship between the proportions of KLRG1⁺/CD28⁻ and KLRG1⁺/CD57⁺ was $r = 0.762$ ($p < 0.001$) and $r = 0.814$ ($p < 0.001$) for CD4⁺ and CD8⁺ T-cells, respectively.

^c The variation in CCR7 expression (28–57%) among KLRG1⁺/CD28⁺ cells (Ibegbu et al., 2005) indicates that this phenotype may overlap both central memory and effector memory T-cells.

^d Identifies naïve T-cells transitioning to a memory phenotype, or an effector memory T-cell transitioning to a terminal phenotype.

correction were performed to detect location of significance. Independent *t*-tests were used to compare physical characteristics and T-cell phenotypes due to latent CMV, EBV or HSV-1 serostatus. All values are presented as the mean ± standard error of the mean (SEM) and all statistical analyses were performed using “Statistical Package for the Social Sciences” (SPSS v17.0, Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. The effects of age and $\dot{V}O_{2max}$ on T-cell phenotypes

The impact of age or $\dot{V}O_{2max}$ on the T-cell subsets were initially determined by univariate regression analysis (Table 3). As expected, a positive relationship was found between age and the proportion of senescent (KLRG1⁺/CD57⁺) CD4⁺ and CD8⁺ T-cells ($r = 0.264$ and 0.363 , respectively; $p < 0.05$). The proportion of senescent CD4⁺ and CD8⁺ T-cells defined as KLRG1⁺/CD28⁻ were also positively associated with age ($r = 0.182$ and 0.377 , respectively; $p < 0.05$ and $p < 0.001$) (Table 3). Similarly, a positive relationship between age and the proportion of CD4⁺ ($r = 0.436$; $p < 0.01$) and CD8⁺ ($r = 0.411$; $p < 0.01$) T-cells with a transitional (CD45RA⁺/CD45RO⁺) phenotype were observed (Table 3). Similar positive relationships were seen between $\dot{V}O_{2max}$ and the frequencies of naïve (KLRG1⁻/CD28⁺) CD4⁺ and CD8⁺ T-cells ($r = 0.171$ and 0.224 , respectively; $p < 0.05$), while $\dot{V}O_{2max}$ was inversely associated with senescent (KLRG1⁺/CD57⁺) and transitional (CD45RA⁺/CD45RO⁺) CD4⁺ ($r = -0.340$ and -0.403 , respectively; $p < 0.01$) and CD8⁺ T-cells ($r = -0.287$ and -0.336 , respectively; $p < 0.01$). Only transitional T-cells were positively associated with BMI ($r = 0.255$ and 0.215 for CD4⁺ and CD8⁺ T-cells, respectively; $p < 0.05$). The associations with T-cell phenotype and BMI were mirrored when percentage body fat was used instead of BMI, but for clarity only the BMI data is shown.

3.2. The impact of $\dot{V}O_{2max}$ on age-associated changes in T-cell phenotype

T-cell phenotypes found to be associated with age and/or $\dot{V}O_{2max}$ were examined by hierarchical multiple regression analysis to determine if these associations were modified when adjusted for

the other (Table 4). The potential confounders BMI, percentage body fat or hip/waist ratio were also included in these models, although for clarity only the BMI results are shown. Age was no longer associated with the proportions of senescent CD4⁺ (KLRG1⁺/CD57⁺) or CD8⁺ (KLRG1⁺/CD28⁻) T-cells when adjusted for $\dot{V}O_{2max}$ ($p > 0.05$). Similarly, proportions of naïve (KLRG1⁻/CD28⁺) CD4⁺ or CD8⁺ T-cells were not associated with age after adjusting for $\dot{V}O_{2max}$ ($p > 0.05$). A moderating association effect of $\dot{V}O_{2max}$ was also found for pan-effector memory (defined as KLRG1⁺)² CD4⁺ and CD8⁺ T-cells ($B = -0.569$ and -1.186 , respectively), although age was still significantly associated ($B = 0.542$; $p < 0.01$). The association between $\dot{V}O_{2max}$ and senescent CD4⁺ (KLRG1⁺/CD57⁺) and CD8⁺ (KLRG1⁺/CD28⁻) T-cells withstood adjustment for age (both $B = -0.953$; $p < 0.05$). Moreover, the association between $\dot{V}O_{2max}$ and naïve (KLRG1⁻/CD28⁺) CD8⁺ T-cells ($B = 1.042$; $p < 0.01$) but not CD4⁺ T-cells ($p > 0.05$) also withstood age adjustment. Similar results, albeit with smaller effect sizes, were observed when self-reported physical activity ratings were used in the models instead of $\dot{V}O_{2max}$ (data not shown). All moderating effects $\dot{V}O_{2max}$ presented in Table 4 withstood adjustments for BMI and percentage body fat ($p < 0.05$). The associations between BMI and transitional (CD45RA⁺/CD45RO⁺) CD4⁺ and CD8⁺ T-cells were eliminated when adjusted for age and $\dot{V}O_{2max}$ ($p > 0.05$). Effects just outside the criterion for statistical significance (i.e., $p = 0.05$ – 0.10) for BMI were found for senescent (KLRG1⁺/CD57⁺) CD8⁺ T-cells ($p = 0.07$) and memory (KLRG1⁺/CD28⁺) CD4⁺ T-cells ($p = 0.05$) after adjusting for age and $\dot{V}O_{2max}$.

