$CD8^+$ T LYMPHOCYTE AND PROGENITOR CELL MOBILIZATION DURING ACUTE PSYCHOLOGICAL STRESS AND BETA-ADRENERGIC STIMULATION

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ABSTRACT

This thesis investigated the effect of acute psychological stress and ß-adrenergic receptor (ßAR) stimulation on the mobilization of $CD8^+$ T lymphocytes (CD8TLs) and progenitor cell (PC) populations. Chapter 2 demonstrated that CD8TL stress- and ßAR- sensitivity increases in parallel with greater effector functions and cell differentiation. As Cytomegalovirus (CMV) infection influences CD8TL differentiation, Chapter 3 compared the mobilization of cytotoxic lymphocytes in CMV seropositive and seronegative individuals; CMV infection enhanced the stress reactivity of CD8TLs, CD4TLs and NKT-like cells. Chapter 4 examined whether antigen-specificity could modulate CD8TL stress- and ßAR-sensitivity. CMV-specific cells demonstrated enhanced mobilization compared to the total-memory CD8TL and the total Epstein-Barr virus (EBV) population. In Chapter 5, we demonstrated that PC subsets, capable of both replenishing leukocyte populations and maintaining endothelial integrity, were also mobilized by acute psychological stress. This result was not replicated by ßAR-agonist infusion suggesting the involvement of ŭAR or non-adrenergic mechanism. In sum, the current findings suggest that stress mobilization serves to protect the host by increasing immune protection and tissue repair mechanisms. However, such a response may also be detrimental dependent on the circumstance, i.e., infection versus inflammation.

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LIST OF PAPERS

This thesis incorporates the following five papers;

1) Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Wallace, G. R., and Bosch, J. A. Acute psychological stress and beta-adrenergic receptor agonist infusion preferentially mobilizes effector memory $CD8^+$ T lymphocytes that exhibit enhanced effector potential. In preparation.
2) Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Moss, P. A., Wallace, G. R., and Bosch, J. A. Cytomegalovirus infection as a determinant of lymphocyte mobilization during stress and beta-adrenergic stimulation. In preparation.
3) Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Moss, P. A., Wallace, G. R., and Bosch, J. A. Mobilization of CMV- and EBV- specific $CD8^+$ T Lymphocytes during acute psychological stress and ß-adrenergic receptor stimulation. In preparation.
4) Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Wallace, G. R., and Bosch, J. A. Progenitor cells are mobilized by acute psychological stress but not beta-adrenergic receptor agonist infusion. In preparation.
5) Bosch, J.A., Riddell, N.E., Burns, V., Moss, P. CD8TLs mobilization by stress is determined by cell differentiation, infleuneced by CMV-infection, and goverened by beta-adrenergic receptor expression. In preparation.

In addition the following presentations arose from material from this thesis;

- Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Moss, P. A., Wallace, G. R., and Bosch, J. A. Selective ß-adrenergic receptor expression on memory $CD8^+$ T lymphocyte subsets regulates cell mobilization and IFN-ɔ production in humans. *9th World Congress on Inflammation,* 2009
- Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Moss, P. A., Wallace, G. R., and Bosch, J. A. Redeployment of effector-memory and CMV-specific CD8TLs during acute stress; Role of $ß_2$-adrenergic receptor expression. *New Developments in Psychoneuroimmunology,* 2009
- Riddell, N.E., Burns, V.E., Edwards, K. M., Drayson, M., Mills, P., Moss, P. A., Wallace, G. R., and Bosch, J. A. ß-adrenergic receptor stimulation selectively mobilizes effector-memory $CD8^+$ T cells and impairs INF-ɔ production. *British Society of Immunology,* 2008

A presentation on material from this thesis was awarded the following academic prize;

- First place in the Young Investigators Award at the International Association of Inflammation Society (IAIS) *9th World Congress on Inflammation,* 2009

During the period of postgraduate studies at the University of Birmingham, the following papers were also published/ submitted;

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

- Anane, L. A., Edwards, K. M., Burns, V.E., Drayson, M., Riddell, N.E., van Zanten, J. J., Wallace, G. R., Mills, P. J., and Bosch, J. A. Mobilization of gammadelta T lymphocytes in response to psychological stress, exercise, and beta-agonist infusion. *Brain Behav Immun* 2009
- Anane, L. A., Riddell, N.E., Burns, V.E., Drayson, M., P. A., Wallace, G. R., and Bosch, J. A. Perforin^{+}CD4^{+} T lymphocytes are mobilized by acute psychological stress and beta-adrenergic stimulation. In preparation.

Finally, this thesis has received the following media coverage;

- ñThe immune system- Donôt die youngò *BBC2* May 2007

CONTENTS

ABBREVIATIONS LIST

ɔŭ T cells, gamma-delta T cells
ŬAR, alpha-adrenergic receptor
ACTH, adrenocorticotropic hormone
ADRB2, beta-2-adrenergic receptor gene
ANOVA, Analysis of Variance
APC, allophycocyanin
APC-Cy7, allophycocyanin-cyanine dye 7
ɓAR, beta-adrenergic receptor
$ɓ_1$AR, beta-1-adrenergic receptor
$ɓ_2$AR, beta-2-adrenergic receptor
$ɓ_3$AR, beta-3-adrenergic receptor
bpm, beats per minute
BMI, body max index
BSA, body surface area
CD4TLs, $CD4^+$ T lymphocytes
CD8TLs, $CD8^+$ T lymphocytes
CLP, common lymphoid progenitors
CM, central memory
CMV, Cytomegalovirus
CMV^{pos}, Cytomegalovirus seropositive
CMV^{tet}, Cytomegalovirus tetramer positive $CD8^+$ T lymphocytes
CMP, common myeloid progenitors
CVD, cardiovascular disease
DBP, diastolic blood pressure
EBV, Epstein-Barr virus
EBV^{pos}, Epstein Barr Virus seropositive
EBV^{tet}, Epstein Barr Virus tetramer positive $CD8^+$ T lymphocytes
ECG, electrocardiography
EDTA, ethylenediaminetetraacetic acid
EM, effector-memory
EMRA, $CD45RA^+$ effector memory
EPC, endothelial progenitor cell
FITC, fluorescein isothiocyanate
GMP, granulocyte/ macrophage progenitors
G-CSF, granulocyte colony-stimulating factor
HSC, haematopoietic stem cell
HR, heart rate
HSV1, Herpes Simple Virus type-1
ICG, impedance cardiography
IFN-ɔ, interferon-gamma
IL-, interleukin-
$KHCO_3$, Potassium bicarbonate
LFA-1, lymphocyte function associated antigen-1
MEP, megakaryocyte/ erythrocyte progenitors
MHC, major histocompatibility complex
MICA, major histocompatibility complex class I-related antigen
MIP-1ɓ, macrophage inflammatory protein-1ɓ
NA, naïve
NH_4Cl, ammonium chloride

NK cells, natural killer cells
NKT cells, natural killer T cells
NKT-like, natural killer-like
NOS3, nitric oxide synthase 3
PB, pacific blue
PBS, phosphate-buffered saline
PC, progenitor cell
PE, phycoerythrin
PE-Cy7, phycoerythrin-cyanine dye 7
PEP, pre-ejection period
PerCP, peridinin chlorophyll protein
PNI, psycho-neuro-immunology
POMS, Profile of Mood States
RA, rheumatoid arthritis
RMSSD, Root Mean Square of Successive Difference
SBP, systolic blood pressure SBP
SD, standard deviation
SEM, standard error of mean
SNS, sympathetic nervous system
TLR, toll-like receptors
UoB, University of Birmingham
UCSD, University of California San Diego
VEGF, vascular endothelial growth factor

PREFACE: BASIC ASSUMPTIONS, AIMS, AND OUTLINE

STRESS AND HEALTH

The basic assumption underlying the research in this thesis is that psychological stress is a risk factor for diseases in which the immune system plays a key role. Animal and human studies support this conjecture and demonstrate an association between stress and infectious diseases, inflammatory and autoimmune conditions, cardiovascular disease, metabolic disorders and wound healing (Alboni and Alboni, 2006; Bose et al., 2009; Chen and Miller, 2007; Chida and Steptoe, 2010; Cohen et al., 2007; Falagas et al., 2010; Glaser and Kiecolt-Glaser, 2005; Godbout and Glaser, 2006; Hamer and Stamatakis, 2008; Kemeny and Schedlowski, 2007; Kiecolt-Glaser et al., 2002; Kivimaki et al., 2006; Melamed et al., 2006; Miller, 2008; Miller et al., 2009; Miller and Blackwell, 2006; Mohr et al., 2004; Rainforth et al., 2007; Walburn et al., 2009). For example, results from meta-analyses demonstrate a strong relationship between stress and wound healing (r=ī 0.42, 95% CI=ī 0.51 toī 0.32, *p*<.01), and yield an adjusted relative risk ratio of coronary heart disease for high verse low job strain of 1.16 (95% CI 0.94ī 1.43) (Hamer and Stamatakis, 2008; Kivimaki et al., 2006; Walburn et al., 2009). It is outside the scope of this thesis to thoroughly review this literature, and the interested reader is referred to the reviews and empirical studies cited in this section. In sum, the evidence for a relationship between stress and health is considered compelling (Glaser and Kiecolt-Glaser, 2005; Miller et al., 2009).

Although it has long been asserted that stress enhances susceptibility to disease, evidence for a biological mechanism did not emerge until the development of psycho-neuro-immunology (PNI) (Bonneau et al., 2007; Kemeny and Schedlowski, 2007; Miller et al.,

2009). PNI research investigates interactions between the brain, behaviour, and the immune system. It is now clear that the immune, neural and endocrine systems ñcommunicateò, and that the immune system may respond to psychological factors (Ader, 2006; Elenkov et al., 2000; Glaser and Kiecolt-Glaser, 2005; Miller et al., 2009; Webster et al., 2002). This modulation of immune function lends biological plausibility to the observed association between stress and impaired health.

THE INFLUENCE OF STRESS ON IMMUNE FUNCTION

What is stress?

The emotion researcher Richard Lazarus proposed a useful way to define psychological stress, by describing it as a process consisting of three distinct steps (Folkman and Lazarus, 1988; Lazarus, 1984). First, a stimulus (i.e., the stressor) has to be present and perceived. Second, the stimulus initiates a conscious or sub-conscious appraisal whereby it is evaluated in relation to available coping options. The situation is perceived as stressful, i.e., seen as a threat, challenge or loss, when it is believed that the demands of the situation are too taxing or plainly outstrip the ability to cope (the stress perception). Thirdly, the stress perception results in a stress response which takes the form of emotional (e.g., anxiety, embarrassment), behavioural (e.g., altering the environment, fight/flight), and biological (e.g., autonomic-endocrine-immune) adaptations. To summarize in simplified terms, stress occurs when a situation exceeds the perceived ability to cope. In line with this definition, in this thesis the term stress refers to a process whereby a situation is perceived as a challenge or threat (i.e., a stressor) which then induces congruent emotional and biological perturbations, such as increases in anxiety and activation of the sympathetic nervous system.

Chronic stress versus acute stress

Meta-analyses demonstrate that chronic stress is associated with immunosuppression (Zorrilla et al., 2001; Segerstrom, 2004). For example, the chronic stress of care-giving for a family member is associated with reduced antibody responses to vaccine and with slower wound healing (Gallagher et al., 2009a, b; Glaser et al., 2000; Kiecolt-Glaser et al., 1996; Kiecolt-Glaser et al., 1995). Such suppressive effects tend to be emphasized in the literature, possibly because of their potential clinical relevance. However, the most significant and life-threatening stressors in the animal kingdom are acute stressors lasting only a matter of minutes, such as escaping a predator or hunting prey (Cannon, 1929; Sapolsky, 1994).Under such conditions, immune-enhancement would seem the most beneficial response, as it appears improbably that eons of evolution would select for, for example, a cardiovascular and metabolic system that helps to rapidly escape the jaws of a predator, only to later succumb to the ñjawsòof an infection (Dhabhar, 2002). Indicative of an evolved ability to rapidly adapt to danger, all vertebrates, including humans, have the remarkable capacity to swiftly alter the leukocyte composition of peripheral blood and tissues in response to acute stress (Benschop et al., 1996; Dhabhar, 2002; Dhabhar and McEwen, 1997; Dhabhar et al., 1995). Further, experimental animal studies have confirmed that acute stress, and the accompanied cell redistribution, predicts stronger delayed-type hypersensitivity responses, enhanced vaccine responses, an increased migration of leukocytes into wounded tissue, and faster wound healing (Dhabhar, 2002; Dhabhar and McEwen, 1996, 1997; Silberman et al., 2003; Viswanathan et al., 2005; Viswanathan and Dhabhar, 2005). Indeed, one of the most comprehensive meta-analyses on stress and immunity in humans to date, reviewing over 300 empirical articles, found that the most robust and replicable findings in PNI are associated with acute stress, thus further suggesting an important role of acute stress responses (Segerstrom and Miller, 2004). For example, the effect size of acute stress responses, such as cell redistribution and salivary antibody secretion rates ranged between r=-.17 and r=.43, whilst the largest effect of chronic stress was much small (e.g. reduced antibody response to immunization; r=ī .22).

ACUTE STRESS AND LEUKOCYTE DISTRIBUTION IN THE BLOOD

During the first few minutes of acute stress the absolute number of granulocytes, monocytes and lymphocytes increases (Benschop et al., 1996; Dhabhar and McEwen, 1999; Dhabhar et al., 1995, 1996; Schedlowski et al., 1993). The acute lymphocytosis response has been extensively studied; it is now know that ßAR-mechanisms govern the detachment of lymphocytes from endothelial cells facilitating cell release into the circulation (Benschop et al., 1996; Dhabhar, 2002; Dhabhar et al., 1995; Dimitrov et al., 2010; Kuhlwein et al., 2001; Mills et al., 1995; Mills et al., 1997; Segerstrom and Miller, 2004; Willemsen et al., 2002; Zorrilla et al., 2001). What is also known is that this lymphocytosis is largely confined to cytotoxic lymphocytes, i.e., which have the ability to lyse infected or transformed (cancerous) cells, such as NK cells, gamma delta (ɔǔ) T cells, and $CD8^+$ T lymphocytes (CD8TLs) (Anane et al., 2009; Segerstrom and Miller, 2004). Within these cytotoxic cell types there is a further subdivision, whereby mobilized cytotoxic cells characteristically exhibit a high tissue migratory potential ($CD11a^{high}$ and $CD62L^{low/neg}$) and a differentiated memory phenotype, as indicated by the expression of NK cell receptors, such as, KLRG1 and $CD57^+$ (Anane et al., 2010; Bosch et al., 2005; Campbell et al., 2009; Gannon et al., 2002; Goebel and Mills, 2000; Kurokawa et al., 1995; Mills et al., 2003; Simpson et al., 2007; Timmons and Cieslak, 2008). These phenotypic characteristics are also associated with the ability to rapidly eliminating foreign antigens in peripheral tissue (Masopust et al., 2004; Sallusto et al., 2004; Weninger et al., 2001).

AIMS OF THIS THESIS:

1) Characterisation of CD8TLs mobilized by acute psychological stress and ßAR-agonist infusion. In these analyses the focus is on describing CD8TLs in terms of memory and differentiation phenotype.

2) The differentiation of CD8TLs is strongly influenced by infection history; in particular, infection with Cytomegalovirus (CMV) is known to drive cell differentiation. Hence, the second aim was to determine whether infection with CMV is associated with stress-induced mobilization of the CD8TL population.
3) Lymphocyte mobilization is driven by activation of beta-adrenergic receptors. These receptors are also expressed on progenitor cells. Therefore the final aim was to determine whether progenitor cells (PCs) were also mobilized by acute stress and βAR-stimulation.

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CHAPTER 1

GENERAL INTRODUCTION

A BRIEF INTRODUCTION TO THE IMMUNE SYSTEM

Despite its immense complexity, there are several ways of categorising cells of the immune system (leukocytes). Distinguishing between innate and adaptive cells is a useful and common way of describing the immune response. Cells of the innate immune system utilize general-purpose recognition strategies to identify their targets; they express receptors that recognize generic molecular patters typical for infectious agents and derailed host cells, such as cancerous cells. Innate immunity can respond when challenged in a relatively short time period (minutes to hours) and thus plays an important role in containing infections and acting as an early warning system. A large proportion of innate cells, such as neutrophils, monocytes (or macrophages once located in the tissue) and dendritic cells, are able to engulf and destroy targets such as bacteria by the process of phagocytosis. Following tissue injury or infection, neutrophils are the predominating cell type to bind to activated endothelium and to extravasate into the tissue, where they contribute to inflammation. Inflammation is a sequence of events, starting with vasodilation and increased capillary permeability, and resulting in further recruitment of leukocytes to the site of tissue damage or infection. The influx of cells promotes antigen clearance and wound healing. The innate immune response also involves Natural killer (NK) cells, which are lymphocytes that kill infected and cancerous cells by releasing toxic substance, such as perforin or granzyme A/B, which induce apoptosis.

The adaptive immune response, involving B lymphocytes and T lymphocytes is initiated within the lymph nodes. Lymphocytes express surface receptors (B cell receptor (BCR) and T cell receptor (TCR) respectively) that are highly selective for a particular antigenic peptide (protein fragment). The BCR can interact directly with free antigen, although cell activation may also require T cell help as discussed shortly. In contrast, the TCR can only interact with antigen bound to a major histocompatibility complex (MHC) expressed by specialized antigen presenting cells (APCs), including dendritic cells, macrophages and B cells. Thus, an important function of innate cells, in particular dendritic cells, is to capture antigen at the site of inflammation and then migrate towards lymph nodes, where they may present processed peptide fragments within MHC molecules to the lymphoid-residing T cells. Interaction with antigen triggers an adaptive immune response, which involves the activation and differentiation of antigen-specific T and B lymphocytes. When activated, lymphocytes proliferate and produce a progeny of clones with the same antigen-specificity. This process efficiently creates a large number of cells able to migrate to the original site of infection/inflammation when they will usually eradicate the infection. After the infection is cleared, most antigen-specific clones will die. The selectivity of the adaptive response helps to prevent bystander damage to the surrounding healthy tissue. The downside of adaptive immunity is the time lapse between initial infection and the production of the antigen-specific population which, during initial antigen encounter, can take up to 6 days. In the mean time the body must rely on the innate immune system to contain the infection. The major advantage of adaptive immunity probably is immunological õmemoryö That is, after the initial immune response, a small number of the antigen-specific lymphocytes remain as long lived memory cells. Upon subsequent antigen encounter, these memory cells are capable of mounting a very rapid immune response (as early as 2 days) which is fast enough to prevent disease symptoms from presenting.