3.3. T-cell phenotypes and age-related $\dot{V}O_{2max}$ classifications

As age and $\dot{V}O_{2max}$ were co-related ($r = -0.563$; $p < 0.001$), we investigated the impact of age-related $\dot{V}O_{2max}$ classifications on the phenotype characteristics of CD4⁺ and CD8⁺ T-cells. All participants were divided into three groups using the age-adjusted $\dot{V}O_{2max}$ classifications provided by the American College of Sports Medicine (ACSM) (Whaley et al., 2006). Participants with an age-adjusted $\dot{V}O_{2max}$ ranked from the 10th to the 39th percentiles were classified as “below average”, whereas participants with an age-adjusted $\dot{V}O_{2max}$ ranked from the 40th to the 69th and 70th to the 99th percentiles were classified as “average” and “above average”, respectively (Fig. 1). The physical characteristics of the participants divided into these groups are presented in Table 5. Participants among the $\dot{V}O_{2max}$ groups did not differ in terms of age ($p < 0.05$), although individuals in the below average group had a significantly greater BMI and % body fat ($p < 0.05$) compared to the other subject groups. However, it was already established by linear regression that $\dot{V}O_{2max}$ was associated with specific T-cell phenotypes even after BMI and % body fat adjustment (Table 4) and were therefore not considered to be confounding factors.

T-cell phenotype comparisons among the three $\dot{V}O_{2max}$ tertiles are presented in Fig. 1. The above average $\dot{V}O_{2max}$ tertile had 21.1% and 22.6% less pan-effector-memory (KLRG1⁺) CD8⁺ T-cells compared to the below average and average tertiles, respectively ($p < 0.05$) (Fig. 1). The proportion of senescent (KLRG1⁺/CD57⁺) CD4⁺ T-cells in the above average tertile was 56.3% and 57.8% lower than the average and below average tertiles, respectively ($p < 0.05$). The above average tertile had 37% less senescent (KLRG1⁺/CD57⁺) CD8⁺ T-cells compared to the average tertile ($p < 0.05$), but no differences were found between the below average and above average tertiles ($p > 0.05$). The above average tertile had a 16.4% and 17.5% higher proportion of naïve (KLRG1⁻/CD28⁺) CD8⁺ T-cells compared to the average and below average tertiles,

² Although we have identified KLRG1⁺ cells as pan effector-memory cells, a small proportion of CD8⁺ T-cells (<3%) may also be KLRG1⁺ central memory cells (Koch et al., 2008).

Table 3
Univariate regression analysis of age, $\dot{V}O_{2max}$ and BMI on T-cell subsets.

T-cell subsets	Pearson's R (p value)			F statistic (p value)		
	Age	$\dot{V}O_{2max}$	BMI	Age	$\dot{V}O_{2max}$	BMI
CD4+ T-cells						
KLRG1+/CD57+ (senescent)	0.264 (0.004)	-0.340 (0.000)	0.028 (0.388)	7.502 (0.007)	13.035 (0.000)	0.081 (0.776)
KLRG1+/CD28- (senescent)	0.182 (0.034)	-0.221 (0.013)	0.073 (0.235)	0.745 (0.39)	5.091 (0.026)	0.528 (0.469)
KLRG1+/CD28+ (memory)	0.052 (0.304)	-0.023 (0.410)	-0.102 (0.154)	0.264 (0.609)	0.052 (0.820)	1.046 (0.309)
KLRG1-/CD28+ (naïve)	-0.275 (0.003)	0.171 (0.044)	0.073 (0.235)	8.120 (0.005)	2.977 (0.088)	0.538 (0.469)
CD45RA+/CD45RO+ (transitional)	0.436 (0.000)	-0.403 (0.000)	0.255 (0.011)	18.267 (0.000)	15.086 (0.000)	5.414 (0.023)
CD8+ T-cells						
KLRG1+/CD57+ (senescent)	0.363 (0.000)	-0.287 (0.002)	-0.043 (0.334)	15.139 (0.000)	8.961 (0.003)	0.186 (0.667)
KLRG1+/CD28- (senescent)	0.377 (0.000)	-0.350 (0.000)	0.024 (0.405)	12.974 (0.000)	13.852 (0.000)	0.058 (0.810)
KLRG1+/CD28+ (memory)	0.368 (0.000)	-0.137 (0.086)	-0.033 (0.371)	15.517 (0.000)	1.897 (0.172)	0.110 (0.741)
KLRG1-/CD28+ (naïve)	-0.189 (0.029)	0.224 (0.012)	0.024 (0.405)	3.655 (0.059)	5.247 (0.024)	0.058 (0.810)
CD45RA+/CD45RO+ (transitional)	0.411 (0.000)	-0.336 (0.001)	0.215 (0.027)	16.094 (0.000)	10.030 (0.002)	3.823 (0.054)

Table 4
Hierarchical multiple regression statistics for age, $\dot{V}O_{2max}$ and BMI on blood T-cell subsets after adjusting for the other factors.

T-cell subsets	Factors	CD4+ T-cells					CD8+ T-cells				
		R ²	B	SEB	F statistic (p value)	t statistic (p value)	R ²	B	SEB	F statistic (p value)	t statistic (p value)
KLRG1+ (pan effector-memory)	Age	0.129	-0.071	0.075	3.599 (0.009)	-0.946 (0.347)	0.359	0.542	0.173	13.609 (0.000)	3.139 (0.002)
	$\dot{V}O_{2max}$		-0.059	0.207		-2.747 (0.007)*		-1.186	0.480		-2.473 (0.015)*
	BMI		-0.477	0.305		-1.566 (0.121)		-0.701	0.706		-0.994 (0.323)
KLRG1+/CD57+ (senescent)	Age	0.147	1.000	1.007	4.166 (0.004)	0.023 (0.982)	0.179	0.204	0.152	5.282 (0.001)	1.345 (0.182)
	$\dot{V}O_{2max}$		-0.953	1.021		-2.375 (0.019)*		-0.690	0.421		-1.638 (0.105)
	BMI		-0.964	1.030		-1.207 (0.230)		-1.153	0.620		-1.861 (0.066)
KLRG1+/CD28- (senescent)	Age	0.048	0.993	1.012	1.205 (0.314)	-0.734 (0.465)	0.178	1.005	1.007	5.205 (0.098)	0.690 (0.492)
	$\dot{V}O_{2max}$		-0.955	1.030		-1.529 (0.130)		-0.953	1.023		-2.247 (0.027)*
	BMI		-0.971	1.045		-0.701 (0.485)		-0.955	1.033		-1.456 (0.149)
KLRG1-/CD28+ (naïve)	Age	0.077	-0.998	1.002	2.015 (0.098)	-1.834 (0.070)	0.116	-1.002	1.005	3.135 (0.018)	0.576 (0.566)
	$\dot{V}O_{2max}$		1.000	1.005		0.092 (0.927)		1.042	1.016		2.665 (0.009)*
	BMI		1.002	1.007		0.359 (0.720)		1.038	1.023		1.581 (0.117)
CD45RA+/CD45RO+ (transitional)	Age	0.238	0.009	0.003	7.916 (0.000)	2.752 (0.007)	0.200	0.010	0.004	6.420 (0.001)	2.901 (0.005)
	$\dot{V}O_{2max}$		-0.004	0.008		-0.540 (0.591)		0.000	0.008		-0.005 (0.996)
	BMI		0.012	0.013		1.086 (0.281)		0.016	0.013		1.188 (0.239)
KLRG1+/CD28+ (memory)	Age	0.045	0.000	0.057	1.137 (0.344)	-0.008 (0.994)	0.157	0.410	0.452	4.478 (0.002)	3.580 (0.001)
	$\dot{V}O_{2max}$		0.033	0.158		0.208 (0.836)		0.452	0.316		1.427 (0.157)
	BMI		-0.452	0.226		-1.998 (0.050)		-0.192	0.455		-0.422 (0.674)

* Significant relationship between $\dot{V}O_{2max}$ and T-cell phenotype after age and BMI adjustment ($p < 0.05$).

respectively ($p < 0.05$). Consistent with the regression analysis, no differences were found for transitional (CD45RA+/CD45RO+) or memory (KLRG1+/CD28+) T-cells within CD4+ or CD8+ subsets among the $\dot{V}O_{2max}$ classifications ($p > 0.05$). Fitted line plots with individual subject data for proportions of senescent (KLRG1+/CD57+) CD4+ and CD8+ T-cells and naïve (KLRG1-/CD28+) CD4+ and CD8+ T-cells in accordance with age-related $\dot{V}O_{2max}$ classification tertiles are presented in Fig. 2.