Adaptive immunity is conferred by three main types of lymphocytes. B cells produce immunoglobulins; these are soluble glycoproteins that can perform a number of functions including, neutralising antigens and hereby prevent binding and entry into a host cell, complement activation, and opsonisation (coating of an antigen by immunoglobulin to increase the efficiency of phagocytosis). Where as all other immune cells need to physically interact with the relevant antigens, B cells can conveniently secrete their antigen-specific antibodies, and thus exert their immunological actions in an endocrine fashion. T cells can be broadly divided into $CD4^+$ T lymphocytes (CD4TLs) also called T-helpers (Th), and $CD8^+$ T lymphocytes (CD8TLs) also called cytotoxic T lymphocytes (CTLs). The main function of CD4TLs is to orchestrate the immune response by releasing cytokines. There is a great diversity of functionally distinct CD4TLs, depending on their pattern of cytokine release. Identified subsets are Th1, Th2, Th17, and Treg, and this list is still expanding. Th1 cells secrete cytokines like IFN-y that stimulate macrophages and CD8TLs, and hereby promote a cell mediated/ phagocytic inflammatory response. Cytokines produced by Th2 cells (e.g. IL-4) promote allergic inflammation, immunity directed towards extracellular pathogens, such as parasites and fungi, and immunoglobulin production. CD8TLs recognise infected cells or otherwise compromised self cells (e.g. transformed cells) and lyse those cells.

CYTOTOXIC LYMPHOCYTES

$CD8^+$ T lymphocytes (CD8TLs)

CD8TLs ($CD8^+CD3^+$ lymphocytes) are lytic cells critical in the detections and elimination of altered self cells. CD8TLs are activated by antigen presented in MHC-I molecules which are expressed by all nucleated cells, thus, in theory, allowing elimination of any altered cell. CD8TL-mediated apoptotic death can induce target (altered) cell lyses by release of cytotoxic proteins, such as perforin, or via interaction with Fas-ligand.

The CD8TL population is extremely heterogeneous and as many as fourteen subsets may exist (Chen et al., 2001; Hamann et al., 1997; Monteiro et al., 2007; Romero et al., 2007; Sallusto et al., 2004). Cells with particular functions can be identified by their expression of cell surface markers, e.g., a propensity for lymph node homing is indicated by the expression of CD62L and/ or CCR7. What makes this a particularly useful way of characterizing cells is that a particular marker, e.g., a lymph node homing marker, also relates to various functional characteristics of the cell. For example, naïve cells migrate between the lymph nodes, in order to find antigen presented by APCs. For these purposes they express homing markers such as CCR7 and CD62L and co-stimulatory molecules like CD28 and CD27, and not markers of effector cells like perforin and Fas-ligand. Thus the presence of one surface molecule (e.g., CCR7) automatically allows inferences about other characteristics of the cells. Another important conclusion that can be drawn from this fact is that migratory potential (e.g., $CCR7^{+}$ verses $CCR7^{-}$) also says something about the specific functional capacity of that cell (e.g., lytic/ effector capability). Thus, cell migration and function are two sides of the same coin (von Andrian and Mackay, 2000).

Over the last ten years, immunologists have developed several systems that allow the phenotyping of functionally different memory CD8TL subsets. A marker commonly used is the leukocyte common antigen isoform CD45RA. CD45RA is one of the three isoforms of the leukocyte surface protein CD45R. This isoform is present on naive T cells and replaced by the isoform CD45RO after antigenic stimulation. However, CD45RA is re-expressed on a subset of so-called ôrevertantômemory cells. Thus CD45RA is not sufficient to distinguish between naive and memory cells, and therefore CD45RA is typically combined with other markers such as the chemokine receptor CCR7 (because naive cells are lymph node homing) or costimulatory receptors CD28 and CD27 (which are present on all naive cells but not all memory cells).

On the basis of such dual surface expression four distinct subsets were identified: Naïve (NA; $CD45RA^{+}$, $CCR7^{+}$); Central Memory (CM; $CD45RA^{-}$, $CCR7^{+}$); Effector Memory (EM; $CD45RA^{-}$, $CCR7^{-}$); and $CD45RA^{+}$ effector memory (EMRA; $CD45RA^{+}$, $CCR7^{-}$) (Sallusto et al., 2004; Sallusto et al., 1999). Naïve and CM cells tend to travel to the lymph nodes, where they interact with APCs and can proliferate and produce a population of antigen specific cells with effector functions. CM cells, which have responded to the infection previously, can produce effector cells in a much shorter time frame to naive cells. In contrast EM and EMRA CD8TL have immediate effector functions; including cytotoxicity, tissue migratory potential, and IFN-ɔ production, thus they preferentially migrate to the peripheral tissues where they may mediate a rapid protective response.

Shortly after the above classification system became established, a second categorization method was introduced which identified three memory subsets on the basis of surface expression of CD28 and CD27 (Appay et al., 2002a). The resulting cell types are denoted as early ($CD28^{+}CD27^{+}$), intermediate ($CD28^{-}CD27^{+}$) or late ($CD28^{-}CD27^{-}$) differentiated cells, according to their position along a linear pathway of increasing effector potential and, possibly, increasing cell differentiation.

Recent technical advances in polychromatic flow cytometry has allowed the development of a classification system that combined the two methods described above, leading to characterisation of nine new CD8TL populations (Chapter 2, Figure 1 and Table 1). First, NA, CM, EM and EMRA cell are identified by CD45RA and CCR7 expression. These subsets are subsequently characterised for simultaneous expression of CD28 and CD27. The NA and CM populations are homogenous with consistent expression of both molecules. However, each effector memory CD8TL population have been found to be heterogeneous (Monteiro, Evaristo et al. 2007; Romero, Zippelius et al. 2007), revealing seven additional subsets:

$CD28^+CD27^+$ EM1 and EMRA1 sub-types; $CD28^-CD27^+$ EM2 and EMRA2 sub-types; $CD28^-CD27^-$ EM3 and EMRA3 sub-types; and the $CD28^+CD27^-$ EM4 sub-type. Characterisation of the novel memory subsets suggest that cells progressively differentiate from EM1/ EMRA1 to EM2/ EMRA2 and finally to EM3/ EMRA3 cells, and that this increasing differentiation is paralleled by enhancing effector functions (Romero et al., 2007).

Other cytotoxic populations studied in this thesis

$CD4^+$ T lymphocytes (CD4TLs)

The CD8TL differentiation pattern described above can be applied to CD4TLs ($CD4^+CD3^+$ lymphocytes) (Baars et al., 1995; Hamann et al., 1996; Jenkins et al., 2001; Sallusto et al., 1999). The common known function of CD4TLs is there role in orchestrating the immune system (Appay, 2004). A significant fraction of the minor EMRA CD4TLs (which form 0.5%-3% of total CD4TL) however, can lyse target cells by expression of perforin and granzymes, and are thus cytotoxic cells (Appay et al., 2002b; Casazza et al., 2006). EMRA CD4TLs exhibit distinct functional properties reminiscent of late-differentiate CD8TLs; in addition to having cytotoxic potential they are highly differentiated ($CD28^-CD27^-$), IFN-ɔ producing and poised for tissue migration (Appay, 2004; Appay et al., 2002b; Casazza et al., 2006; Fletcher et al., 2005).

Natural Killer T (NKT) cells

Natural killer T cells (NKT cells) are a small (~0.1-0.5% of peripheral blood leukocytes) heterogeneous T cell populations that are characterized by the co-expression of TCR and various NK cell receptors, including CD16, CD56, CD161, CD94, CD158a and CD158b (Godfrey et al., 2000). The exact role of NKT cells at sites of inflammation is not known, however their rapid release of cytokines that may promote or suppress different immune response, has earned NKT cells the moniker of the ñdouble-edged swordò(Cui et al., 1999; Godfrey and Kronenberg, 2004; Mempel et al., 2002; Shi et al., 2001; Smyth and Godfrey,

2000; van der Vliet et al., 2004). Further, NKT cells can exhibit lytic capacity in both a Fas and perforin dependent manner (Arase et al., 1994; Smyth et al., 2000). ñTrueò NKT cells express a unique semi-invariant TCR and thus are called invariant NKT cells. This specialised TCR facilitates recognition of the non-polymorphic CD1d molecule, an antigen-presenting molecule that binds self- and foreign lipids and glycolipids. NKT cells can thus be identified by CD1d-tetramers loaded with glycosphingolipid antigen Ŭ-galactosylcermide or by anti-VŬ24/Vƀ11 that directly binds to invariant TCR (Berzins et al., 2005). NKT-like cells can be identified by simultaneous expression of CD3 and CD56 (Atanackovic et al., 2006; Dimitrov et al., 2010; Sondergaard et al., 1999). However, late-differentiated CD8TLs/ CD4TLs and gamma-delta (ɔŭ) T cells also express NK cell receptors, thus results obtained using this non-specific identification method may only partially represent the NKT population.

Natural Killer cells (NK cells)

Natural killer (NK) cells are innate lymphocytes identified by expressing CD56 and lack of a T cell receptor, as indicated by lack of the TCR accessory molecule, CD3 (Cooper et al., 2001a). Two functionally distinct NK cell subsets exist: $CD56^{low}$ cells function as cytotoxic cells, whereas $CD56^{high}$ cells have an immunomodulatory role as they secrete large amounts of cytokines (Cooper et al., 2001a; Cooper et al., 2001b). $CD56^{low}$ NK cells predominant in the peripheral circulation (90% of circulating NK cells) and contain high concentrations of preformed cytolytic granules in their cytoplasm, such as perforin (Cooper et al., 2001a). Similar to effector memory T cells, $CD56^{low}$ NK cells express adhesion molecules that support homing to peripheral tissue (Frey et al., 1998; Lima et al., 2001). Following migration into inflamed tissues, $CD56^{low}$ NK cells may exert their cytotoxic function and lyse target cells (Cooper et al., 2001a; Moretta et al., 2002).

INFECTION HISTORY AND CELL DIFFERENTATION

Viral Infection and CD8TL differentiation

The precise pathway and mechanisms of CD8TL differentiation and development of distinct memory subsets after initial antigen encounter have not been fully elucidated. It is thought, that the frequency of antigen encounter and/ or stimulation of the cell by homeostatic cytokines, such as IL-7 and IL-15 are key factors (Kaech et al., 2003; Schluns and Lefrancois, 2003; Weng et al., 2002). Studies of CD8TLs demonstrate that differentiation is related to particular viral antigens and, accordingly, different subsets appear to provide protection against different groups of antigens (Appay et al., 2002a; van Lier et al., 2003). For example, immunological control of Cytomegalovirus (CMV), a common herpes virus carried by 50-70% of western populations, is largely provided by the late-differentiated effector-memory subsets ($CD45RA^{+/\bar{I}}\ CCR7^{\bar{I}}\ CD28^{\bar{I}}\ CD27^{\bar{I}}$). CD8TLs directed against Epstein-Barr virus (EBV), another very common (~90% infected) latent herpes virus, mostly exhibit an early or intermediate ($CD45RA^{\bar{I}/+}\ CCR7^{\bar{I}}\ CD28^{+/\bar{I}}\ CD27^{+}$) effector-memory phenotype (Appay et al., 2002a; Monteiro et al., 2007; van Lier et al., 2003). A final example is immunity towards influenza, a virus that does not result in chronic infections. Influenza-specific CD8TLs are mostly of a CM phenotype ($CD45RA^{\bar{I}}\ CCR7^{+}CD28^{+}CD27^{+}$). The reasons for this viral specificity are unknown. It is likely due to a combination of factors such as, the different functional properties necessary to control each virus, the distinct microenvironment at the time of T cell priming, and the frequency of reactivation (Appay et al., 2002a). It is also possible that viruses are able to directly block or divert T cell differentiation as part of their evasion of host immunity (Appay et al., 2002a).

Viral infections and CD4TLs, NKT cells and NK cells

Homeostasis of memory CD4TLs is regulated in a similar fashion to the CD8TL memory population, that is the long-lived memory pool is maintained by cytokines, mainly IL-7, and

potentially antigen-specific stimulation (Seder and Ahmed, 2003). However, the outcomes of antigen-specific activation are less dramatic than those seen for the CD8TL population. For example, CMV infection does induce the accumulation of differentiated effector-like perforin$^+$ CD4TLs but to a much lesser extent than is seen for the CD8TL population (Gamadia et al., 2004; Pourgheysari et al., 2007; Saez-Borderias et al., 2006). Accumulation of highly differentiated CD56$^+$ NKT cells is also observed during chronic antigen activation (Tarazona et al., 2000). No such affect is observed in the NK cell population. However, CMV infection may alter the NK cell repertoire of NK cells (Guma et al., 2004).

CELL MIGRATION, IMMUNE FUNCTION, AND BLOOD

Cell migration is the directed movement of cells between the blood, lymph organs and peripheral tissue. This continuous recirculation increases the chance that a cell will encounter a particular antigen and thus the development of an immune response. Cell migration is regulated by the interaction of Cell Adhesion Molecules (CAMs) expressed on leukocytes and tissues and by gradients of tissue-derived chemokines that interact with corresponding receptors expressed on immune cells. For example, the entrance of a naïve cell into a lymph node is facilitated by interactions between CD62L, expressed on lymphocytes, and glycoproteins (e.g. MAdCAM-1) expressed on specialized blood vessel endothelia (high endothelial venules, HEV) leading into lymph nodes (Berg et al., 1993). Such interactions are further facilitated through the actions of chemokines (von Andrian and Mackay, 2000). Chemokines are small polypeptides that control the adhesion, chemotaxis and activation of leukocytes. Dysfunction of cell migration (e.g., altered adhesion molecule or chemokine receptor expression, degradation of extra-cellular matrix) is a key pathological element in diseases such as cancer, atherosclerosis, and rheumatoid arthritis (Itoh, 2006; Konttinen et al., 1998; Pap et al., 2000; Rajavashisth et al., 1999; Seiki, 2003).

The blood is the main conduit for lymphocyte trafficking: these cells migrate in and out of the blood in order to sample the various lymphoid and peripheral tissues. This continuous redeployment is thus key to immunesurveillance (Dhabhar et al., 1995; Sprent and Tough, 1994; von Andrian and Mackay, 2000). Whereas the blood contains less than 2% of all leukocytes, sampling its cellular content provides a window on migratory behaviour between tissues and the activation status of the immune system (Dhabhar et al., 1995). The fact that acute stressors rapidly increase leukocyte numbers in peripheral blood may thus meaningfully be interpreted as a heightened immunosurveillance and readiness to respond to a challenge. For example, increased numbers of cells expressing CAMs and chemokine receptors that are associated with tissue migration may indicate an enhanced capacity to mount an inflammatory response. Indeed, both animal and human studies have shown that enhanced lymphocyte mobilization during stress (e.g., pre-operative anxiety, immobilization) is associated with increased lymphocyte infiltration into the tissues and better surgical recovery (Dhabhar, 2002; Dhabhar and McEwen, 1996, 1997; Rosenberger et al., 2009; Viswanathan et al., 2005; Viswanathan and Dhabhar, 2005).

NEUROENDOCRINE MODULATORS OF IMMUNE FUNCTION

Psychological stress can manifest within the host and cause immune alterations by two major pathways; the sympathetic-adrenal medullary (SAM) and the hypothalamic-pituitary-adrenal (HPA) axes (Black, 1994; Padgett and Glaser, 2003; Segerstrom and Miller, 2004). Perceived stress induces the release of neurotransmitters, including norepinephrine, serotonin, and acetylcholine, which stimulate the production of corticotrophin (CRF) from the paraventricular nucleus of the hypothalamus (Lightman and Young, 1989; Tsagarakis et al., 1988). CRF, the coordinator of the stress response, then acts in two ways. The first is

induction of norepinephrine secretion from sympathetic fibres which innervate many organs including primary (bone marrow and thymus) and secondary (spleen and lymph nodes) lymphoid tissue (Dunn and Berridge, 1990; Elenkov et al., 2000; Melia and Duman, 1991). Central activation of the sympathetic nervous system (SNS) is also transmitted to chromaffin cells within the adrenal medulla which stimulates the release of epinephrine into the circulation. The second function of CRF is to induce production of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland (Black, 1994). ACTH, in turn, stimulates the adrenal cortex to synthesize and secrete glucocorticoids (GC) which, together with the catecholamines (CA), norepinephrine and epinephrine, are the major stress hormones.

All leukocytes express functional receptors for these stress hormones, thereby rending immunity under the direct influence of products produced by the HPA and SAM axes (Rabin, 1999). Modulatory effects of the major stress hormones, include; immune cellularity, migration and proliferation, antibody secretion, cytotoxic activity and cytokine production (Black, 1994; Liao et al., 1995; Munck and Naray-Fejes-Toth, 1994; Padgett and Glaser, 2003; Wiegers et al., 1995). Further, immune cells produce cytokines, such as interleukin-1 (IL-1), which can stimulate production of CRH by the hypothalamus (Yang and Glaser, 2000). Therefore, not only can neuro-endocrine mediators alter immune function, the immune system can regulates the release of such mediators and influence its own function by ñcommunicatingò with the brain (Elenkov et al., 2000).

Lymphocytosis, mobilization of lymphocytes from stores, such as marginal pools or the spleen, into peripheral circulation, is dependent on beta-2-adrenergic receptor (β_2AR) mechanisms (Benschop et al., 1996b). Since acute stressors, such as short-term psychological stress or exercise, induce a similar lymphocytosis to epinephrine infusion, and in the view that both these stressors are associated with the release of norepinephrine and epinephrine, it was predicted that lymphocytosis was CA-mediated. Further, lymphocytes

express adrenergic receptors, and in particular, the ß$_2$AR. Blockade of ßARs prior to infusion with epinephrine completely abrogates lymphocytosis and demonstrates the involvement of ßARs in the response (Gader, 1974). Similarly, ßAR-antagonist also inhibits lymphocytosis induced by exercise or psychological stress (Ahlborg and Ahlborg, 1970; Benschop et al., 1996a; Benschop et al., 1994a). ß$_2$AR-selective antagonists, but not ß$_1$AR-selective antagonists, inhibit the mobilization of NK cells during CA infusion, further confirming the role of the ß$_2$AR in particular. In addition, ß$_2$AR-antagonists can prevent epinephrine induced NK cell detachment from endothelia cells in culture (Benschop et al., 1994b). Although activation of ß$_2$ARs upon the endothelial cells may play a negligible role, stimulation of ß$_2$ARs present on NK cells is sufficient to cause endothelial cell detachment, thus, mobilization is likely caused by stimulation of receptors present on the lymphocytes themselves. More recently, CD8$^+$ T lymphocytes (CD8TLs), gamma delta (ɔŭ) T cells and NKT-like cells were found to detach from endothelial cells following addition of epinephrine *in-vitro* (Dimitrov et al., 2010). Finally, enhanced cell mobilization corresponds to greater expression of the ß$_2$AR subtype thus suggesting that differential mobilization of lymphocyte subsets is governed by the level of receptor expression; NK cells demonstrate the greatest mobilization and the highest receptor expression, CD8TLs have intermediate receptor expression levels and exhibit intermediate mobilization, whilst CD4TLs and B cells do not mobilize and express the lowest level of ß$_2$AR. Together these findings indicate that stress-lymphocytosis is mediated by stimulation of the ß$_2$AR subtype present upon the cell surface of lymphocytes, which induces detachment from endothelial cells and subsequent cell mobilization into the blood.

PROGENITOR CELLS

What are progenitor cells?

Progenitor cells (PCs) are non-lineage committed cells that have unique cellular characteristics such as, strong ability for clonal expansion (i.e., to multiply) and resistance to cellular stress (Urbich and Dimmeler, 2004). They act as a repair system for the host, replenishing specialized somatic cells and also maintaining the normal turnover of regenerative organs, such as the blood or skin. Recent evidence suggests that PCs may also promote angiogenesis and vascular regeneration (Asahara et al., 1997; Khakoo and Finkel, 2005; Takakura et al., 2000).

Haematopoietic stem cells (HSCs)

Distinct PC subsets can be identified in the peripheral circulation (Chapter 4, Table 1 and Figure 1). Virtually all unipotent PCs express CD34 antigen (Sutherland et al., 1993). Haematopoietic stem cells (HSCs), that give rise to the common myeloid progenitor (CMP) subset and the common lymphoid progenitor subset (CLP), are $CD34^+$ PCs that express low levels of the common leukocyte antigen CD45 and have low granularity (as indicated by a low side scatter (SSC) profile) (Barnett et al., 1999; Gajkowska et al., 2006; Hirschi et al., 2008; Ribatti, 2007; Sutherland et al., 1994). CMPs, which further give rise to megakaryocyte/ erythrocyte progenitors (MEPs) and granulocyte/ macrophage progenitors (GMPs), can be identified from the HSC population by differential expression of the leukocyte activation antigen CD38, CD123 (the IL-3RŬ chain associated with cell cycle progression and differentiation), the CD45 isoform, CD45RA (Akashi et al., 2000; Manz et al., 2002). CLPs are HSCs that are $CD7^+$ (an antigen that appears early in the development of lymphoid lineages) and may or may not express CD38 (Galy et al., 1995; Hao et al., 2001; Hoebeke et al., 2007; Kondo et al., 1997; Terstappen et al., 1991). A less extensive way to identify HSCs which have multi-lineage potential (early HSCs) or lineage committed HSCs (late HSCs) is by lack of CD38 expression and positive CD38 expression, respectively (Terstappen et al., 1991).

Endothelial progenitor cells (EPC)

Research has been unable to establish an unique identifying marker for EPCs (Hirschi et al., 2008). Combined with the low abundance of these cells in normal adult circulation, EPCs are particularly difficult to study (Fadini et al., 2008). However, researchers have utilised different combinations of markers commonly used to identify HSCs and mature endothelial cells (Timmermans et al., 2009). Thus, EPCs have been identified by positive expression of CD34 in combination with: 1) markers associated with mature progenitor populations, such as CD38; 2) markers expressed on mature endothelial cells, such as KDR (a receptor for vascular endothelium growth factor (VEGF), often referred to as VEGFR); 3) markers that are not expressed by mature EC such as CD133; and/ or, 4) markers that are expressed by leukocytes, such as CD45. Recently, it was demonstrated that isolated $CD34^{+}CD45^{\bar{I}}$ cells can form endothelial cell colonies (Case et al., 2007; Timmermans et al., 2007). The $CD34^{+}CD45^{\bar{I}}$ cells often expressed KDR but were negative for CD133 expression (Timmermans et al., 2007). Thus, EPCs may be identified as $CD34^{+}CD45^{\bar{I}}$ cells that may also express KDR but not CD133 (Fadini et al., 2008; Hristov et al., 2009; Timmermans et al., 2009).

Adrenergic sensitivity of progenitor cells (PCs)

Results suggest that exercise can induce PC mobilization (Barrett et al., 1978; Bonsignore et al., 2002; Goussetis et al., 2009; Morici et al., 2005; Rehman et al., 2004; Schmidt et al., 2007). Further, PCs are reported to be mobilized by myocardial infarction, which is an alternative form of physiological acute stress (Turan et al., 2007; Wojakowski et al., 2006). Thus it is reasonable to consider that PC mobilization during physiological stress may be induced by activation of the SNS, similar to lymphocytosis. First both murine and human PCs express functional adrenergic receptor subtypes, $\breve{U}_1$, $\breve{U}_2$ and $ß_2$ (Muthu et al., 2007; Spiegel et al., 2007). Second the bone marrow, the major reservoir for adult PCs, is highly innervated by sympathetic nerve fibres (Elenkov et al., 2000). Third, there is experimental evidence that

physiologic stimuli and infectious challenge can increase the turnover of norepinephrine in the bone marrow and that injury induced adrenergic stimuli can upregulate erythropoiesis and monocytopoiesis (Fonseca et al., 2004; Tang et al., 2001; Tang et al., 1999). The latter finding can be reversed by sympathetic ablation of nerve terminals and therefore convincingly demonstrates the modulation of PCs by adrenergic mechanisms (Cohen et al., 2004; Tang et al., 2001). Finally, sympathetic stimulation regulates murine PC mobilization from bone marrow into the peripheral blood, and this finding can be replicated by administration of ß$_2$AR-agonist (Katayama et al., 2006; Spiegel et al., 2007). Together these results provide convincing evidence of the functional influence that sympathetic stimulation can exhibit on the PC populations, in particular their mobilization.

THESIS OVERVIEW AND SUMMARY

There is sound empirical and mechanistic/biological basis to expect a modulatory effect of acute stress and ßAR-stimuli on blood cell composition. Various neuro-endocrine systems, including the sympathetic nervous system, respond to stress resulting in the release of neurotransmitters (such as norepinephrine) and stress hormones (such as epinephrine). Lymphocytes are sensitive to these changes via the expression of cognate neuro-endocrine receptors. Cytotoxic T cells respond very sensitively to such changes, possibly due to distinct ß$_2$AR expression by different T cell phenotypes (Dimitrov et al., 2009; Holmes et al., 2005). The aim of the current thesis was to characterize this ßAR-responsivity further, in particular concentrating on the effects of CMV on T cell mobilization.

Chapter 2 describes the stress and ßAR-agonist-induced mobilization of CD8TL memory subsets. As hypothesised, late differentiated ñeffector-likeòCD8TLs that were recently found to express high levels of ß$_2$AR, demonstrated the greatest mobilization during ßAR-stimuli.

As CMV infection is thought to cause the accumulation of stress sensitive late-differentiated CD8TLs, in Chapter 3 the mobilization of CD8TLs in CMV infected (positive) and CMV negative individuals was compared; CD8TL mobilization was greater in CMV positive individuals. By examining the mobilization of antigen-specific (CMV and EBV) CD8TLs, Chapter 4 investigated whether this previous finding was solely explained by the accumulation of late-differentiate cells within CMV positive individuals, or whether CMV-specific cells were intrinsically more stress-sensitive. For example, would early-differentiated CMV-specific cells mobilize greater than early-EBV specific cells? Experimental studies have recently shown that stem cells, in both humans and mice, also express adrenergic receptors, suggesting that these cells may also demonstrate sensitivity to stress. Thus, Chapter 5 describes progenitor cell responses to psychological stress and ßAR-stimulation. Chapter 6, the final chapter, provides a summary and discussion of the results.

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CHAPTER 2

ACUTE PSYCHOLOGICAL STRESS AND BETA-ADRENERGIC RECECPTOR AGONIST INFUSION PREFERENTIALLY MOBILIZE EFFECTOR MEMORY CD8$^+$ T LYMPHOCYTES THAT EXHIBIT ENHANCED EFFECTOR POTENTIAL

ABSTRACT

Objective: Beta-adrenergic receptor (ϐAR) stimuli, such as acute psychological stress and exercise, induce mobilization of lymphocytes into the peripheral circulation, in particular CD8$^+$ T lymphocytes (CD8TLs) and Natural Killer (NK) cells. Recent evidence suggests that CD8TL mobilization is largely attributed to the selective increase in cells exhibiting immediate effector potential, i.e., effector-memory cells. The current study further tested this hypothesis utilizing multicoloured flow cytometry and an advance CD8TL subset classification system. **Methods:** Two separate studies compared CD8TL mobilization in response to an acute speech stress task (*n*=28) and ϐAR-agonist infusion at ~ 1µg/min/1.73 m^2 BSA (*n*=16). A sub-group of volunteers completed the infusion protocol under two conditions; ϐAR-antagonist administration and placebo (*n*=8). CD8TLs were classified into naïve (NA), central memory (CM), effector memory 1-3 (EM1, EM2 and EM3) or CD45RA$^+$ effector memory 1-3 (EMRA1, EMRA2 and EMRA3) subsets using simultaneous expression of the surface markers CCR7, CD27, CD28 and CD45RA. **Results:** A stepwise increase in CD8TL subset mobilization that paralleled an increase in effector potential was found (EMRA3/ EM3 > EMRA2/ EM2 > EMRA1/ EM1 > CM/ NA). During both the stress task and ϐAR-agonist infusion the greatest increase in cell numbers was observed for the EMRA3 CD8TLs (+144%

and +186% respectively) and the smallest for naïve cells (+11% and -17% respectively). Prior administration with the ϐAR-antagonist inhibited mobilization during the ϐAR-agonist infusion thus, confirming the involvement of ϐAR mechanisms in the response. **Conclusion:** The increase in CD8TL numbers during stress and ϐAR-stimuli can be explained by the mobilization of effector memory subsets, in particular the cells with the greatest effector potential. By increasing the peripheral numbers of cytotoxic and tissue migratory CD8TLs, stress and ϐAR-stimulus may evoke enhanced immune surveillance in anticipation of wounding and concurrent infection.

INTRODUCTION

Lymphocytes continuously traffic from the blood, into various tissues, and back into the blood. This continuous migration is essential to maintain an effective immune-defence network (von Andrian and Mackay, 2000). Characterizing how stress affects this lymphocyte redeployment may help explain how stressors affect the ability of the immune system to protect its host (Bosch et al., 2005). Stressors like heat, psychological stress, and physical exercise, have the remarkable capacity to acutely change the proportions of different leukocyte populations in the blood and tissue (Benschop et al., 1996; Bouchama et al., 1992; Pedersen and Hoffman-Goetz, 2000). These changes are associated with the release of epinephrine and norepinephrine that bind adrenergic receptors, in particular the beta-2-adrenergic receptor ($ß_2$AR) subtype, which is expressed on lymphocytes (Benschop et al., 1996). Stimulation of $ß_2$ARs mediates endothelial detachment and subsequent recirculation of lymphocytes into peripheral blood (Benschop, Nijkamp et al. 1994). Although stress-leukocytosis is a robust and well-known phenomenon, described as early as 1910 (Schulz, 1983), its exact immunological functions are not yet understood. A further phenotypic characterization of this response may enhance understanding on how stress may alter immune function.

Research has shown that cytotoxic lymphocytes are more readily mobilised by stress than other lymphocyte subsets (Anane et al., 2009; Atanackovic et al., 2006; Bosch et al., 2005; Campbell et al., 2009). For example, psychological stress rapidly increases NK cell numbers and, to a somewhat lesser extent, gamma-delta T cells and $CD8^+$ T lymphocyte (CD8TL) numbers (Anane et al., 2009). Within each cytotoxic population there is greater mobilization of subsets that show a high cytotoxic ability (e.g., perforin expression) and tissue migrating potential (Millsl et al. 2000; Bosch et al. 2003; Bosch, et al. 2005). Stress strongly mobilizes the cytotoxic and tissue-migrating $CD56^{low}$ NK cells, while the regulatory and lymph node

homing $CD56^{high}$ NK subset do not become mobilized (Bosch et al. 2005). A similar pattern has been shown for CD8TLs in response to psychological stress, exercise or ßAR-agonist infusion; preferentially mobilized cells display markers associated with tissue migration (e.g., $CD11a^{+}$, $CD62L^{-}$) (Mills, Karnik et al. 1997; Goebel and Mills 2000; Mills, Goebel et al. 2000; Bosch, Berntson et al. 2003) and cytotoxicity (e.g., perforin, CD57) (Atanackovic et al., 2006). These alterations have often been interpreted as reflecting rapid changes in surface molecule expression and/ or functional capacity. More recently, it has emerged that these individual observations may instead reflect exclusive mobilization of specific subsets of memory cells which constitutively exhibit the above characteristics (Campbell et al., 2009).

Over the last ten years, immunologists have developed an elaborate system for phenotyping functionally different CD8TL subsets. Initially, the CD8TL population was categorised into subsets using one of two different classification systems (Appay et al., 2002; Sallusto et al., 2004; Sallusto et al., 1999). In the first, CD8TLs are divided into four functionally distinct subsets identified by CD45RA and CCR7 expression: Naïve (NA; $CD45RA^{+}$, $CCR7^{+}$); Central Memory (CM; $CD45RA^{-}$, $CCR7^{+}$); Effector Memory (EM; $CD45RA^{-}$, $CCR7^{-}$); and RA effector memory (EMRA; $CD45RA^{+}$, $CCR7^{-}$) (Sallusto et al., 2004; Sallusto et al., 1999). NA and CM cells tend to travel to the secondary lymphoid organs where they interact with antigen presenting cells. The NA and CM cells do not express effector mediators, such as perforin or IFN-ɔ. In contrast, EM and, in particular, EMRA cells have effector functions including cytotoxicity, tissue migratory potential, and IFN-ɔ production. Therefore, these cells migrate into the peripheral tissue where they may encounter antigen and mediate a rapid protective response. In the second classification system, three subsets are identified by the expression of surface molecules CD28 and CD27 (Appay et al., 2002). The cells are defined as early ($CD28^{+}CD27^{+}$), intermediate ($CD28^{-}CD27^{+}$) or late ($CD28^{-}CD27^{-}$) according to their position along a linear pathway of increasing effector potential and, possibly, increasing cell differentiation.

More recently, polychromatic flow cytometry has allowed the combination of these two classification methods, and has led to the characterisation of nine new CD8TL populations (Figure 1 and Table 1). First, NA, CM, EM and EMRA cell are identified by CD45RA and CCR7 expression. These subsets are subsequently characterised for simultaneous expression of CD28 and CD27. The NA and CM populations are homogenous with consistent expression of both molecules. However, each effector memory CD8TL population have been found to be heterogeneous (Monteiro, Evaristo et al. 2007; Romero, Zippelius et al. 2007), revealing seven additional subsets:$CD27^{+}CD28^{+}$ EM1 and EMRA1 sub-types; $CD27^{+}CD28^{-}$ EM2 and EMRA2 sub-types; $CD27^{-}CD28^{-}$ EM3 and EMRA3 sub-types; and the $CD27^{-}CD28^{+}$ EM4 sub-type. It has been hypothesised that the cells progressively differentiate from EM1/ EMRA1 to EM2/ EMRA2 and finally to EM3/ EMRA3 cells (Romero et al., 2007). A stepwise increase in effector gene mRNA expression (granzyme B, perforin, IFN-y, and NK receptor CD94) was found from EM1/ EMRA1 to EM3/ EMRA3 cells. In addition, comparisons of cytolytic activity revealed that the EM1 population had a ten times lower ex-*vivo* lytic capacity than the EM3 population (Romero et al., 2007). Thus, it appears that the EM3/ EMRA3 cells represent differentiated effector-like memory cells.

The aim of the current study was to investigate the mobilization of these newly identified CD8TL effector-memory subsets utilizing multicoloured flow cytometry. The study aimed to further test the hypothesis that stress and ßAR-agonist infusion selectively redistributes effector-memory cells with immediate effector functions.

METHODS

Participants

Participants were recruited from community volunteers and staff and students attending the University of Birmingham (UoB), UK or University of California San Diego (UCSD), US. All volunteers reported to be in good health and were non-medicated with the exception of the contraceptive pill. Participants were instructed not to engage in strenuous physical exercise and to refrain from consuming alcohol or non-prescription drugs 24 hours before their experimental session, and to abstain from smoking and caffeine on the day of the experiment. Student volunteers performing the psychological stress task and all volunteers undertaking the infusion procedure received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the appropriate institutional review board (UoB or UCSD).

Psychological stress study

Procedure

Upon participant (age 38.3 years, SD±13.4; 17 female/ 11 males) arrival at the UoB: (1) electrodes for electrocardiography (ECG) and impedance cardiography (ICG) were attached; (2) a 20-gauge intravenous cannula (Becton-Dickinson) was placed in a palpable vein of the lower arm; and (3) an occluding cuff was placed over the brachial artery of the other arm for blood pressure measurements. Subsequently, while seated in a comfortable upright position, participants filled out several questionnaires and engaged in leisure reading. After 20 minutes, a baseline blood sample was obtained and the procedure for the laboratory stressor was initiated.

Public Speaking Task

To induce stress, participants performed two back-to-back speeches, each with 2 minutes of preparation and 4 minutes of speech delivery (Bosch et al., 2005; Bosch et al., 2003b) Social stress was enhanced by recording the speeches on videotape and by the attendance of an audience of three. For the first speech, the participant had to defend him/herself after being

falsely accused of shoplifting (Saab et al., 1989) and, for the second speech, the participant gave a presentation about his or her best and worst personal characteristics (van Eck et al., 1996). Instructions for the task were presented via a DVD recording, which ensured standardization of instructions and timing of the tasks. Including instructions, the task lasted 15 minutes. A blood sample was obtained during the second presentation, 13 minutes after initiation of the task. Following the task, the participants again engaged in leisure reading, and a final blood sample was obtained after 15 minutes of recovery.

Cardiovascular assessment

Assessment of cardiovascular responses focused on cardiac sympathetic and vagal control as previously described (Berntson et al., 1993; Bosch et al., 2003a). In brief; indices of sympathetic and parasympathetic drive were obtained by analysis of ECG and ICG signals. The thoracic ICG and ECG signals were recorded from six Ag-AgClspot-electrodes (AMI type 1650-005, Medtronic) using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) device. The ECG and ICG complexes were ensemble averaged with reference to the ECG R-wave across 30-sec periods. From these 30-sec ensembles, average levels were computed for heart rate (HR) and pre-ejection period (PEP). These means were further averaged over a 6-min pre-task baseline and over each 6-min task. Changes in PEP were used to index changes in cardiac sympathetic drive, whereas heart rate variability, or Root Mean Square of Successive Difference (RMSSD), was used to index changes in cardiac vagal tone.

βAR-agonist infusion study

Procedure

Isoproterenol infusion was performed according to a standardized protocol (Goebel et al., 2000; Mills et al., 2002; Mills et al., 2000; Mills et al., 1997). In brief, upon arrival at the UCSD, participant (age 35.9 years, SD±9.3; 8 female/ 8 males) height and weight were taken

to confirm correct calculation of body surface area (BSA) used to calculate the isoproterenol infusion rate according to standard hospital procedures. Subsequently, participants were asked to lie in a semi-supine position for 15 minutes following placement of; 1) two 22-gauge intravenous cannulas (Becton-Dickinson), one for drawing blood samples and one for isoproterenol infusion, inserted into a palpable vein in opposite lower arms; 2) three spot ECG electrodes; and 3) an occluding cuff on the non-infusion arm.