3.4. The impact of latent CMV, EBV and HSV-1 infections

As the moderating effects of $\dot{V}O_{2max}$ on age-associated changes in T-cell phenotype may be confounded by latent viral infections, we determined CMV, EBV and HSV-1 serostatus on the Houston subject cohort ($n = 36$). Due to the greater prevalence of these viruses with age, subjects were age-matched in accordance with their serostatus and compared for physical characteristics and T-cell phenotypes. Neither CMV, EBV or HSV-1 serostatus was associated with $\dot{V}O_{2max}$, PA-R, BMI or percentage body fat (data not shown; $p > 0.05$). Latent CMV infection was associated with a greater proportion of senescent (KLRG1+/CD57+; $20.2 \pm 1.9\%$

versus $10.9 \pm 0.8\%$) and lower proportion of naïve (KLRG1-/CD28+; $51 \pm 3.3\%$ versus $61 \pm 2.2\%$) CD8+ T-cells compared to the non-infected. Similarly, CMV infection was associated with a greater proportion of senescent (KLRG1+/CD57+; $3.4 \pm 0.4\%$ versus $1.9 \pm 0.3\%$) CD4+ T-cells. However, the proportions of naïve (KLRG1-/CD28+; $82.5 \pm 1.3\%$ versus $82.8 \pm 1.2\%$) CD4+ T-cells were not associated with CMV serostatus ($p > 0.05$). T-cell phenotypes were not associated with latent EBV or HSV-1 infection (data not shown; $p > 0.05$).

4. Discussion

Increased proportions of memory, effector-memory and senescent T-cells are striking features of an aging immune system and a hallmark of the immune risk profile (IRP). This study examined for the first time the impact of maximal aerobic capacity ($\dot{V}O_{2max}$) as a measure of aerobic fitness on the proportions of naïve, memory, effector-memory and senescent blood T-cell populations that are known to undergo profound age-associated changes. As expected, the proportions of senescent CD4+ and CD8+ T-cells increased with advancing age at a respective rate of

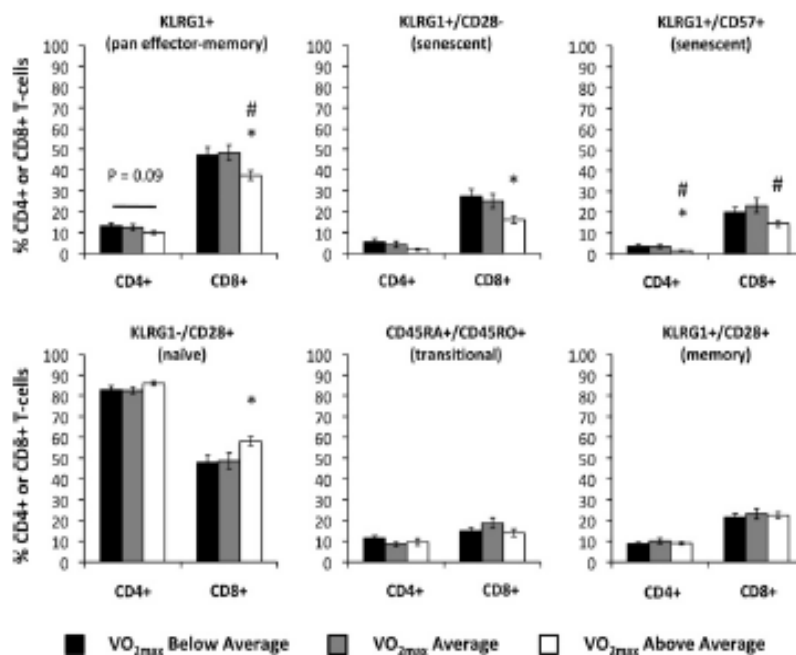


Fig. 1. A comparison of the proportions of pan-effector-memory (KLRG1+), senescent (KLRG1+/CD57+; KLRG1+/CD28-), naïve (KLRG1-/CD28+), transitional (CD45RA+/CD45RO+) and memory (KLRG1+/CD28+) cells within all CD4+ and CD8+ T-cell subtypes among the age-adjusted $\dot{V}O_{2max}$ tertiles (mean \pm SE). *Statistically significant difference from below average ($p < 0.05$); # statistically significant difference from average ($p < 0.05$).

Table 5

Subject's physical characteristics classified according to their age-adjusted $\dot{V}O_{2max}$ (mean \pm SD).