ßAR-agonist was then infused at incremental rates (0.1µg, 0.5µg and 1µg /min/1.73 m^2 BSA for 5 minutes) for 15 minutes until the participants heart rate had increased by ~20 beats per minute (bpm) compared to resting heart rate. The final infusion rate was maintained for 10 minutes. On average, the maximal dose reached 1µg/min/1.73 m^2 BSA. Blood was taken prior to initiation of isoproterenol infusion (ßbaselineÓ) and in the final minutes of the infusion. ECG, heart rate and systolic blood pressure (SBP)/ diastolic blood pressure (DBP) were monitored throughout the infusion. The half-life of isoproterenol is approximately 2ï 3 min (Goebel et al., 2000). The infusion was generally well-tolerated and all participants successfully completed the protocol.

ßAR-antagonist (blockade) procedure

A sub group of 8 participants (age 34.6 years, SD±11.5; 2 female) performed the ßAR-agonist infusion procedure under two conditions: 1) following five consecutive days of administration with 80mg of the ßAR-antagonist propranolol; and 2) following 5 days administration of a placebo. Drug administrations were counter balanced and single blinded. Only volunteers with a normal resting heart rate greater than 50 bpm were selected to perform the blockade treatment.

Questionnaires

In all studies, health and lifestyle variables were assessed by self-report questionnaire. These variables included recent symptoms of illness, exercise behaviour, alcohol consumption, caffeine consumption, smoking, and use of recreational drugs. Affective responses to the psychological stress task were assessed using the short-form of the Profile of Mood States (POMS) (McNair et al., 1992). Participants completed the POMS at baseline, immediately post-task and at 15-minutes recovery.

Flow cytometry

Whole blood was collected into EDTA tubes, maintained at room temperature and processed within 4 hours after collection. Whole blood (100µl) was incubated for 20 minutes at 4°C in the dark with a cocktail of fluorescent-labelled monoclonal antibodies: CD28-FITC, CD3-PerCP, CD27-APC, CD8-APC-Cy7 (obtained from Becton-Dickinson, Oxford, UK), CCR7-PE (eBioscience, purchased from Insight Biotechnology Ltd, Middlesex, UK) and CD45RA-PB (Invitrogen, Paisley, UK). Subsequently, red blood cells were lysed and removed by centrifugation (283G, 7-min at room temperature) following incubation with FACS Lysing solution (Becton Dickenson, Oxford, UK). The remaining cell pellet was re-suspended in 250µl PBS containing 1.5% paraformaldehyde, and stored in the dark at 4°C until analyses. Preparations were read within 18 hours. Approximately 80,000 gated lymphocytes were acquired from each preparation using a triple-laser (Cyan ADP, DAKO, Cambridgeshire, UK) or a dual-laser flow cytometer (FACS-Canto II, Becton Dickinson, Oxfordshire UK). Data were analyzed using Flowjo 7.4 (Treestar Inc, Ashland, OR, USA). A complete white blood cell count was obtained for each blood sample using a haematology analyzer (Coulter ACT^{diff}, Beckman Coulter, High Wycombe, UK or Coulter GEN-S haematology analyser, Beckman-Coulter, Miami, USA).

Data analysis

Repeated-measures Analysis of Variance (ANOVA) was used to assess the effects of the psychological stress task on mood and cardiovascular parameters and to examine the effects of the psychological stress task, ß-agonist infusion and ßAR-antagonist procedure on immunological measures. Variations in degrees of freedom reflect occasional missing data. Where means are presented, standard deviation is given in brackets. Percentage changes in cell numbers between time points and differences in the magnitude of mobilization between CD8TL subsets were examined by the one sample T-test and the Student paired T-test respectively. Data were analyzed using SPSS 16 for windows (SPSS Inc, Chicago, Illinois).

RESULTS

Psychological stress study

Anxiety and cardiovascular responses

Increases in the tension-anxiety POMS subscale confirmed that the speech tasks were perceived as stressful (+7.7 (SD = 4.5); $F_{(2, 54)} = 45.1$, $p<.001$). A physiological stress response was confirmed by the significant increases in SBP (+26.6 mmHg (SD = 9.8); $F_{(2, 54)} =156.7$, $p<.001$), DBP (+17.5 mmHg (SD = 8.0); $F_{(2, 54)} = 75.4$, $p<.001$), and HR (+20.8 bpm (SD = 11.8); $F_{(2, 54)} = 81.9$, $p<.001$), reflecting an increase in sympathetic drive as evidenced by a decrease in PEP (ï 10.5 ms (SD = 10.5); $F_{(2, 46)} = 15.3$, $p<.001$). Additionally, there was a significant decrease in RMSSD (ï 17.1 ms (SD = 23.9); $F_{(2, 48)} = 17.7$, $p<.001$), reflecting vagal withdrawal. At 15-min recovery, all cardiovascular and autonomic measures had returned to baseline values.

Preferential mobilization of effector-memory CD8TL during psychological stress

As can be seen in Table 2, the speech task induced a moderate but statistically significant increase in lymphocyte and total CD8TL numbers from baseline to stress (+27% and +31% respectively, both $p<.001$), indicating that the laboratory task effectively induced immune cell mobilization. Further, as can be seen in Figure 2, substantial and significant increases were found in EM2, EMRA2, EM3 and EMRA3 subsets, all of which are effector-like memory cells. The EM3 and EMRA3 responses were significantly greater than the EM2 (q%; $t_{(27)} = 3.6$, $p<.001$) and EMRA2 (q% $t_{(27)} = 5.1$, $p<.001$) responses, respectively. A small, but still significant, mobilization was also observed for the less-differentiated EM1 and CM subsets. Finally, the percentage change in cell numbers of the EMRA populations were also significantly greater than the equivalent EM subsets (EMRA2 to EM2; q%; $t_{(27)} = 3.4$, $p=.002$ and EMRA3 to EM3; q%; $t_{(27)} = 3.9$, $p=.001$). In most participants, the number of EM4 CD8TLs was too small to reliably calculate changes in cell number. Age, BMI and gender were included as potential modifiers in our statistical model. Controlling for these variables yielded comparable results.

Predictors of CD8TL stress reactivity

The relationship between CD8TL mobilization (increase in CD8TL number: æCD8TL) and baseline values of each CD8TL subset was examined using Pearsonôs correlation coefficient. As demonstrated in Figure 3, there was a strong positive correlation between heightened CD8TL reactivity and larger baseline values for EM3 cells ($r_{(26)} =.68$, $p<.001$) and EMRA3 cells ($r_{(26)} =.46$, $p=.014$).

βAR-agonist infusion study

βAR-agonist preferentially mobilized effector-memory CD8TLs and βAR-antagonist administration inhibited the response

Overall, the results of this study replicate the findings of the stress study. Table 3 demonstrates that the ϐAR-agonist infusion induced a pronounced lymphocytosis and CD8TL mobilization. It also increased all CD8TL effector memory subsets, apart from the EMRA1, and unexpectedly, the EM3 subtype, and induced a decrease in circulating NA and CM cell numbers. As illustrated by Figure 4, EMRA3 showed the greatest mobilization, followed by EM2 and EMRA2, and then the EM1 and EMRA1 subsets (in pairwise comparisons, all $p<.01$). Age, BMI and gender were again included as potential modifiers in our statistical model and similarly controlling for these variables yielded comparable results.

As can be seen in Table 3 and Figure 4, administration of the ϐAR-antagonist abrogated the effects of ϐAR-agonist infusion. These findings further implicate ϐAR mechanisms in CD8TL mobilization.

Again, a strong positive correlation between heightened CD8TL reactivity and larger baseline values for EM3 cells ($r_{(14)} =.48$, $p<.063$) and EMRA3 cells ($r_{(14)} =.85$, $p<.001$) was demonstrated.

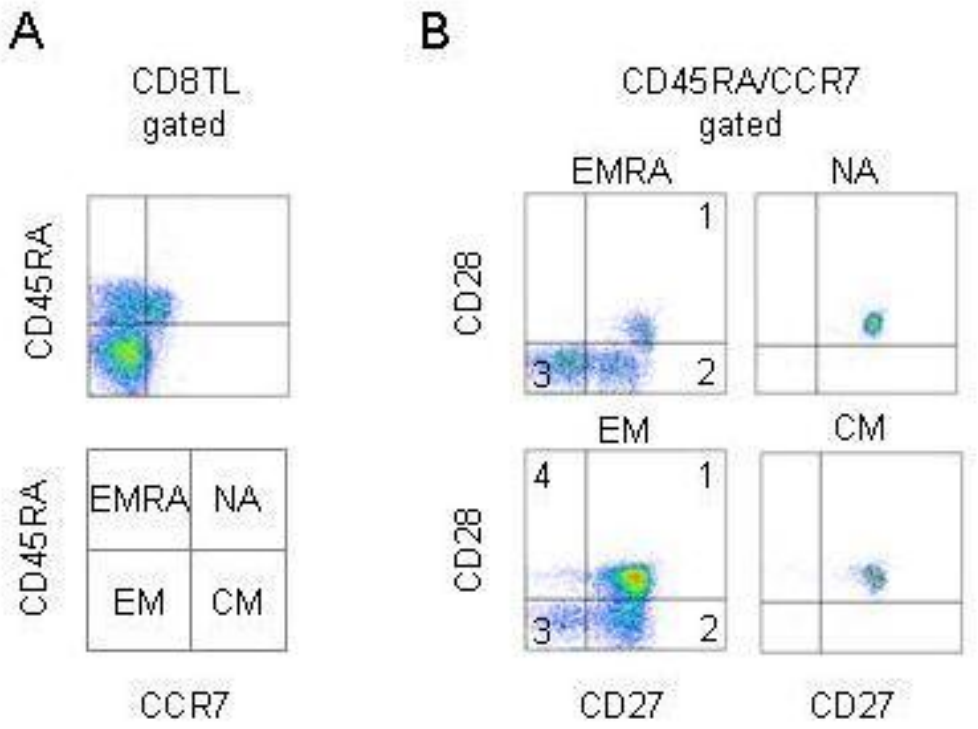

Figure 1. Identification of CD8TL subsets. A) $CD3^{+}CD8^{+}$ gated lymphocytes (CD8TLs) were separated into four subsets (NA, CM, EM and EMRA) based on surface expression of CD45RA and CCR7. B) The four subsets were further characterized for CD27 and CD28 expression, yielding nine populations of CD8TLs (NA, CM, EM1-4, EMRA 1-3)

Table 1. Description of CD8TL subsets grouped by phenotypic and functional similarities

CD8TL subtype	*Phenotype*	*Cell characteristics*	*Cytotoxic potential*	*Tissue migration potential*	*Antigen experience/ replicative history*
NA	$CD45RA^{+}CCR7^{+}CD27^{+}CD28^{+}$	Mature cell that has not yet contacted cognate antigen in the peripheral lymphoid organs	-	-	-
CM	$CD45RA^{-}CCR7^{+}CD27^{+}CD28^{+}$	Primed memory cell, low expression of effector mediators, rapidly proliferate and differentiate to effector cells following antigenic stimulation, short replicative history	+	+	+
EM1	$CD45RA^{-}CCR7^{-}CD27^{+}CD28^{+}$	Central memory-like cells: low expression of effector mediators; short replicative history	++	++	++
EMRA1	$CD45RA^{+}CCR7^{-}CD27^{+}CD28^{+}$			++	
EM4	$CD45RA^{-}CCR7^{-}CD27^{-}CD28^{+}$		++	++	
EM2	$CD45RA^{-}CCR7^{-}CD27^{+}CD28^{-}$	Primed memory cells, intermediate effector functions and replicative history, migrate to inflamed tissue	+++	+++	++
EMRA2	$CD45RA^{+}CCR7^{-}CD27^{+}CD28^{-}$			+++	
EM3	$CD45RA^{-}CCR7^{-}CD27^{-}CD28^{-}$	Primed effector-like memory cells, high effector mediator expression and cytolytic activity, extensive replicative history, inflamed tissue migratory	++++	++++	+++
EMRA3	$CD45RA^{+}CCR7^{-}CD27^{-}CD28^{-}$		+++++	++++	++++

Table 2. Mean (SD) cell numbers at each time point during the psychological stress study, with results from repeated measures ANOVA

Cell type (cells/µl)	*Baseline*	*Task*	*Recovery*	*F(df)*	*p*
Lymphocytes	1789 (426)	2271 (781)	1925 (579)	$F_{(2,54)} = 17.6$	<.001
CD8TLs	390.3 (122)	502.5 (184)	424.4 (135)	$F_{(2,54)} = 12.9$	<.001
NA	139.6 (93)	150.6 (100)	142.1 (86)	$F_{(2,54)} = 1.9$	=.155
CM	17.0 (7.7)	19.7 (12)	17.7 (9.0)	$F_{(2,54)} = 4.0$	=.025
EM1	96.0 (38)	109.7 (44)	104.6 (45)	$F_{(2,54)} = 4.0$	=.023
EM2	22.2 (17)	32.5 (22)	25.4 (19)	$F_{(2,54)} = 18.1$	<.001
EM3	15.2 (21)	32.6 (52)	19.2 (27)	$F_{(2,54)} = 7.7$	=.001
EMRA1	30.8 (21)	33.34(21)	31.6 (22)	$F_{(2,54)} = 2.3$	=.111
EMRA2	28.7 (20)	45.8 (38)	32.7 (26)	$F_{(2,54)} = 16.3$	<.001
EMRA3	30.9 (37)	64.1 (79)	39.5 (48)	$F_{(2,54)} = 12.6$	<.001

Table 3. Mean (SD) cell numbers at each time point during the βAR-agonist infusion and βAR-antagonist administration, with results from repeated measures ANOVA

	βAR-agonist and placebo				**βAR-agonist and βAR-antagonist**			
Cell type (cells/μl)	*Baseline*	*1st Infusion Rate*	*F(df)*	*p*	*Baseline*	*1st Infusion Rate*	*F(df)*	*p*
Lymphocytes	1722 (522)	2241 (676)	$F_{(1,11)} = 8.8$	=.013	1584 (369)	1627 (303)	$F_{(1,2)} = 8.3$	=.103
CD8TLs	405.9 (174)	549.6 (253)	$F_{(1,11)} = 7.2$	=.022	394.6 (147)	402.6 (132)	$F_{(1,2)} = 32.2$	=.030
NA	116.3 (87)	93.3 (75)	$F_{(1,11)} = 12.0$	=.005	119.6 (100)	110.7 (84)	$F_{(1,2)} = 29.4$	=.032
CM	18.2 (12)	15.5 (9.3)	$F_{(1,11)} = 15.7$	=.002	16.2 (7.2)	16.0 (7.7)	$F_{(1,2)} =7.0$	=.117
EM1	103.5 (57)	102.5 (52)	$F_{(1,11)} = 1.3$	=.278	87.9 (32)	87.5 (31)	$F_{(1,2)} = 3.8$	=.191
EM2	17.7 (12)	28.1 (18)	$F_{(1,11)} = 37.1$	<.001	20.9 (14)	23.9 (17)	$F_{(1,2)} = 3.4$	=.207
EM3	11.8 (13)	29.2 (34)	$F_{(1,11)} = 4.0$	=.070	8.3 (6.8)	10.8 (13)	$F_{(1,2)} = 0.2$	=.737
EMRA1	34.4 (31)	37.4 (38)	$F_{(1,11)} = 2.9$	=.119	30.1 (28)	28.9 (25)	$F_{(1,2)} = 0.6$	=.533
EMRA2	24.6 (12)	52.8 (31)	$F_{(1,11)} = 15.9$	=.002	28.8 (14)	30.4 (13)	$F_{(1,2)} = 2.3$	=.272
EMRA3	53.1 (51)	157.6 (158)	$F_{(1,11)} = 8.1$	=.016	53.1 (52)	62.3 (63)	$F_{(1,2)} = 0.4$	=.599

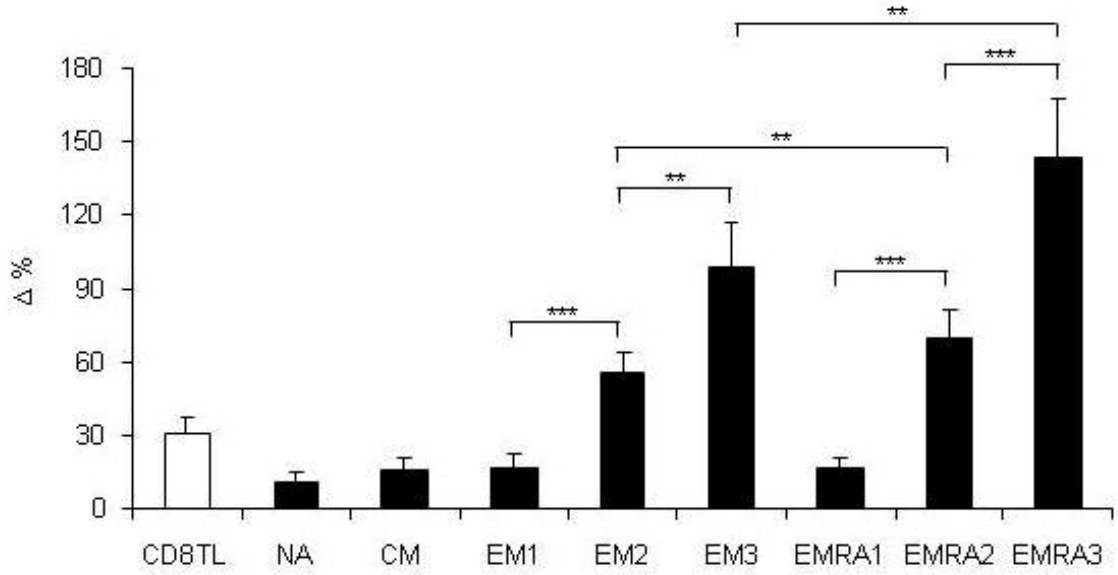

Figure 2. Effects of a psychological stressor on the mobilization of CD8TL subsets. Relative change in cell number (æ%; SEM) of the eight CD8TL subsets in response to stress. *p<.05, **p<.01, ***p<.001 indicates mobilization is significantly different between the individual CD8TL subsets.

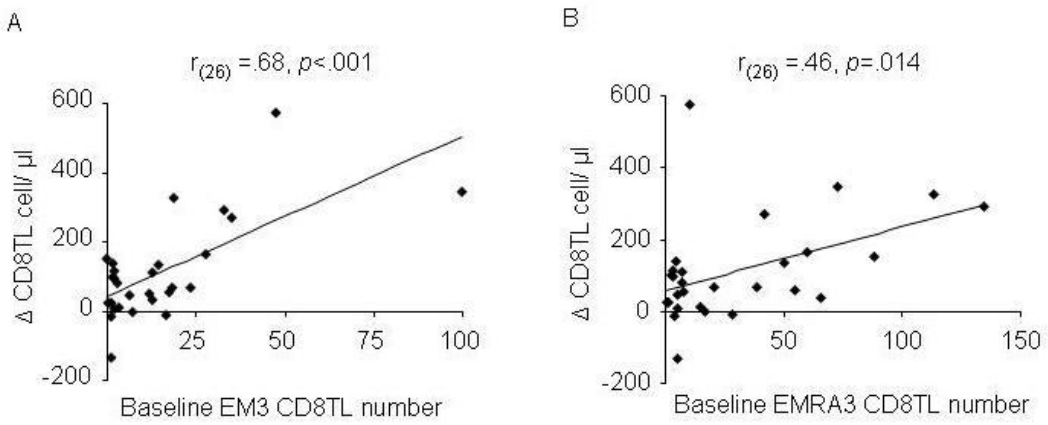

Figure 3. Scatter plots showing the relationship between CD8TL reactivity and baseline values of EM3 and EMRA3 CD8TL subsets.

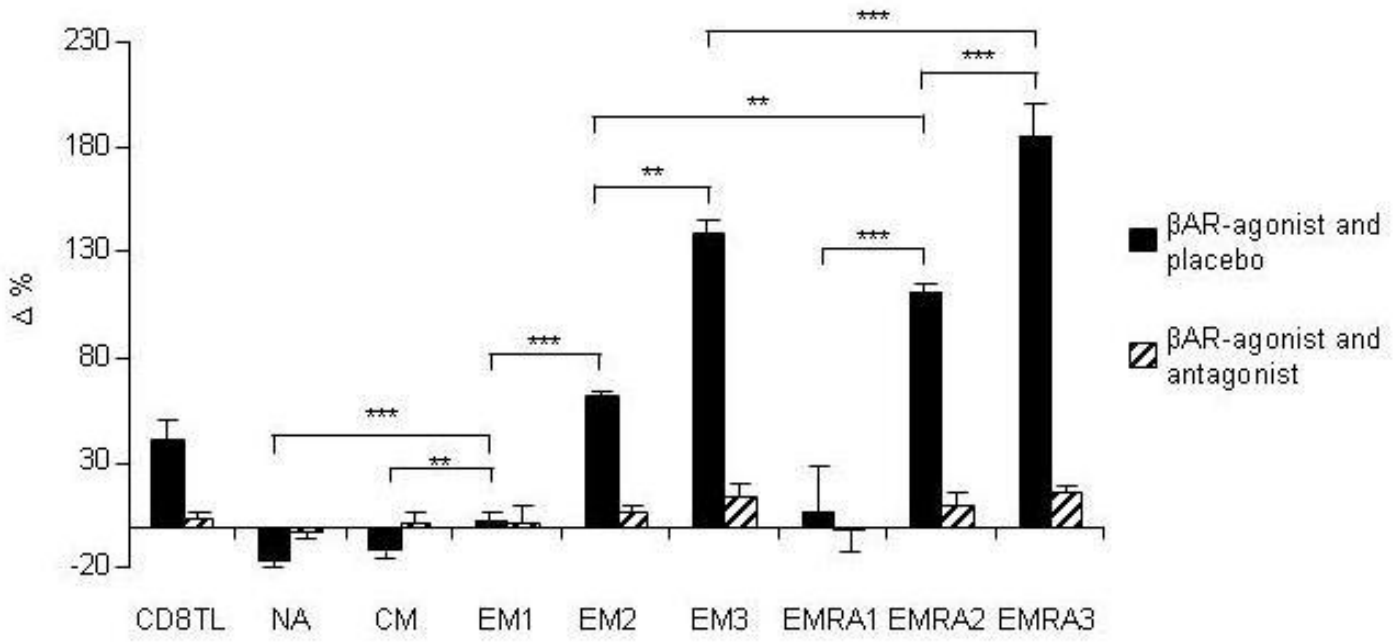

Figure 4. Effects of ßAR-agonist and ßAR-antagonist administration on the mobilization of CD8TL subsets. Relative change in cell number (æ%; SEM) of the eight CD8TL subsets in response to stress. *p<.05, **p<.01, ***p<.001 indicates mobilization is significantly different between the individual CD8TL subsets.

DISCUSSION

Acutely stressful events rapidly mobilize $CD8^+$ T cells (CD8TLs) into peripheral blood. In the present study, a detailed phenotypic analysis of this response found a marked specificity in the mobilization of certain memory phenotypes associated with the progressive loss of CCR7, CD28 and CD27. The largest increase during stress was seen for the memory subsets that exhibit a strong effector potential ($CCR7^-CD28^-CD27^-$ EM3 and EMRA3 cells); these are characterised by high tissue migratory ability, high cytotoxicity, and greater production of IFN-ɔ (Monteiro et al., 2007; Romero et al., 2007). Indeed, >67% of CD8TL stress reactivity may be explained by the combined mobilization of EM3 and EMRA3 subsets. Effector memory cells with a lesser effector potential (i.e. EM2 and EMRA2 cells) were mobilized to a lesser extent, and little or no change was seen for subsets that have little or no immediate effector potential (NA, CM, EM1 and EMRA1). This pattern of response was replicated by infusion of the ɓ-adrenergic receptor (ɓAR) agonist, isoproterenol, suggesting mediation by ɓAR mechanisms. Further, preferentially mobilized cells have demonstrated higher $ɓ_2$AR expression, suggesting that increase levels of this receptor may provide the selectivity of the mobilization response (Dimitrov et al., 2009; Holmes et al., 2005). Finally, the results showed that different markers can be used to identify sensitivity of CD8TL subsets to stress and ɓ-adrenergic stimulation; lack of CCR7 expression was common to all mobilized CD8TLs, whereas lack of CD28 expression identified the most sensitive cells.

The current datum confirms the proposition that stress and beta-adrenergic stimulation selectively mobilizes highly differentiated, effector-like, memory cells (Anane et al., 2010; Campbell et al., 2009; Dimitrov et al., 2010). Recent studies have provided a detailed characterization of the immunological properties of these mobilized subsets (Monteiro et al., 2007; Romero et al., 2007). Hence it is known that the mobilized cells have short telomere length (a marker of extensive replicative history) and decreased telomerase activity. These

cells also display other features of a senescent profile, such as a loss of a proliferative response to mitogens and impaired proliferation to antigen (however they may proliferate in response to homeostatic cytokines such as IL-7 and IL-15), reduced IL-2 production, and expression of NK cell receptors (CD57 and NKG2D) (Azuma et al., 1993; Brenchley et al., 2003; Champagne et al., 2001; Hamann et al., 1997; van Leeuwen et al., 2002; Voehringer et al., 2002). Further, the EM3 and EMRA3 subtypes have a ten-fold greater ex-vivo cytolytic capacity than the non-responsive EM1 subset, express the apoptosis inducer Fas-ligand, and are the main producers of pro-inflammatory mediators like IFN-y, RANTES, and macrophage inflammatory protein-1ß (Monteiro et al., 2007; Romero et al., 2007). The mobilized CD8TL subsets also display adhesion molecule (e.g., CD11a) and chemokine receptor (e.g., CCR5) profiles that are associated with a tissue homing potential, paralleled by low expression of lymph node homing markers, such as CD62L and CCR7 (Atanackovic et al., 2006; Dimitrov et al., 2010; Monteiro et al., 2007). Finally, the stress sensitive subsets are often enriched by cells specific for latent herpes viruses, such as Cytomegalovirus (Appay et al., 2002; van Lier et al., 2003). In summary, redistribution of CD8TLs by stress and ß-adrenergic stimuli is highly selective for late-differentiated cells that are characterised by vast and immediate effector function, tissue migration potential, altered proliferative capacity and cytokine production, and may often provide immunity to latent herpes viruses.

The current study provides an encompassing interpretation of previous observations of phenotypic and functional immune changes in response to acute stress (e.g. adhesion molecule expression, chemotaxis, proliferative capacity). Rather than reflecting changes in individual cells, our findings suggest the more parsimonious explanation that stress selectively mobilises cells already exhibiting exactly those specific phenotypic and functional characteristics. For example, instead of mobilized T cells ñalteringòtheir expression of adhesion molecules, stress preferentially mobilizes cells that already exhibit this (i.e., $CD62L^{low}$ and $CD11a^{high}$) receptor profile (Bosch et al., 2003a; Bosch et al., 2005; Mills et al.,

2000). This explanation can also be applied to previous observations that stress and adrenergic stimuli mobilize cells that are senescent (Simpson et al., 2007), express cytotoxic granules (Atanackovic et al., 2006), have potential for tissue migration, and express inflammatory mediators (Bosch et al., 2003a); these seemingly independent observations can be subsumed under the mobilisation of effector memory CD8TLs that exhibit this exact constellation of characteristics. Thus, selective mobilization of effector-memory CD8TLs unifies, and appears to at least in part account for, a large number of earlier observations. This would suggest that future studies examining functional immune changes during acute stress would do well to account for selective mobilisation, before making inferences about functional changes to individual cells.

It has been proposed that stress lymphocytosis enhances immune surveillance during perceived threat when the risk of injury and concurrent infection are heightened (Benschop et al., 1996; Dhabhar and McEwen, 1997; Segerstrom and Miller, 2004). However, some stressors, such as heatstroke, evoke lymphocytosis but are not typically accompanied by an increased risk of infection (Bouchama et al., 1992; Bruunsgaard et al., 1999; Karandikar et al., 2002; Simpson et al., 2007). It is plausible, therefore, that lymphocytosis may have an alternative role beyond antigen-specific immunosurveillance. Although CD8TLs typically require recognition of cognate antigen in order to extravasate and rapidly exert effector function in the tissue, there is evidence that they can ñsenseò non-specific signals from the environment (Kundig et al., 1996a; Kundig et al., 1996b; Ogasawara and Lanier, 2005). As expression of NK cell receptors are limited to differentiated CD8TL subsets, only stress sensitive cells exhibit innate-like cell functions (Bauer et al., 1999; Weng et al., 2009). Damaged or stressed tissue (e.g. caused by wounding, heat shock, malignant transformation or infection) induces the expression of cellular ñstressòsignals, such as MHC class I-related antigen (MICA), which can activate the NK cell receptors when present upon CD8TLs (Eagle et al., 2009; Ogasawara and Lanier, 2005). Through expression of NK cell receptors, stress

mobilized CD8TLs may therefore be able to enhance infectious and tumour immunity, and/or aid wound healing and the clearance of damaged tissue using both specific and non-specific pathways (Agaiby and Dyson, 1999; Diefenbach et al., 2001; Ogasawara and Lanier, 2005; Toulon et al., 2009). Indeed, stress induced immune cell redistribution is associated with improved wound healing following surgery (Rosenberger et al., 2009). It is important to note, however, that mobilization of cytotoxic CD8TLs with innate characteristics may also have detrimental effects. Increasing the surveillance of CD8TLs that are not regulated by antigen-specific activation could exacerbate inflammation and autoimmune disorders (Bosch et al., 2003a; Nyklicek et al., 2005; Straub et al., 2005; Weng et al., 2009). For example, stress increases leukocyte infiltration into inflamed areas (Sanders and Straub, 2002), and it is plausible that expression of NKG2D ligand in the affected tissue could interact with the effector-like CD8TLs (Eagle et al., 2009; Meresse et al., 2004; Ogasawara and Lanier, 2005). In addition, late-differentiated ($CD28^-$) CD8TLs have increased lymphocyte function associated antigen-1 (LFA-1) expression which lowers the T cell activation threshold and further predispose cells to the breakage of self-tolerance and the induction of autoimmunity (Weng et al., 2009; Yung et al., 1996). In summary, one may speculate that stress-induced CD8TL redistribution could have clinically relevant implications; however, the specific health outcome will likely depend on the type of immune response involved and the duration and timing of stress (Dhabhar, 2001; Dhabhar, 2009).

Some limitations should be noted. First, the ability of mobilized cells to exhibit effector functions was not assessed. However, the characteristics of the novel CD8TL subsets have been extensively described elsewhere (Monteiro et al., 2007; Romero et al., 2007). Secondly, the role of adrenergic receptor stimulation in the stress response could only be inferred from correlations with changes in cardiac sympathetic drive. However, the subsequent infusion and blockade study strongly supported this proposition. Additional

pharmacological studies (e.g., selective ßAR- antagonist infusion during stress) may further strengthen this evidence.

In conclusion, the present study demonstrates a preferential mobilization of effector memory CD8TL subsets during acute psychological stress and ßAR-agonist infusion, whilst naïve and regulatory memory CD8TL numbers remain largely unchanged. It is proposed that this immune alteration may serve a protective function although, dependent on the circumstance, this response may not always be beneficial. Further research into the antigen-specificity of these mobilised cells is warranted. In sum, the highly selectively and robust redistribution of effector memory CD8TLs is consistent with the notion that stress and sympathetic activation create a circulatory environment that is conducive to inflammation and microbial clearance.

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CHAPTER 3

CYTOMEGALOVIRUS INFECTION AS A DETERMINANT OF LYMPHOCYTE MOBILIZATION DURING STRESS AND BETA-ADRENERGIC STIMULATION

ABSTRACT

Objective: Recently we demonstrated that $CD8^+$ T Lymphocyte (CD8TL) subsets with a differentiated, effector-like phenotype are selectively mobilized by stress, exercise and ß-agonist infusion. Latent infection with Cytomegalovirus (CMV) dramatically increases the number and proportions of these responsive late-differentiated CD8TLs. Therefore, the current study tested if infection with CMV increased CD8TL responsivity to acute stress and ß-adrenergic stimulation. **Methods:** In two studies, we compared CD8TL mobilization in response to an acute stress (speech task; *n*=32) and ßAR-agonist infusion (isoproterenol infusion without (*n*=20) or with (*n*=8) prior administration of a ßAR-antagonist). CMV infection was measured by ELISA. CD8TLs, CD4 T lymphocytes (CD4TLs), NKT-like, and NK cells were determined by flow cytometry. The response of each lymphocyte subset was compared for CMV positive (CMV^{pos}) and CMV negative (CMV^{neg}) individuals. **Results:** CMV infection increased the mobilization of CD8TLs during stress ($F_{(1,30)} = 5.2$, p=.029), and correlated with greater baseline numbers of late differentiated cells ($r_{(18)} = .49$-$.82$, ps<.05). The enhanced CD8TL mobilization was replicated during ßAR-agonist infusion, and also found for cytotoxic CD4TL and NKT-like cells. Administration of the ßAR-antagonist propranolol abrogated the responses to infusion. **Conclusion:** This is the first study to demonstrate that infection

history is a potent determinant of immunological responses to stress and adrenergic stimulation.

INTRODUCTION

Stimuli that activate the sympathetic nervous system, such as acute psychological stress and exercise, rapidly invoke a robust mobilization of lymphocytes into the circulation via stimulation of lymphocyte ß$_2$-adrenergic receptors (ß$_2$AR) (Benschop et al., 1996a; Benschop et al., 1994a; Benschop et al., 1994b; Benschop et al., 1996b; Dimitrov et al., 2010; Pedersen and Hoffman-Goetz, 2000; Schedlowski et al., 1996; Segerstrom and Miller, 2004). This lymphocytosis predominantly involves cells exhibiting relatively high levels of ß$_2$AR and a cytotoxic ability, such as natural killer (NK) cells, gamma-delta (ɔǔ) T cells, and CD8$^+$ T lymphocytes (CD8TLs) (Anane et al., 2009; Campbell et al., 2009; Elenkov et al., 2000). Further, among these, only differentiated subsets with enhanced effector and tissue migrating potential are mobilized (Anane et al., 2010; Atanackovic et al., 2006; Bosch et al., 2005; Campbell et al., 2009; Dimitrov et al., 2010). These stress-sensitive CD8TL subsets also express heightened levels of the ß$_2$AR suggesting that enhanced expression may provide a mechanism for their selective mobilization (Dimitrov et al., 2009). Thus, stress enriches the blood with ß$_2$AR^{high} lymphocytes that are prone to engage in tissue migration, target cell killing and production of inflammatory cytokines (Benschop et al., 1996b; Bosch et al., 2005; Dhabhar and McEwen, 1997; Segerstrom and Miller, 2004).

The CD8TL population is highly heterogeneous and we recently reported (Chapter 2) that distinct subsets vary greatly in the ability to mobilize during stress. Stress-induced mobilization gradually becomes stronger in phenotypes that have accumulated more effector characteristics (e.g., cytotoxicity, IFN-y production) and a stronger tissue-migrating ability. This differentiation can roughly be monitored by the sequential loss of the surface markers CCR7, CD28, and CD27 (Monteiro et al., 2007; Romero et al., 2007; Sallusto et al., 1999). Thus, highly differentiated CD8TLs (EM3 or EMRA3 cells; CD45RA$^{+/-}$CCR7$^-$CD28$^-$CD27$^-$) show a substantially larger mobilization than intermediate-differentiated cells (EM2 or

EMRA2 cells; $CD45RA^{+/-}CCR7^{-}CD28^{-}CD27^{+}$), while early-differentiated cells (EM1 or EMRA1; $CD45RA^{+/-}CCR7^{-}CD28^{+}CD27^{+}$), central memory cells (CM; $CD45RA^{-}CCR7^{+}CD28^{+}CD27^{+}$) and naïve CD8TLs (NA; $CD45RA^{+}CCR7^{+}CD28^{+}CD27^{+}$) demonstrate little to no mobilization.

The composition of the CD8TL memory pool, and therefore potentially the CD8TL response to stress and adrenergic stimulation, is driven to a large extent by infection history (Appay et al., 2002a; van Lier et al., 2003). For example, latent infection with Cytomegalovirus (CMV) dramatically increases the number and proportions of late-differentiated, and stress-responsive, CD8TLs (Gillespie et al., 2000; Khan et al., 2002). CMV is a herpes virus that is carried by 30-70% of western populations, and infection rate is increased by age and with various social-demographic factors (e.g., higher infection rates with lower socio-economic status) (Dowd et al., 2009; Gillespie et al., 2000; Khan, 2007; Khan et al., 2002). After primary infection, the virus remains dormant in the body (latency), where it is believed to intermittently reactivate (van Lier et al., 2003). The resulting repeated activation of CMV-specific CD8TLs is thought to underlie differentiation of these cells into a so-called late differentiated phenotype (i.e., $CCR7^{-}CD27^{-}CD28^{-}$) (Appay et al., 2002a; Khan et al., 2007; Romero et al., 2007; van Lier et al., 2003). Thus, the primary hypothesis tested in the current study is that CD8TL mobilization in response to stress and adrenergic receptor stimulation will be amplified in CMV infected individuals. Further, it was hypothesised that the increased response will be associated with greater numbers of late differentiated (EM3 and EMRA3) CD8TLs.

CMV infection does not only alter the composition of the CD8TL memory pool, but also causes accumulation of cytotoxic $perforin^{+}$ CD4TLs, a minor subset of CD4TLs, and natural killer-like T cells (NKT-like, i.e., $CD56^{+}$ T cells) (Gamadia et al., 2004; Pourgheysari et al., 2007; Saez-Borderias et al., 2006; Tarazona et al., 2000). Similar to CMV-specific CD8TLs,

CMV-specific CD4TLs are often highly differentiated ($CD28^{-}CD27^{-}$) $perforin^{+}$ cells that likewise exhibit an enhanced sensitivity to stress and adrenergic stimulation (Anane, In preparation; Appay et al., 2002b; Casazza et al., 2006; Fletcher et al., 2005). NKT-like cells can be mobilized by adrenaline infusion and thus are also expected to be mobilized by acute stress and βAR-stimulation (Dimitrov et al., 2010). Thus a second hypothesis was that mobilization of NKT-like and $perforin^{+}$ CD4TLs will also be amplified by CMV infection. This study presents the first investigation into the effects of viral infection on immunological sensitivity to stress and neuroendocrine stimulation.