	$\dot{V}O_{2max}$ below average	$\dot{V}O_{2max}$ average	$\dot{V}O_{2max}$ above average
n	36	25	41
Age (yr old)	40.7 \pm 2.3	40.2 \pm 1.9	37.1 \pm 1.9
BMI	28.4 \pm 0.6	25.2 \pm 0.5*	23.2 \pm 0.4*#
% Body fat	25.5 \pm 1	19.4 \pm 0.6*	15.4 \pm 0.7*#
$\dot{V}O_{2max}$	34.4 \pm 0.8	43 \pm 0.6*	47.3 \pm 0.9*#
PA-R	3.6 \pm 0.3	5.5 \pm 0.2*	6.4 \pm 0.2*#

* Statistically significant difference from below average ($p < 0.05$).

Statistically significant difference from average ($p < 0.05$).

10% and 10.2% per decade. This was accompanied by a per decade reduction in the proportions of naïve CD4+ and CD8+ T-cells of 10% and 9.9%, respectively. Supporting our hypothesis, we found that those with above average $\dot{V}O_{2max}$ scores had less senescent CD4+ and CD8+ T-cells and more naïve CD8+ T-cells than those with below average $\dot{V}O_{2max}$ scores, even after adjusting for age, body mass index and percentage body fat. Strikingly, we found that the well-accepted association between age and senescent T-cells no longer existed when age was adjusted for $\dot{V}O_{2max}$, indicating that aerobic fitness may be a stronger determinant of T-cell phenotypic shifts than chronological age. This effect was limited to the senescent cells as $\dot{V}O_{2max}$ was not associated with memory (KLRG1+/CD28+) cells after adjusting for age. This is the first study to show that aerobic fitness is associated with a moderation of the natural age-related accumulation of senescent T-cells in peripheral blood, highlighting the beneficial effects of maintaining a physically active lifestyle on the aging immune system.

Increased proportions of effector-memory and senescent T-cells have been found in blood due to aging (Simpson et al., 2008), psychological adverse working conditions (Bosch et al., 2009) and persistent viral infections (Thimme et al., 2005). Although recent studies indicate that a sedentary lifestyle promotes biological

aging and telomere shortening (Cherkas et al., 2008; Ludlow et al., 2008; LaRocca et al., 2010; Song et al., 2010), this is the first study to our knowledge to report a moderating association of aerobic fitness on the age-related accumulation of senescent T-cells. We found that, on average, a difference in $\dot{V}O_{2max}$ of 10 mL kg⁻¹ min⁻¹, which corresponds to a transition from the below average to the above average $\dot{V}O_{2max}$ category, was associated with a 9.5% difference in the proportion of senescent CD8+ T-cells independently of age. As the average proportion of senescent CD8+ T-cells was found to increase by 10% per decade, this indicates that aerobic fitness could play a major role in shaping the aging T-cell compartment. Indeed, senescent T-cells are known to have critically short telomeres (Olovnikov, 1973; Karlseder et al., 2002) and have been associated with poor vaccine efficacy, impaired immune vigilance and greater morbidity and mortality as a result of infectious disease (Wikby et al., 1998). As such, the present data bolsters the intuition that regular physical activity may exert preventative and/or rejuvenating properties on the aging immune system by dampening the age-related accumulation of senescent T-cells and preventing early transition to the IRP category in later life (Simpson and Guy, 2010).

Aerobic fitness was also associated with a moderation of the proportions of naïve CD8+ T-cells, which were positively associated with $\dot{V}O_{2max}$ scores even after adjusting for age. Remarkably, the associations observed between age and naïve and senescent T-cells did not withstand adjustment for $\dot{V}O_{2max}$, indicating that aging may be secondary to changes in aerobic fitness at shaping T-cell phenotypic shifts. This seems plausible as $\dot{V}O_{2max}$ is also known to decline with age, although not conspicuously so until after 40 yr of age (Jackson et al., 2009). We postulate that maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells that have limited antigenic specificity, whilst also maintaining adequate numbers of naïve T-cells capable of recognizing and responding to novel pathogens. Moreover, because many of the proposed therapeutic interventions (i.e., gene, cytokine, hormone and monoclonal antibody therapy) that have been proposed to