METHODS

Participants

Participants were recruited from community volunteers and staff and students attending the University of Birmingham (UoB), UK, or the University of California San Diego (UCSD), USA. All study volunteers reported to be in good health and were non-medicated with the exception of the contraceptive pill. Participants were instructed not to engage in strenuous physical exercise and to refrain from consuming alcohol or non-prescription drugs 24 hours before their experimental session, and to abstain from smoking and caffeine on the day of the experiment. Student volunteers performing the psychological stress task and all volunteers undertaking the infusion procedure received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the appropriate institutional review boards (UoB or UCSD).

Psychological stress study

Procedure

32 volunteers took part (age 25.8 years, SD ±8.0; 23 female). Upon arrival at the Exercise and Behavioural Immunology Laboratory, UoB: (1) electrodes for electrocardiography (ECG) and impedance cardiography (ICG) were attached; (2) a 20-gauge intravenous cannula (Becton-Dickinson) was placed into a palpable vein in the lower arm; and (3) an occluding cuff was placed over the brachial artery of the other arm for systolic (SBP) and diastolic (DBP) blood pressure measurements. Subsequently, while seated in a comfortable upright position, participants filled out several questionnaires and engaged in leisure reading. After 20 minutes, a baseline blood sample was obtained and the procedure for the laboratory stressor was initiated.

Public Speaking Task

To induce stress, participants performed two back-to-back speeches, each with 2 minutes of preparation and 4 minutes of speech delivery (Bosch et al., 2005; Bosch et al., 2003b). Social stress was enhanced by recording the speeches on videotape and by the attendance of an audience of three. For the first speech, the participant had to defend him/herself from being falsely accused of shoplifting (Saab et al., 1989) and, for the second speech, the participant discussed his or her best and worst personal characteristics (van Eck et al., 1996). Instructions for the task were presented via a DVD recording, which ensured standardization of instructions and timing of the tasks. Including instructions, the task lasted 15 minutes. A blood sample was obtained during the second presentation, 13 minutes after initiation of the task. Following the task, the participants again engaged in leisure reading, and a final blood sample was obtained after 15 minutes of recovery.

Cardiovascular assessment

Assessment of cardiovascular responses focused on cardiac sympathetic and vagal control as previously described (Berntson et al., 1993; Bosch et al., 2003a). In brief, indices of sympathetic and parasympathetic drive were obtained by analysis of ECG and ICG signals.

The thoracic ICG and ECG signals were recorded from six Ag-AgClspot-electrodes (AMI type 1650-005, Medtronic) using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) device. The ECG and ICG complexes were ensemble averaged with reference to the ECG R-wave across 30-sec periods. From these 30-sec ensembles, average levels were computed for heart rate (HR) and pre-ejection period (PEP). These means were further averaged over a 6-min pre-task baseline and over each 6-min task. Changes in PEP were used to index changes in cardiac sympathetic drive, whereas heart rate variability, or Root Mean Square of Successive Difference (RMSSD), was used to index changes in cardiac vagal tone.

βAR-agonist infusion study

Procedure

20 volunteers took part (age 35.9 years, SD±9.3; 8 female). βAR-agonist (isoproterenol) infusion was performed according to a standardized protocol (Mills et al., 2000; Mills et al., 1997). In brief, upon arrival at the laboratory, UCSD, participant height and weight were taken to ensure correct calculation of body surface area (BSA) used to determine the βAR-agonist infusion rate. Subsequently, participants were in a semi-supine position for 15 minutes following placement of: 1) two 22-gauge intravenous cannulas (Becton-Dickinson) of which one was for drawing blood samples and one was for βAR-agonist infusion, inserted into a palpable veins in opposite lower arms; 2) three spot ECG electrodes; and 3) an occluding cuff on the non-infusion arm.

βAR-agonist was infused at incremental rates (0.1µg, 0.5µg and 1µg /min/1.73 m^2 BSA for 5 minutes each) for 15 minutes until the participants heart rate had increased by ~20 beats per minute (bpm) compared to resting heart rate. The final infusion rate was maintained for 10 minutes. On average, the maximal dose reached 1µg/min/1.73 m^2 BSA. Blood was taken prior to initiation of isoproterenol infusion (β baseline) and in the final minutes of the infusion.

ECG, heart rate and blood pressure were monitored throughout the infusion. The half-life of isoproterenol is approximately 2ï 3 min (Goebel et al., 2000). The infusion was generally well-tolerated and all participants successfully completed the protocol.

βAR-antagonist (blockade) procedure

A sub group of 8 participants (age 34.6 ±11.5 years, 2 female) underwent the infusion twice following a 5-day course of either of 80mg of the ßAR- antagonist propranolol or placebo. Condition was counter balanced and single blinded.

Questionnaires

In both studies, health and lifestyle variables were assessed by self-report questionnaire. Variables included recent symptoms of illness, exercise behaviour, alcohol consumption, caffeine consumption, smoking, and use of recreational drugs. Affective responses to the psychological stress task were assessed using the short-form of the Profile of Mood States (POMS) (McNair et al., 1992). Participants completed the POMS at baseline, immediately post-task and at 15-minutes recovery.

Flow cytometry

Blood was collected into EDTA tubes, maintained at room temperature and processed within 4 hours after collection. Whole blood (100µl for CD8TL enumeration, 60µl for CD4TL enumeration and 25µl for NK/ NKT-like cell enumeration) was incubated for 20 minutes at 4°C in the dark with a cocktail of fluorescent-labelled monoclonal antibodies: CD27-FITC, CD28-FITC, CD56-PE, perforin-PE, IgG2b-PE, CD3-PerCP, CD4-APC, CD27-APC, CD8-APC-Cy7, (obtained from Becton-Dickinson, Oxford, UK), CCR7-PE (eBioscience, purchased from Insight Biotechnology Ltd, Middlesex, UK) and CD45RA-pacific blue (Invitrogen, Paisley, UK). Subsequently, red blood cells were lysed and removed by centrifugation (283G, 7-min at room temperature) following incubation with FACS Lysing

solution (Becton Dickenson, Oxford, UK). The remaining cell pellet was re-suspended in 100-250ul PBS containing 1.5% paraformaldehyde, and stored in the dark at 4°C until analyses. Preparations were read within 18 hours using either a dual-laser flow cytometer (FACS-Canto II; Becton Dickinson). Data were analyzed using Flowjo 7.4 (Treestar Inc, Ashland, OR, USA). A complete white blood cell count was obtained for each blood sample using a haematology analyzer (Coulter ACT^{diff} (Beckman Coulter, High Wycombe, UK).

Assessment of CMV serostatus

Anti-CMV Immunoglobulin G (IgG) in EDTA plasma was measured using a commercially available Enzyme-linked Immunosorbent Assay (ELISA) (Biocheck Inc, Forster City, CA) according to the manufacturerõs instructions. The presence of CMV-binding IgG (seropositive, CMV^{pos}) indicates that an individual has been infected with CMV.

Statistical analysis

Repeated-measures Analysis of Variance (ANOVA) was used to assess changes in mood, cardiovascular parameters, and immunological measures. For pairwise comparisons, student t-tests were used. Variations in degrees of freedom reflect occasional missing data. Where means are presented, standard deviations (SD) were added in brackets while Standard Errors of Mean (SEM) are presented in Figures. Data were analyzed using SPSS 16 for Windows (SPSS Inc, Chicago, Illinois).

RESULTS

Psychological stress study

Anxiety and cardiovascular responses

Increases in the tension-anxiety POMS subscale confirmed that the speech tasks were perceived as stressful (+7.8 (±4.6); $F_{(1, 34)} = 99.2$, $p<.001$). As Figure 1a demonstrates, a physiological stress response was shown by the significant increases in SBP (+25.7 (±8.2) mmHg; $F_{(1,35)} = 353.7$, $p<.001$), DBP (+16.8 (±8.3) mmHg; $F_{(1,35)} = 146.5$, $p<.001$), and heart rate (+21.2 (±12.0) bpm; $F_{(1, 35)} = 113.5$, $p<.001$), reflecting an increase in sympathetic drive as evidenced by a decrease in PEP (ï 11.6 (±12.1) ms; $F_{(1, 24)} = 23.2$, $p<.001$). Additionally, there was a significant decrease in RMSSD (ï 23.7 (±21.9) ms; $F_{(1,25)} = 25.5$, $p<.001$), reflecting vagal withdrawal. After a 15-min post-task recovery, all cardiovascular and autonomic measures had returned to baseline values. Further analyses confirmed that the task evoked a similar cardiovascular response within both serostatus groups (F values range between .03 - . 196, all *p*ôs >.05).

CMV serostatus is associated with enhanced mobilization of CD8TLs during psychological stress

As can be seen in Figure 1b, the speech task induced a statistically significant increase in lymphocyte, total CD8TL, NK cell and NKT-like cell numbers. Repeated measures ANOVA yielded a significant time by serostatus interaction for total CD8TLs ($F_{(1,30)} = 5.2$ $p=.029$), related to a larger increase in seropositive individuals. Further, a similar enhanced mobilization pattern was observed for lymphocytes ($F_{(1,30)} = 3.1$, $p=.090$) and NKT-like cells ($F_{(1,16)} = 2.68$ $p=.121$) within the CMV^{pos} group. Within the various CD8TL subsets, shown in Table 1, a significant time by serostatus effect was also seen for the EM3 and the EMRA3 CD8TL subsets. After adjustment for baseline values, this interaction remained significant for total CD8TLs and EM3 CD8TLs. The CMV^{pos} and CMV^{neg} groups were similar in terms of age, BMI, sex, and smoking distribution, and, unsurprisingly, the above results remained essentially unaltered when these variable were entered as covariates.

Figure 2 demonstrates significant results of bivariate correlations; an association existed between greater CD8TL stress reactivity and baseline values for NA ($r_{(18)}$ = -.33, p=.017), EM3 ($r_{(18)}$ =.82, p<.001) and EMRA3 CD8TLs ($r_{(18)}$ =.49, p=.027).

βAR-agonist infusion study

CMV serostatus is associated with enhanced mobilization of CD8TLs, NKT-like, and cytotoxic CD4TL cells during βAR-agonist infusion

Overall, the results of the βAR-agonist infusion study replicated the findings of the stress study. Figure 3 demonstrates that the βAR-agonist infusion induced a pronounced lymphocytosis and mobilization of CD8TLs, NK cells and NKT-like cells. Analyses of time by serostatus interactions confirmed a significantly greater mobilization for CMV^{pos} individuals of lymphocytes ($F_{(1,17)}$ = 4.9, p=.041), CD8TLs ($F_{(1,17)}$ = 24.1, p<.001) and NKT-like cells ($F_{(1,17)}$ = 12.9, p=.002). The CD8TL and NKT-like cell results remained unaltered after correcting for baseline numbers. Table 2 compares the change in cell number between the CMV^{pos} and CMV^{neg} group for CD8TL and CD4TL subsets. A significant time by serostatus interaction was found for EM3 CD8TLs, EMRA2 CD8TLs and EMRA3 CD8TLs. Subsequent analyses was performed on CD4TL subsets, despite not finding a main effect of time for total CD4TLs, as we have recently demonstrated that minor cytotoxic CD4TL subsets are stress sensitive (Anane, In preparation). A significant time by serostatus interaction was found for $CD27^{\bar{I}}$ CD4TLs, both $perforin^{+}$ and $perforin^{\bar{I}}$. After adjusting for baseline values, these interaction effects remained significant for EM3 CD8TLs, EMRA2 CD8TLs, total $CD27^{\bar{I}}$ CD4TLs and $perforin^{\bar{I}}$ $CD27^{\bar{I}}$ CD4TLs. Mean BMI, sex, and smoking distribution were again similar in the CMV^{pos} and CMV^{neg} group but, on this occasion, the CMV^{pos} were significantly older than the CMV^{neg} group ($F_{(1,18)}$ = 5.6, p=.03). Therefore, all analyses were repeated with age as a covariate and it was found that results were unaffected.

Figure 2 demonstrates significant results of bivariate correlations. Similar to the stress study, an association existed between greater CD8TL stress reactivity and baseline levels of EM3 CD8TLs ($r_{(15)}$ =.46, p<.001) and EMRA3 CD8TLs ($r_{(15)}$ =.86, p=.027).

Administration of the ßAR-antagonist uniformly abrogated the effects of ßAR-agonist infusion (data not shown) with the exception of NKT-like cell mobilization ($F_{(1,6)}$ = 8.9, p=.025), however the percentage change in cell number was small. Further, there was no difference in mobilization of NKT-like cells between the CMV^{pos} and CMV^{neg} groups ($F_{(1,6)}$ = 5.2, p=.063).

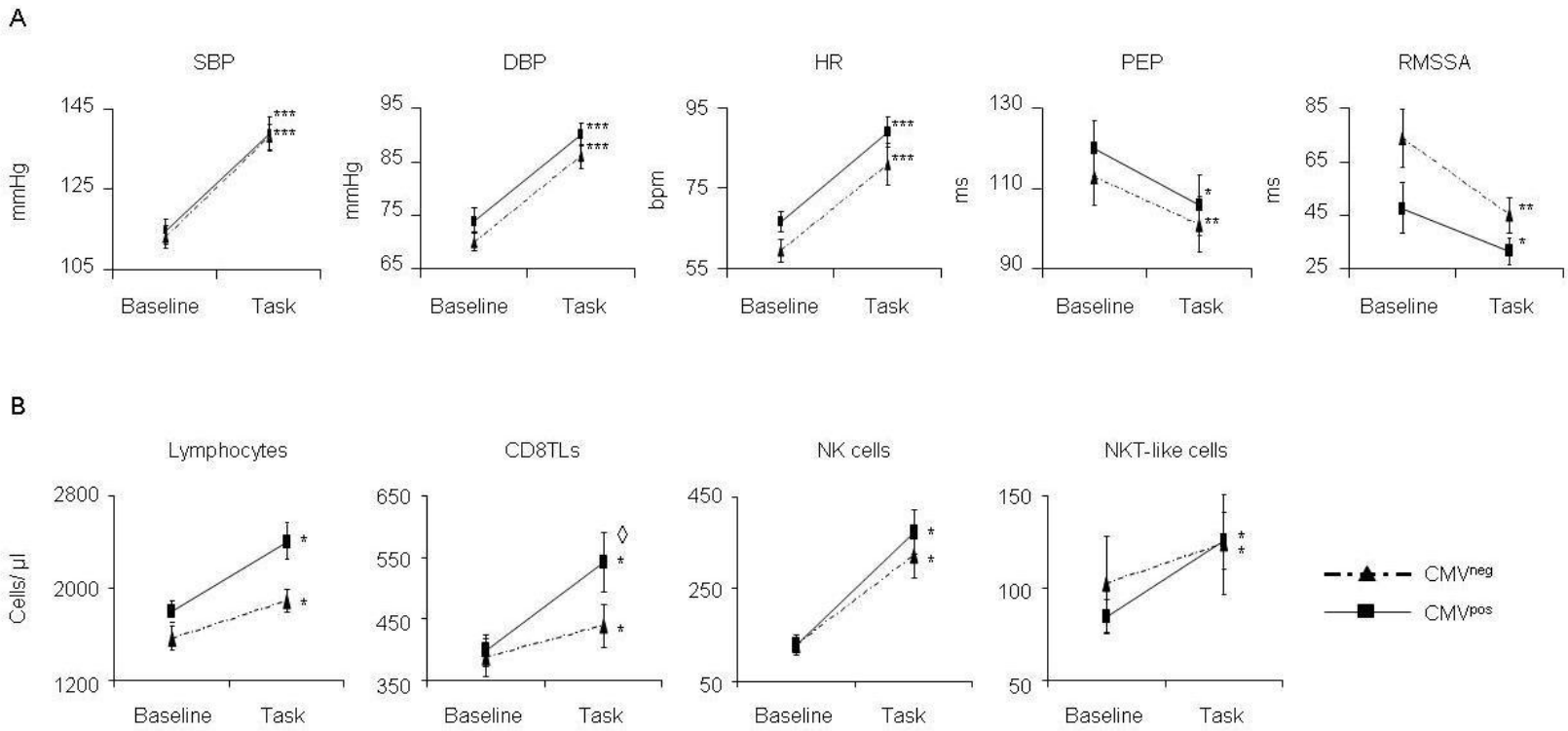

Figure 1. Effects of psychological stress on cardiovascular parameters (A) and on the mobilization of lymphocyte subsets (B). Lines indicate means (±SEM). **p*<.05, **p*<.01 and ****p*<.001 indicates main effects of time for each group (CMV^{pos} and CMV^{neg}). ö *p*<.05 indicates time by serostatus interaction. Results are from repeated measures ANOVA.

Table 1. Mean (±SD) $CD8^+$ T lymphocyte (CD8TL) subsets in CMV negative and CMV positive individuals at baseline, during the psychological stress task and following a 15 minute recovery, with results from repeated measures ANOVA.

	CMV negative		*CMV positive*		*Time * CMV*	
Cells/ µl	Baseline	Task	Baseline	Task	F(df)	*p*
CD8TL Subsets						
NA	157 (27)	158 (87)	128 (84)	150 (109)	$F_{(1,19)} = 2.77$	=.112
CM	17.1 (4)	18.3 (5)	18.9 (8.8)	24.8 (18)	$F_{(1,19)} = 2.79$	=.111
EM1	99.9 (31)	111 (39)*	116 (35)	137 (54)	$F_{(1,19)} = 0.67$	=.424
EM2	16.6 (9)	25.1 (16)***	30.4 (16)	42.5 (17)	$F_{(1,19)} = 0.64$	=.435
EM3	3.77 (3.4)	7.94 (8.8)**	36.2 (28)	78.7 (8.8)*	$F_{(1,19)} = 6.69$	=.018
EMRA1	29.9 (12)	32.4 (11)	26.2 (9.4)	28.0 (8.6)	$F_{(1,19)} = 0.15$	=.705
EMRA2	21.5 (8.6)	33.4 (14)***	27.3 (15)	39.6 (10.4)**	$F_{(1,19)} = 0.01$	=.909
EMRA3	5.86 (21)	13.9 (4.5)***	46.9 (24)	91.8 (46)**	$F_{(1,19)} = 12.86$	=.002

*p<.05, **p<.01 and ***p<.001 indicates a main effect for time within the participant group (CMV positive or CMV negative).