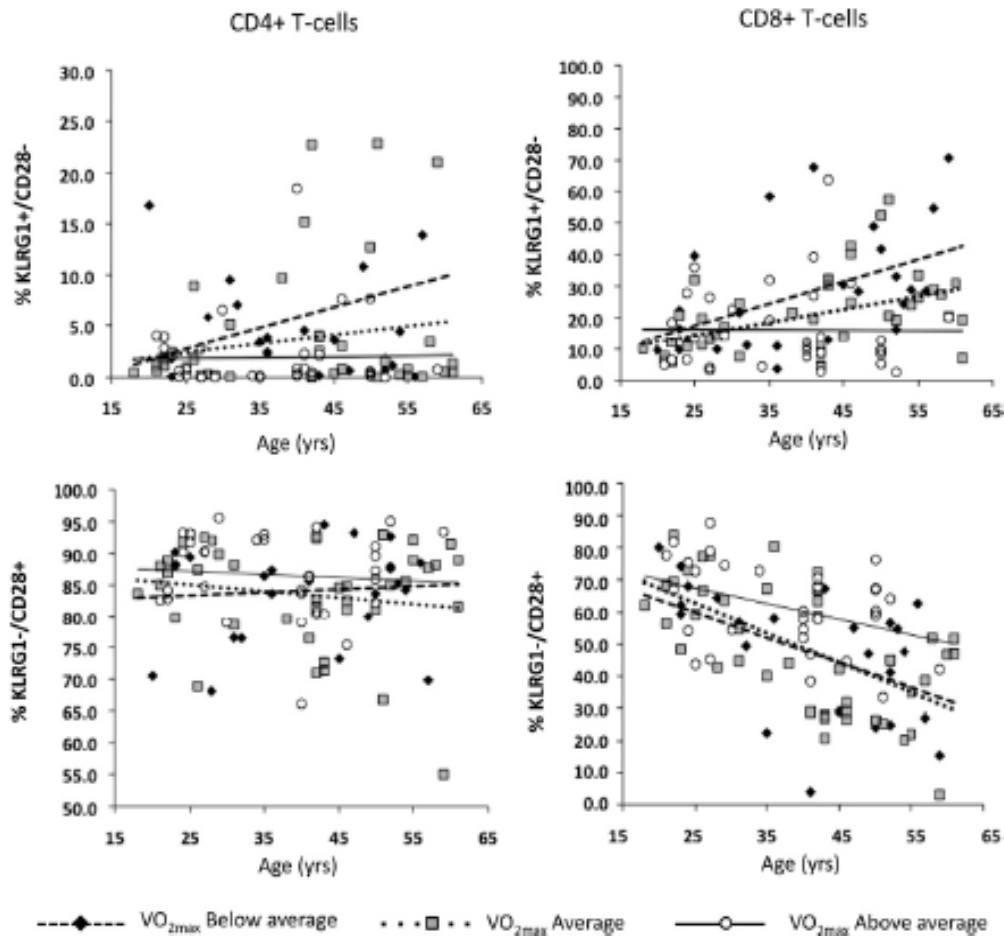


Fig. 2. Fitted line plots with individual subject data showing the changes in the percentage of all CD4+(left column) and CD8+(right column) T-cells expressing surface markers of senescent and naive T-cells as a function of age. Changes with age in accordance with $\dot{V}O_{2max}$ classification are also shown.

negate aging immunity (Greenstein et al., 1987; Kendall et al., 1990; Phillips et al., 2004; Rosenberg et al., 2006) are risky and bear a large number of undesired side effects and ethical complications, physical exercise could be considered a safer alternative strategy to combat age-related immunosenescence. It is acknowledged, however, that the presently reported links between $\dot{V}O_{2max}$ and T-cell phenotypes have been obtained using cross-sectional data and a longitudinal study is required to determine if increases in $\dot{V}O_{2max}$ are accompanied by changes in the proportions of naive and senescent T-cells.

The association between $\dot{V}O_{2max}$ and the moderation of the age-related changes in T-cell subsets withstood adjustment for, BMI, percentage body fat and waist/hip ratio, suggesting that the $\dot{V}O_{2max}$ associations were not secondary to differences in body composition. However, previous studies have documented negative associations linking BMI, % body fat, and biological markers of adiposity such as leptin with leukocyte telomere length (Valdes et al., 2005; Lee et al., 2011) and reduced thymic output (Yang et al., 2009). Although our measures of adiposity were not related with immunosenescence, this was not surprising as very few subjects (7%) in the present cohort were classified as obese (BMI > 30) as our aim was to determine the association of aerobic fitness on blood T-cell phenotypes independently of BMI and % body fat, which are known confounders. It remains possible, and very likely, therefore, that excess adiposity will be related with increased senescent blood T-cells when more severe classifications of obesity

are examined. Indeed, BMI was marginally associated with the proportions of memory (KLRG1+/CD28+) CD4+ T-cells and senescent (KLRG1+/CD57+) CD8+ T-cells after age and $\dot{V}O_{2max}$ adjustment.

The mechanisms by which regular exercise exerts positive effects on the aging immune system remain to be established. We have suggested that the beneficial effects of exercise may come from either prevention and/or rejuvenation perspectives (Simpson and Guy, 2010). Firstly, from a prevention standpoint, exercise may elicit secondary effects on the immune system due to its well-known stress reducing properties (Salmon, 2001), thus limiting the potential for stress-induced latent viral reactivation and telomere erosion. Secondly, from a rejuvenation standpoint, regular exercise may lead to the destruction of excess viral specific T-cell clones via apoptosis, freeing up "immune space" for naive T-cells to occupy and expand the antigenic T-cell repertoire (Simpson, 2011). Furthermore, it was shown recently that exercise elicits Interleukin-7 release from active skeletal muscle (Haugen et al., 2010). This cytokine is known to play an important role in naive T-cell homeostasis and the output of recent thymic emigrants (Schluns et al., 2000) and may therefore be involved in the aetiology of exercise-induced immune enhancement.

As latent herpesvirus infections, particularly CMV, are associated with a greater number and proportion of senescent T-cells (Ouyang et al., 2004), we considered CMV, EBV and HSV-1 serostatus as potential confounders of the moderating associations of $\dot{V}O_{2max}$. Although CMV, but not EBV or HSV-1, seropositivity was associated

with a greater proportion of senescent CD4+ and CD8+ T-cells, none of these latent viruses were associated with $\dot{V}O_{2max}$ scores. We therefore considered $\dot{V}O_{2max}$ to be associated with blood T-cell phenotypes independently of these persistent viral infections. It is acknowledged, however, that the sample size used for our serostatus determination was relatively small and the possibility that relationships exist between aerobic fitness and latent viral infections cannot be ruled out if a larger cohort is examined. Additional limitations of the present study include the non-inclusion of women and the elderly (i.e., over 65 yr), and, because our subject recruitment was limited to students and members of academic staff at institutions of higher education, participants of lower socioeconomic standing were less likely to be recruited and may explain why $\dot{V}O_{2max}$ was not related to latent viral infections in our study.

The term "senescence" has also come under recent scrutiny because it is used interchangeably with the term "exhaustion", both of which control T-cell proliferation activity and function via independent pathways (Akbar and Henson, 2011). While senescent cells have undergone permanent growth cycle arrest, the proliferative activity of "exhausted" cells can be restored by KLRG1 blockade (Henson et al., 2009), indicating that KLRG1 regulates exhaustion-related pathways (Akbar and Henson, 2011). It is plausible, therefore, that the cell types we have identified as "senescent" could display functional characteristics of "exhaustion". However, it is possible that KLRG1 blockade only restores proliferation in exhausted but pre-senescent cells, and may still be involved in pathways regulating senescence (Akbar and Henson, 2011). Indeed, whether or not KLRG1 blockade can restore proliferation in CD57 co-expressing "senescent" cells remains to be determined.

In conclusion, aerobic fitness was associated with a lower proportion of senescent and a higher proportion of naïve cells, particularly within the CD8+ T-cell compartment in healthy adult men. These findings withstood adjustment for a number of potential confounders (i.e., age, BMI, % body fat and latent viral infections) indicating that high levels of aerobic fitness could independently exert protective effects on the aging immune system. Future longitudinal studies are required to determine if changes in $\dot{V}O_{2max}$ due to exercise training will be accompanied by changes in the composition of naïve and senescent T-cells. It will be of particular interest to determine if exercise can improve immunity in those displaying biomarkers associated with the IRP, as this would help to establish if regular exercise has immune restorative properties.

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Brief Commentary

Can we jog our way to a younger-looking immune system?

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The effects of acute and chronic/regular exercise on the immune system¹ have been extensively studied (Walsh et al., 2011). These results have drawn attention in the field of psychoneuroimmunology, as indicated by a steadily increasing number of published articles on this topic in this very journal, *Brain, Behavior, and Immunity* (BBI). Recent literature has also highlighted the anti-inflammatory (Petersen and Pedersen, 2005) and anti-aging (Safdar et al., 2011) properties of exercise. In this issue of the journal, Simpson and colleagues report intriguing data that suggest aerobic fitness impedes immunological aging (Spielmann et al., 2011).