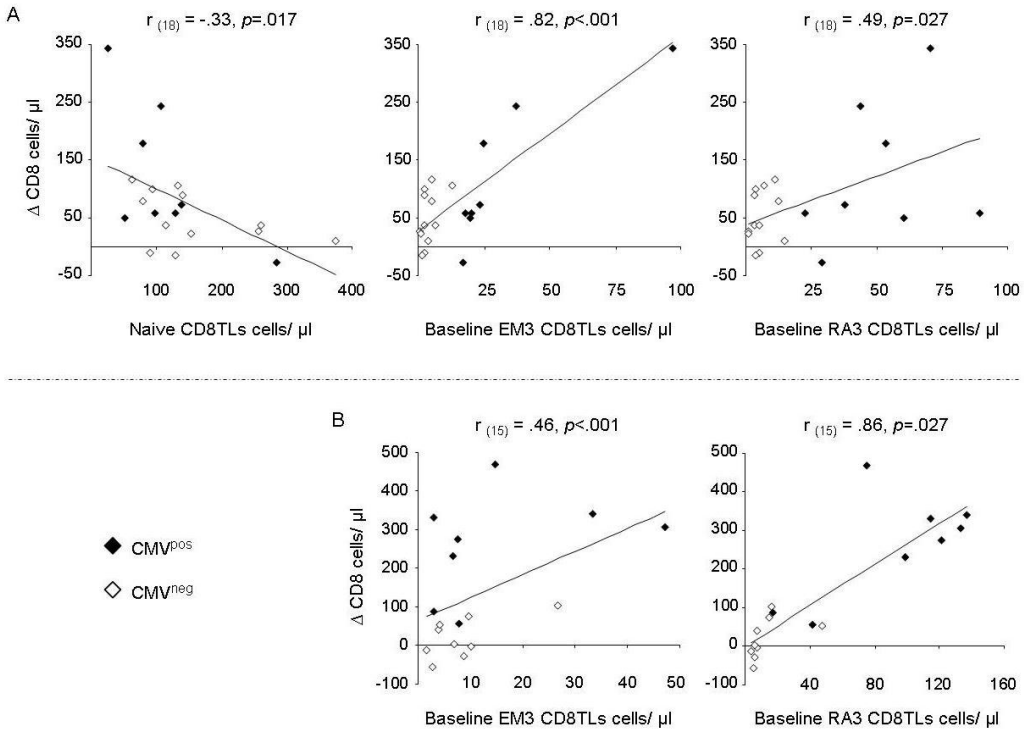

Figure 2. Scatter plots showing significant relationships between CD8TL reactivity and baseline values of CD8TL subsets during psychological stress (A) and ϐAR-agonist infusion (B).

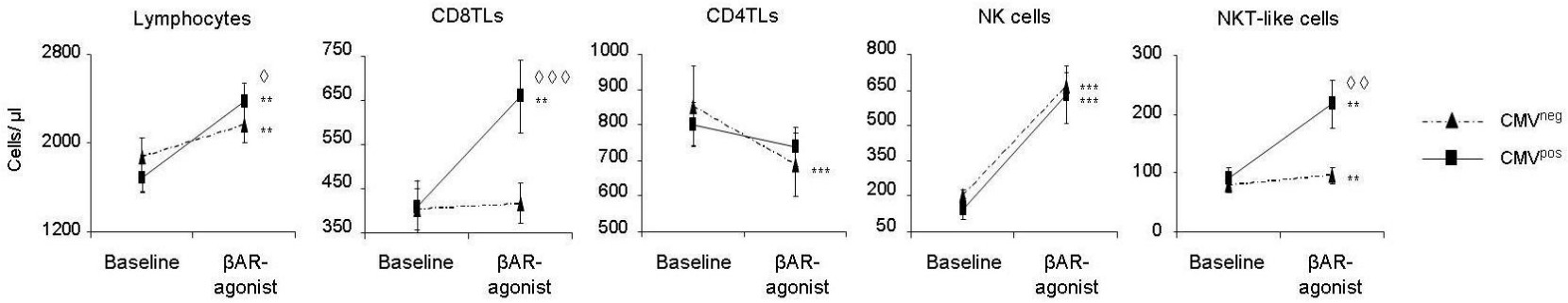

Figure 3. Effects of ß-adrenergic receptor (ßAR) agonist infusion on the mobilization of lymphocyte subsets. Lines indicate means (±SEM). **p*<.05, **p*<.01 and ****p*<.001 indicates main effects of time for each group (CMV^{pos} and CMV^{neg}). ö*p*<.05, öö*p*<.01 and ööö*p*<.001 indicates time by serostatus interaction.

Table 2. Mean (±SD) $CD8^+$ T lymphocyte (CD8TL) and $CD4^+$ T lymphocytes (CD4TL) in CMV negative and CMV positive individuals at baseline and during ß-AR agonist infusion, with results from repeated measures ANOVA.

	CMV negative		*CMV positive*		*Time*CMV*	
Cells/ µl	Baseline	1st Infusion	Baseline	1st Infusion	F(df)	*p*
CD8TL Subsets						
NA	122 (63)	95.7 (43)*	111 (107)	95.6 (101)*	$F_{(1,16)} = 0.30$	=.875
CM	21.3 (13)	18.9 (10)	16.1 (11)	13.7 (8.9)*	$F_{(1,16)} = 0.14$	=.709
EM1	134 (75)	127 (66)	88.0 (46)	88.5 (41)	$F_{(1,14)} = 1.60$	=.227
EM2	25.5 (14)	36.8 (20)**	11.9 (5.9)	20.8 (11)**	$F_{(1,14)} = 0.06$	=.807
EM3	8.2 (7.6)	15.6 (15)*	15.4 (16)	42.5 (42)*	$F_{(1,14)} = 8.26$	=.012
EMRA1	34.9 (30)	34.2 (30)	34.0 (32)	40.3 (45)	$F_{(1,14)} = 0.94$	=.348
EMRA2	21.7 (9.6)	37.2 (17)***	27.4 (13)	68.0 (36)**	$F_{(1,14)} = 6.54$	=.023
EMRA3	12.9 (14)	31.1 (29)**	92.4 (44)	282 (130)**	$F_{(1,14)} = 26.49$	<.001
CD4TL Subsets						
$CD27^+$	800 (354)	641 (287)***	697 (181)	608 (160)*	$F_{(1,16)} = 0.90$	=.356
$CD27^-$	53.4 (21)	46.3 (22)**	105 (63)	131 (67)*	$F_{(1,16)} = 8.82$	=.009
$CD27^-$ $perforin^+$	0.39 (.31)	0.50 (0.33)	2.28 (1.4)	5.59 (3.4)**	$F_{(1,16)} = 17.07$	=.001
$CD27^-$ $perforin^-$	53.0 (21)	45.8 (22)**	103 (62)	126 (66)*	$F_{(1,16)} = 6.30$	=.023

*p <.05, **p<.01 and ***p<.001 indicates a main effect of time within the participant group (CMV^{pos} vs. CMV^{neg}).

DISCUSSION

The present study investigated the effects of CMV serostatus on the mobilization of lymphocyte subsets. During acute psychological stress, CMV^{pos} individuals demonstrated an enhanced mobilization of CD8TLs. This enhanced reactivity was unrelated to autonomic, cardiovascular or psychological responses, and withstood correction for initial values (total CD8TLs at baseline), gender, age, and BMI. Further, the results were replicated by ß-AR-agonist infusion, and blocked by ß-AR-antagonist administration, indicating involvement of ß-adrenergic mechanisms. To the best of our knowledge, this is the first study to demonstrate that infection history is a determinant of immune system responses to acute stress and neuro-endocrine stimulation.

Replicating prior findings (Gillespie et al., 2000; Khan et al., 2002), we found that CMV serostatus was associated with substantial differences in the composition of the CD8TL memory pool. For example, CMV^{pos} individuals had between 7 and 11 times more EM3 and EMRA3 CD8TLs than did CMV^{neg} individuals. As these subsets express greater levels of $ß_2$AR and respond sensitively to adrenergic stimuli such as stress (Chapter 2) and exercise (Campbell et al., 2009), it was anticipated that CMV^{pos} individuals would show an enhanced CD8TL response to stress and beta-adrenergic stimulation. Further, greater numbers of stress sensitive late-differentiated cells at baseline predicted an enhanced CD8TL mobilization, whilst high numbers of stress-unresponsive naive CD8TLs correlated with low mobilization. These analyses suggest that the enhanced CD8TL reactivity among CMV^{pos} individuals, although unrelated to the total number of CD8TLs, is largely explained by a greater proportion of these CD8TLs being of a stress-responsive late-differentiated phenotype. The results of the ß-AR-agonist infusion similarly showed a substantially larger increase of EM3, EMRA2 and EMRA3 CD8TLs in seropositive individuals. In sum, CMV infection does substantially enhance the reactivity of the CD8TL population during stress and

βAR stimulation. Accumulation of late-differentiated CD8TL due to the latent infection may to a large extent explain this phenomenon.

Further exploration of our data showed that NKT-like ($CD56^{+}CD3^{+}$) cells were successfully mobilized by acute psychological stress and βAR-stimulation and that $CD27^{-}$ CD4TL numbers were increased by βAR-agonist infusion. Further, CMV infection enhanced the βAR-induced mobilization of NKT-like cells and $CD27^{-}$ CD4TLs. Consistent with the literature, we found that CMV infection increased the number of $perforin^{+}$ and $perforin^{-}$ $CD27^{-}$ CD4TLs (Casazza et al., 2006; Fletcher et al., 2005; Pourgheysari et al., 2007; Saez-Borderias et al., 2006). We recently demonstrated that the cytotoxic CD4TL subset sensitively mobilizes to stress (Anane, In preparation). On that basis, we anticipated that the mobilization of $perforin^{+}CD27^{-}$ CD4TLs would also be particularly enhanced in CMV^{pos} individuals. However, the $perforin^{+}CD27^{-}$ and the $perforin^{-}$ $CD27^{-}$ CD4TL subsets demonstrated greater mobilization in CMV^{pos} participants. Thus, the enhanced response of $CD27^{-}$ CD4TLs in the CMV^{pos} group may be explained by differentiation of CD4TLs (as demonstrated by loss of CD27 expression) as a result of the infection, but is not necessarily related to cytotoxicity of the cell.

Although still speculative at this point, the increased numbers of stress-sensitive T cells due to CMV infection, and subsequent higher stress-reactivity, may impact the efficiency of immunological processes, such as inflammation. $CD28^{-}$ T cells are readily activated by inflammation and can thereafter perpetuate and amplify the inflammatory response (Goronzy and Weyand, 2003; Weng et al., 2009). For example, late-differentiated T cells express stimulatory NK receptors, such as CD161 and CD144, which reduce the threshold of antigen-specific activation and, under certain circumstance, may render cell activation independent of recognition of cognate antigen (Groh et al., 2003; Groh et al., 2001; Meresse et al., 2004; Nakajima et al., 2002; Saez-Borderias et al., 2006; Speiser et al., 2001; Verneris et al., 2004). The inflammatory potential of mobilized cells is consistent with the idea that acute

stress may promote the migration of inflammatory cells and cause exacerbation of conditions such as atherosclerosis (Bosch et al., 2003a; Bosch et al., 2003b; Marsland et al., 2002; Nyklicek et al., 2005).

The dramatic effects of CMV on the memory T cell population may, apart from CD8TL mobilization, influence other immunological parameters frequently studied in psycho-neuro-immunology (PNI). This may include *in-vivo* measures such as vaccine responses, as well as *in-vitro* tests, such as mitogen-induced proliferation. For example, both CMV infection and the associated accumulation of differentiated T cells are linked with poorer vaccines responses (Saurwein-Teissl et al., 2002; Trzonkowski et al., 2003). Likewise, late-differentiated T cells lack the ability to proliferate to mitogen (Effros, 1997). Thus CMV infection may be a relevant confounding factor in PNI research, in particular since a number of characteristics, such as socio-economic status and ethnicity, have been associated with CMV infection and correlate with both psychological and physical health (Aiello et al., 2009; Czernochowski et al., 2008; Gallagher et al., 2009; Glaser et al., 2000; Gouin et al., 2008; Lupien et al., 2007; Nelson et al., 2007; Pawelec et al., 2005; Trzonkowski et al., 2003; Wikby et al., 2005; Zajacova et al., 2009). Taking CMV serostatus into account may thus potentially partly explain previous observations in this field.

A few limitations of the study are noted. Firstly, the enhanced NKT-like cell mobilization in CMV^{pos} individuals did not quite reach significance in the psychological stress study. It is likely that this is due to a lack of power, as the pattern of responses between the two studies was remarkably consistent. Second, from the data obtained, any potential mechanism that may change mobilization potential in CMV infected individuals, other than altered baseline numbers, can not be determined. For example, it is possible that CMV-specific cells have greater stress sensitivity. Third, CD4TL mobilization was only assessed in the ßAR-agonist infusion study and thus, it may only be inferred that psychological stress may induce a similar

heightened $CD27^{-}$ CD4TL mobilization in CMV^{pos} individuals. Finally, only young (<48 years) healthy participants were included in the study. On the basis of the knowledge that cell differentiation, and thus cell mobilization, is related to age and CMV infection, it would be interesting to assess difference in cell mobilization in an older population.

In sum, the current study demonstrates that CMV infection enhances the stress reactivity of T cell subsets. The result may have implications for health outcomes such as progression of inflammatory diseases. Although CMV is often studied due to its immunodominance, it should be remembered that it is one of many latent viruses that may alter T cell immunity. As such, it is possible that a number of infections, such as Epstein-Barr virus or Varicella virus, may also alter the stress reactivity of lymphocytes. These infectious agents are consider to be harmless to immunocompetent individuals, but the current results suggest that chronic viral infections may affect immunity, and stress mediated alterations in immunity, which in turn, may have implications for host health.

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CHAPTER 4

MOBILIZATION OF CMV- AND EBV-SPECIFIC CD8$^+$ T LYMPHOCYTES DURING ACUTE PSYCHOLOGICAL STRESS AND β-ADRENERGIC RECEPTOR STIMULATION

ABSTRACT

Objective: Recently we demonstrated that CD8$^+$ T Lymphocyte (CD8TL) subsets with a differentiated, effector-like phenotype become selectively mobilized by adrenergic stimuli such as stress, exercise and ßAR-agonist infusion. CD8TL differentiation is related to the antigen-specificity of the cell; for example, Cytomegalovirus (CMV)-specific CD8TLs show a further degree of differentiation than, for example, Epstein-Barr virus (EBV)-specific CD8TLs. Further, recent findings demonstrate that infection with CMV can increase the stress-reactivity of the CD8TL population. Therefore, the current study tested if antigen-specificity is a determinant of CD8TL responsivity to acute stress and ß-adrenergic stimulation and further, whether CMV-specific cells demonstrate heightened mobilization. **Methods:** In two studies, we compared CD8TL mobilization in response to an acute stress (speech task; *n*=29) and ßAR-agonist infusion (isoproterenol infusion with (*n*=6) or without (*n*=4) prior administration of a ßAR-antagonist). CMV-specific and EBV-specific CD8TLs were identified using MHC-class I tetramers and were further classified into early-, intermediate- or late-differentiated cells using the surface markers CD45RA, CD28, and CD27. **Results:** During acute stress, CMV tetramer positive (CMV^{tet+}) CD8TLs (+94 q9%) showed a larger mobilization response than EBV tetramer positive (EBV^{tet+}) CD8TLs (+54 q9%, p<.05) and the

total memory CD8TL populations (+61 ±9%, $p<.01$). Subsequent analyses revealed that $CD28^{I}$ (intermediated- and late-differentiated) cells mobilized more than $CD28^{+}$ (early) cells regardless of antigen-specificity. However, early and intermediate CMV^{tet+} cells and early EBV^{tet+} CD8TLs demonstrated enhanced stress responsiveness when compared to the corresponding phenotype within the total CD8TL population (i.e. intermediate with intermediate). Infusion of a βAR-agonist replicated the stress findings, and agonist-induced mobilization was abrogated by βAR-antagonist administration. **Conclusion:** In conclusion, cell differentiation is a major determinant of CD8TL mobilization but antigen-specificity may also explain the distinct stress- and βAR-sensitivity of antigen-specific cells. Such differential sensitivity could have implications for the control of viral reactivation during stress.

INTRODUCTION

Lymphocytosis is one of the most replicated effects of stress upon the immune system and is largely driven by an increase in cytotoxic cell numbers, such as $CD8^+$ T lymphocytes (CD8TLs), gamma-delta (ɔŭ) T cells, and natural killer (NK) cells (Anane et al., 2009; Benschop et al., 1996b; Campbell et al., 2009; Dimitrov et al., 2010; Segerstrom and Miller, 2004). It has been proposed that stress lymphocytosis enhances immunosurveillance, in parallel with increasing cardiovascular and metabolic activity, in the face of potential threat (Benschop et al., 1996b; Bosch et al., 2005; Dhabhar and McEwen, 1997; Dopp et al., 2000). The essential role of sympathetic activation in mobilization has been conclusively demonstrated, and involves stimulation of beta-2-adrenergic receptors ($ƃ_2$AR) that are expressed upon the surface of lymphocytes (Benschop et al., 1996a; Benschop et al., 1996b; Dimitrov et al., 2010).

Cytotoxic lymphocyte mobilization displays a marked selectivity for differentiated memory CD8TLs that have developed an effector phenotype and express enhanced $ƃ_2$AR expression (Anane et al., 2010; Campbell et al., 2009; Dimitrov et al., 2010). For example, the magnitude of stress mobilization is greater in late-differentiated CD8TLs ($CD28^ī CD27^ī$) than in intermediate-differentiated cells ($CD28^ī CD27^+$), while early-differentiated cells ($CD28^+CD27^+$) demonstrate the smallest response. The greater expression of $ƃ_2$ARs on late-differentiated cells compared to early-differentiated CD8TLs may provide a mechanism for enhanced stress and ƃAR-stimuli. As CD8TLs differentiate into effector memory phenotypes, they gain effector functions such as cytotoxicity ($perforin^+$, $granzyme^+$), a tissue migratory profile ($CCR7^ī CD62L^ī CD11a^+$), and increased production of IFN-ɔ (Monteiro et al., 2007; Romero et al., 2007). Similar to stress sensitivity, the effector potential of CD8TLs increases from early-differentiation, to intermediate, and finally to late (Monteiro et al., 2007; Romero et al., 2007). Further, late-differentiated cells express NK cell receptors, such as NKG2D, and

can therefore exhibited innate effector functions (Ogasawara and Lanier, 2005; Weng et al., 2009). CD8TLs differentiation is caused by repeated cell activation and extensive rounds of cell division and thus, stress sensitive cells are also characterized by short telomeres and decreased telomerase activity (Akbar and Vukmanovic-Stejic, 2007; Effros, 2004; Monteiro et al., 2007; Romero et al., 2007). In sum, acutely stressful events rapidly mobilize highly differentiated memory CD8TLs that can migrate into inflamed tissue and have immediate effector potential.