Immune dysregulation during aging, collectively termed immunosenescence, is signified by increased susceptibility to infectious diseases and other pathological immune-related conditions. The markers that have been consistently utilized for characterizing aging of the immune system include telomere shortening, decreased numbers of naive T cells, and increased numbers of highly differentiated T cells. Cellular surface markers for identifying senescent T lymphocytes include CD28, CD27, CD62L, and CCR7, which are downregulated on effector memory or highly differentiated cells. For example, the proportion of CD28–CD8+ T cells increases in the elderly accompanied by a decreased percentage of CCR7+CD8+ central memory T cells that can home to lymph nodes (Derhovanessian et al., 2009). Hence, there is an accumulation of terminally differentiated T cells that are resistant to programmed cell death (Pawelec et al., 2006), which leads to a limited T cell repertoire with proliferative potential. The Simpson group (Spielmann et al., 2011) investigated the proportions of immunosenescent T cells in relation to aerobic fitness in 102 healthy men between 18 and 61 years of age. Senescent T cells were detected based on the expression of KLRG1+, CD57+ and CD28–. Age and aerobic fitness levels, as determined by estimated peak oxygen consumption ($\text{VO}_{2\text{max}}$), were positively and negatively associated with senescent T cell proportions, respectively. However, after accounting for fitness chronological age was no longer associated with senescent T cell proportions. When age-adjusted fitness groups were compared, the above-average fitness group exhibited significantly smaller proportions of senescent T cells (by about 35–58%) and greater naive T cell proportions (about 17%).

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¹ A clear distinction between acute and transient immune responses to a single bout of exercise (as a physical stressor in certain instances) and immunological changes following chronic exercise training or regular physical activity is necessary. In this Commentary "exercise" primarily refers to regular physical activity unless noted otherwise.

Despite the large age range of participants, with 50% younger than 42 years of age, these findings provide a number of important implications for the preventive benefits of exercise against aging-associated immunosenescence. Firstly, given that senescent T cell proportions increase by 10% per decade of advancing age in these healthy/asymptomatic men, it would be reasonable to postulate that higher physical fitness levels and regular exercise may prevent or arrest accumulation of senescent T cells for up to several decades. Secondly, physical fitness and activity levels are major moderating factors in the age-immunosenescence relationship and are independent predictors of "immunological age". Thirdly, the therapeutic value of exercise in enhancing immunocompetence of the asymptomatic aging population and immunocompromised clinical populations (e.g., HIV+) is potentially immense.

The findings from the study by Spielmann et al. should be considered with the understanding that incongruence and complexity remain in characterizing and phenotyping of T cells based on surface molecule expression patterns. Of note, Spielmann et al. characterized "senescent T cells" based on the markers KLRG1+/CD28– and KLRG1/CD57+, which were highly correlated, for both CD4+ and CD8+ T cells. Differing views exist not only in the markers identifying subpopulations of T cells but also in categorizing them according to the cellular functions. For example, among antigen-experienced CD8+ memory T cells, T cell subpopulations are variably categorized in the literature as resting memory vs. effector, central vs. effector memory, and early, intermediate vs. late differentiated T cells based on the expression of CD45RA, CD27, CD28 or CCR7 (Appay et al., 2008). Also, this naming of T cell subsets according to the profile of surface markers often does not correspond to their "effector" functions, such as cytotoxicity and cytokine production. Recent evidence challenges the classic lineage differentiation model of hematopoiesis by showing that a range of progenitors can give rise to the T cell lineage dependent upon stimulation by factors such as Notch and IL-7 (Schlenger and Rodewald, 2010). This plasticity in early T cell development offers a basis for the consideration that T cell proliferation and differentiation are rather malleable processes. This plasticity is reflected by heterogeneous profiles of cellular markers of differing functional roles (costimulatory molecules, lymphoid vs. peripheral tissue homing molecules, cytokine and chemokine receptors, adhesion molecules, etc.) (Reiner et al., 2007). Thus, the T cell differentiation pathway should perhaps be viewed as a dynamic up- and down-regulation spectrum of cellular molecules along the continuum of functional diversity rather than as discrete stages that are defined by a distinct set of markers or functions.

The underlying mechanisms for the protective effects of exercise against the accumulation of senescent T cells remain unclear. Certain latent viral infections, especially cytomegalovirus, contribute to an expansion of highly differentiated virus-specific T cells potentially via constant antigenic challenge (Pawelec et al., 2005). Although Spielmann et al. reported no association between serostatus for chronic viral infections and fitness levels in a subset of the participants, the role of fitness in the viral serostatus and immunosenescence association was not fully examined. We previously reported that regular physical activity (Hong et al., 2004) and fitness levels (Hong et al., 2005) are associated with attenuated T cell mobilization responses to acute stressors that are accompanied by smaller catecholamine responses. Diminished adrenergic responses to acute stressors among the physically fit, therefore, may in part contribute to keeping the reactivation of latent viruses under control. Future studies to examine whether neurohormonal, metabolic, or hemodynamic changes/adaptations from regular exercise directly affect cellular proliferation and differentiation pathways or indirectly via mediating factors will shed light on implementing a safe yet effective exercise regimen to turn back the aging immune system.

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