The differentiation of CD8TLs is largely driven by infectious history. It is thought that repeated antigen exposure results in concomitant rounds of activation and cell division and gradually drives antigen-specific CD8TLs towards a late-differentiated phenotype (Appay et al., 2002; van Lier et al., 2003). For example, Cytomegalovirus (CMV), a latent herpes virus carried by 50-70% of western populations, may never reach true latency and has the strongest impact on differentiation; CD8TLs directed against CMV-infected cells typically exhibit a late-differentiated phenotype. In contrast, less vigorously reactivating latent viruses, such as Epstein-Barr virus (EBV), another very common (~90% infected) latent herpes virus, typically generate EBV-specific CD8TLs of an early- or intermediate-memory phenotype (Appay et al., 2002; Monteiro et al., 2007; van Lier et al., 2003). The total CD8TL population is mainly constituted by stress-unresponsive naïve cells and early-memory cells. On this basis, it would be predicted that CMV-specific CD8TLs would display the strongest sensitivity to acute stressors and that EBV-specific CD8TLs mobilization would be similar to the total CD8TL response. Normal CD8TL immunity is paramount in controlling latent viruses as demonstrated by the increased risk of CMV reactivation in stem cell transplant recipients who have dysfunctional CMV-specific T cell responses (Gratama et al., 2008; Lilleri et al., 2008; Morita-Hoshi et al., 2008; Ozdemir et al., 2002). As such, this phenomenon may have relevance for the association between latent virus reactivation and stress (Sarid et al., 2001, 2004; Stowe et al., 2001). In addition, the results may determine whether the enhanced

CD8TL response within CMV infected individuals, as described in chapter 3, is due to a enhanced mobilization of CMV-specific cells, or whether phenotype (i.e., irrespective of antigen specificity) is a determinant of mobilization.

Virus-specific CD8TLs can be identified using major histocompatibility complex (MHC)-class I tetramers. The CD8TL T cell receptor (TCR) can bind to specific viral peptides if the peptides are presented within a MHC-class I molecule. MHC-class I molecules that contain peptides from a chosen virus can be synthesised and combined to form a tetramer structure that is conjugated to a fluorochrome (Altman et al., 1996). Tetramers bind to peptide-specific CD8TLs *in-vitro* and the bound cells can be detected by flow cytometry. The aim of the current study was to determine if viral specificity, as well as cell differentiation, could predict the stress sensitivity of CD8TLs. A secondary aim was to determine whether any effect of stress could be reproduced by βAR-stimulation. We analysed the mobilization of CMV-specific and EBV-specific CD8TLs using flow cytometry and MHC-class I tetramers. Viral-specificity was determined simultaneously with expression of the differentiation markers CD45RA, CD28 and CD27. This study represents the first detailed investigation on stress-induced mobilisation of virus-specific CD8TLs.

METHODS

Participants and virus-specific CD8TL screening

Participants were recruited from community volunteers and staff and students attending the University of Birmingham (UoB), UK or University of California San Diego (UCSD), US. During an initial visit to the laboratory, participants gave informed consent and provided a 2ml EDTA blood sample by venepuncture from a palpable lower arm vein. Blood was analysed for the presence of CMV tetramer positive (CMV^{tet+}) and EBV tetramer positive

(EBV^{tet+}) CD8TLs (method described in Flow cytometry section). Participants found to have CMV^{tet+} and/or EBV^{tet+} CD8TLs were then invited to partake in the psychological stress or βAR-agonist infusion study. All study volunteers reported to be in good health and were non-medicated with the exception of the contraceptive pill. Participants were instructed not to engage in strenuous physical exercise and to refrain from consuming alcohol or non-prescription drugs 24 hours before their experimental session, and to abstain from smoking and caffeine on the day of the experiment. Student volunteers performing the psychological stress task and all volunteers undertaking the infusion procedure received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the appropriate institutional review board (UoB or UCSD).

Psychological stress study

Procedure

Upon participant (age 32.6 years, SD±14.5; 14 female/ 15 males) arrival at the UoB: (1) electrodes for electrocardiography (ECG) and impedance cardiography (ICG) were attached; (2) a 20-gauge intravenous cannula (Becton-Dickinson) was placed into a palpable vein in the lower arm; and (3) an occluding cuff was placed over the brachial artery of the other arm for blood pressure measurements. Subsequently, while seated in a comfortable upright position, participants filled out several questionnaires and engaged in leisure reading. After 20 minutes, a baseline blood sample was obtained and the procedure for the laboratory stressor was initiated.

Public Speaking Task

To induce stress, participants performed two back-to-back speeches, each with 2 minutes of preparation and 4 minutes of speech delivery (Bosch et al., 2005; Bosch et al., 2003b) Social stress was enhanced by recording the speeches on videotape and by the attendance of an audience of three. For the first speech, the participant had to defend him/herself after being

falsely accused of shoplifting (Saab et al., 1989) and, for the second speech, the participant gave a presentation about his or her best and worst personal characteristics (van Eck et al., 1996). Instructions for the task were presented via a DVD recording, which ensured standardization of instructions and timing of the tasks. Including instructions, the task lasted 15 minutes. A blood sample was obtained during the second presentation, 13 minutes after initiation of the task. Following the task, the participants again engaged in leisure reading, and a final blood sample was obtained after 15 minutes of recovery.

Cardiovascular assessment

Assessment of cardiovascular responses focused on cardiac sympathetic and vagal control as previously described (Berntson et al., 1993; Bosch et al., 2003a). Indices of sympathetic and parasympathetic drive were obtained by analysis of ECG and ICG signals. The thoracic ICG and ECG signals were recorded from six Ag-AgClspot-electrodes (AMI type 1650-005, Medtronic) using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) device. The ECG and ICG complexes were ensemble averaged with reference to the ECG R-wave across 30-sec periods. From these 30-sec ensembles, average levels were computed for heart rate (HR) and pre-ejection period (PEP). These means were further averaged over a 6-min pre-task baseline, each 6-min task and a 6 minute post task recovery. Changes in PEP were used to index changes in cardiac sympathetic drive, whereas heart rate variability, or Root Mean Square of Successive Difference (RMSSD), was used to index changes in cardiac vagal tone.

β-agonist infusion study

Procedure

Isoproterenol infusion was performed according to a standardized protocol (Mills et al., 2000; Mills et al., 1997). Upon arrival at the laboratory, UCSD, participant (age 40.3 years, SD±9.1; 2 female/ 4 male) height and weight were taken to confirm correct calculation of body surface

area (BSA) used to determine the βAR-agonist infusion rate. Subsequently, participants were in a semi-supine position for 15 minutes following placement of: 1) two 22-gauge intravenous cannulas (Becton-Dickinson), one for drawing blood samples and one for βAR-agonist infusion, inserted into a palpable vein in opposite lower arms; 2) three spot ECG electrodes; and 3) an occluding cuff on the non-infusion arm.

βAR-agonist was then infused at incremental rates (0.1µg, 0.5µg and 1µg /min/1.73 m^2 BSA for 5 minutes each) for 15 minutes until the participants heart rate had increased by ~20 beats per minute (bpm) compared to resting heart rate. The final infusion rate was maintained for 10 minutes. On average, the maximal dose reached 1µg/min/1.73 m^2 BSA. Blood was taken prior to initiation of isoproterenol infusion (β"baseline") and in the final minutes of the infusion. ECG, heart rate and blood pressure were monitored throughout the infusion. The half-life of isoproterenol is approximately 2ï 3 min (Goebel et al., 2000). The infusion was generally well-tolerated and all participants successfully completed the protocol.

βAR-antagonist (blockade) procedure

A sub group of 4 participants (age 43.3 ±10.0 years, 1 female) performed the βAR-agonist infusion procedure twice; once following five consecutive days of administration with 80mg of the βAR-antagonist propranolol and once following 5 days administration of a placebo. Drug administrations were counter-balanced and single-blinded.

Questionnaires

In all studies, health and lifestyle variables were assessed by self-report questionnaire. These variables included recent symptoms of illness, exercise behaviour, alcohol consumption, caffeine consumption, smoking, and use of recreational drugs. Affective responses to the psychological stress task were assessed using the short-form of the Profile

of Mood States (POMS) (McNair et al., 1992). Participants completed the POMS at baseline, immediately post-task and at 15-minutes recovery.

Flow cytometry

Whole blood was collected into EDTA tubes, maintained at room temperature and processed within 4 hours after collection. In brief, 9 ml of ammonium chloride lysis solution (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) was added to 1 ml of whole blood to remove red blood cells. After 10 minutes of gentle rotation on ice, 5 ml of phosphate-buffered saline (PBS) was added to stop the reaction, and the samples were centrifuged (283G, 7-min at room temperature). After supernatant removal, the sample was incubated for 25 minutes at room temperature with MHC-I tetramers, conjugated with streptavidin phycoerythrin (PE) directed against CMV or EBV epitopes (Table 1). Tetramers used were restricted to common HLA class I subtypes (population coverage ~ 10-55% each) and directed against highly immunodominant viral epitopes (Khan et al., 2002a; Longmate et al., 2001; Mori et al., 1997; Price et al., 2005; Sette and Sidney, 1999; Steven et al., 1997). The PE-streptavidin was purchased from Invitrogen, Paisley UK and the tetramers were synthesized in house (Institute for Cancer Studies, University of Birmingham, UK; (Altman et al., 1996)). Subsequently, samples were incubated for 20 min at room temperature with a cocktail of fluorescent-labelled monoclonal antibodies: CD45RA-FITC, CD3-PerCP, CD27-APC, CD8-APC-Cy7 (Becton-Dickinson, Oxford, UK) and CD28-PE-Cy7 (eBioscience, Insight Biotechnology Ltd, Middlesex, UK). Cell suspensions were then washed and re-suspended in 500µl PBS containing 1.5% paraformaldehyde, and stored in the dark at 4°C until analysis. Preparations were read within 18 hours. Approximately $1x10^6$ gated lymphocytes were acquired from each preparation using a dual-laser flow cytometer (FACS-Canto II, Becton Dickinson, Oxfordshire UK). Data were analyzed using Flowjo 7.4 (Treestar Inc, Ashland, OR, USA). A complete white blood cell count was obtained for each blood sample using a

haematology analyzer (Coulter ACT^{diff}, Beckman Coulter, High Wycombe, UK or Coulter GEN-S haematology analyser, Beckman-Coulter, Miami, USA).

Data analysis

CD8TL mobilization

The aim of this study was to compare the mobilization of the total CD8TL population with that of the total CMV^{tet} and EBV^{tet+} CD8TL populations. All antigen-specific cells are stress sensitive (CCR7^{-}) memory cells (Atanackovic et al., 2006). To ensure fair comparisons were made, antigen-specific CD8TL mobilization was compared to the ñtotal-memory CD8TLò response by removing non-stress responsive naïve (CD45RA^{+}CD28^{+}CD27^{+}) CD8TLs from the total CD8TL analysis. For the same reason, naïve CD8TLs were also removed from the early CD8TL population and the response of the remaining ñearly-memory CD8TLsòwas then compared to that of early EBV^{tet+} and early CMV^{tet} cells.

Statistical analysis

The Kolmogorov-Smirnov test indicated that the immunological data was not normally distributed. Therefore non-parametric analysis (Friedman test) was used to examine the effects of the psychological stress task on cardiovascular, psychological and immunological parameters and the effect of ß-agonist infusion and ßAR-antagonist procedure on cell numbers. For pairwise comparisons, Wilcoxon signed rank tests were used. Where medians are presented, interquartile range is given in brackets. Variations in degrees of freedom reflect occasional missing data. Data were analyzed using SPSS 16 for windows (SPSS Inc, Chicago, Illinois).

RESULTS

Psychological stress study

Anxiety and cardiovascular responses

Increases in the tension-anxiety POMS subscale confirmed that the speech tasks were perceived as stressful (+9.0 (6.5); $\chi^2_{(27)} = 44.7$, $p<.001$). A physiological stress response was demonstrated by the significant increases in SBP (+30.0 (13.1) mmHg; $\chi^2_{(27)} = 51.1$, $p<.001$), DBP (+17.3 (9.0) mmHg; $\chi^2_{(27)} = 42.6$, $p<.001$), and HR (+ 23.1 (13.0) bpm; $\chi^2_{(27)} = 43.5$, $p<.001$), reflecting an increase in sympathetic drive as evidenced by a decrease in PEP (ï 7.5 (10.6) ms; $\chi^2_{(27)} = 21.4$, $p<.001$). Additionally, there was a significant decrease in RMSSD (ï 12.8 (26.5) ms; $\chi^2_{(27)} = 21.6$, $p<.001$), reflecting vagal withdrawal. At 15-min recovery, all cardiovascular and autonomic measures had returned to baseline values.

Preferential mobilization of CMV-specific CD8TLs during psychological stress

As can be seen in Table 2, the speech task effectively induced significant mobilization of lymphocytes and all CD8TL populations. Figure 2 shows the total CMV^{tet+} CD8TL response (+94 qþ%) was significantly greater than the total EBV^{tet+} CD8TL mobilization (+54 qþ%; z = -2.2, $p=.025$) and total memory CD8TL response (+61 qþ%; z = -2.7, $p=.008$). The mobilization of the total memory CD8TL population was not different from the total EBV^{tet+} CD8TL population.

Figure 2 demonstrates that, consistent with previous findings, stress responsiveness increased from the early phenotype, to intermediate and then to late, in each of the CD8TL populations, apart from the CMV-specific cells; no difference was observed between the intermediate and late CMV^{tet+} subsets. Not all cells at the same stage of differentiation but with different antigen-specificity mobilized to the same extent; early EBV^{tet+} and early CMV^{tet} CD8TLs had greater stress sensitivity than the total early-memory CD8TL population (qþ%; z

= -3.0, *p*=.003 and q%; z = -2.8, *p*=.004 respectively). Further, intermediate CMV^{tet+} CD8TLs mobilized to a greater extent than the total intermediate CD8TL subset (q%; z = -2.4, *p*=.016). No other subset differences were found between the mobilizations of distinct antigen-specific CD8TLs.

βAR-agonist infusion study

βAR-agonist mobilized CMV-specific CD8TLs and βAR-antagonist administration inhibited the response

As shown in Figure 3, the βAR-agonist infusion data replicated the findings of the psychological stress study, although the difference in mobilization between CMV^{tet+} CD8TLs and the total memory CD8TL population was not significant, perhaps due to the small sample size. However, the findings do confirm that the CMV^{tet+} CD8TL mobilization is mediated by βAR-mechanisms as demonstrated by successful mobilization of CMV^{tet+} cells during the βAR-agonist infusion and the subsequently abrogation of this effect by βAR-antagonist. In addition, Figure 3 demonstrates that the mobilization of early CMV^{tet+} CD8TLs was significantly greater than the early-memory CD8TL subset response (q%; z = -2.2, *p*=.028). No differences were found between intermediate and late CMV^{tet+} subset mobilization when compared to the total intermediate and total late CD8TL subset responses respectively.

Table 1. MHC class I restricted peptides used.

Virus	*Viral Antigen*	*HLA-Restriction*	*Sequence Positions*	*Peptide Sequence*
CMV	pp50	A1	245-253	VTEHDTLLY
	Lower matrix protein pp65	A2	495-503	NLVPMVATV
	Lower matrix protein pp65	B7	265-275	RPHERNFGTVL
	Lower matrix protein pp65	B7	417-426	TPRVTGGGAM
	Intermediate early protein-1	B8	199-207	ELKRKMIYM
EBV	LMP2 protein (latent cycle)	A2	426-434	CLGGLLTMV
	BMLF1 protein (lytic cycle)	A2	280-288	GLCTLVAML
	BZLF-1 protein (lytic cycle)	B8	190-197	RAKFKQLL

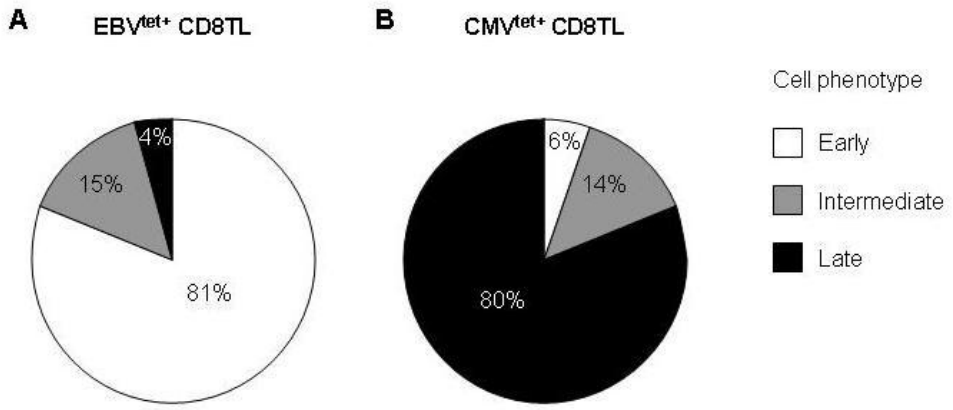

Figure 1. Pie charts showing the subset constitution of antigen-specific CD8TLs. Consistent with the literature, the majority of EBV^{tet+} CD8TLs were of the low stress-responding early phenotype (A) and a large proportion of CMV^{tet+} CD8TLs were stress sensitive late differentiated cells (B).

Table 2. Median (interquartile range) of lymphocytes and CD8TL subset numbers at each time point during the psychological stress study.

Cell type (cells/μl)	*Baseline*	*Task*	*Recovery*	*n*
Lymphocytes	1700 (550)	2400 (1000)	2000 (850)	29
Total CD8TLs	392.2 (207)	580.2 (322)	474.4 (308)	29
Total Memory CD8TLs	236.9 (194)	356.5 (444)	267.0 (247)	19
Naïve CD8TLs	132.2 (78)	134.3 (107)	134.7 (101)	19
Early-memory CD8TLs	110.3 (82)	129.1 (79)	125.6 (85)	19
Intermediate CD8TLs	43.68 (53)	89.82 (104)	56.44 (66)	19
Late CD8TLs	72.12 (118)	159.99 (448)	61.48 (254)	19
Total EBV^{tet+} CD8TLs	2.180 (3.2)	3.201 (5.1)	2.527 (3.6)	20
Early EBV^{tet+} CD8TLs	1.313 (1.8)	1.914 (2.8)	1.468 (2.4)	12
Intermediate EBV^{tet+} CD8TLs	0.604 (1.1)	0.853 (2.4)	0.648 (1.4)	12
Late EBV^{tet+} CD8TLs	0.105 (0.1)	0.219 (0.2)	0.140 (0.2)	12
Total CMV^{tet+} CD8TLs	5.431 (25)	7.306 (55)	4.605 (31)	17
Early CMV^{tet+} CD8TLs	1.866 (17)	3.103 (27)	1.913 (13)	11
Intermediate CMV^{tet+} CD8TLs	5.619 (18)	10.68 (44)	6.514 (26)	11
Late CMV^{tet+} CD8TLs	13.80 (29)	31.70 (93)	19.72 (50)	11

All p-values were <.035 when analysed using the non-parametric Friedman test (σ^2-values ranged between 6.7 and 41.3) and when analysed using the parametric repeated measures ANOVA on the Ln (1+ cell number) (F-values ranged between 7.77 and 90.9).

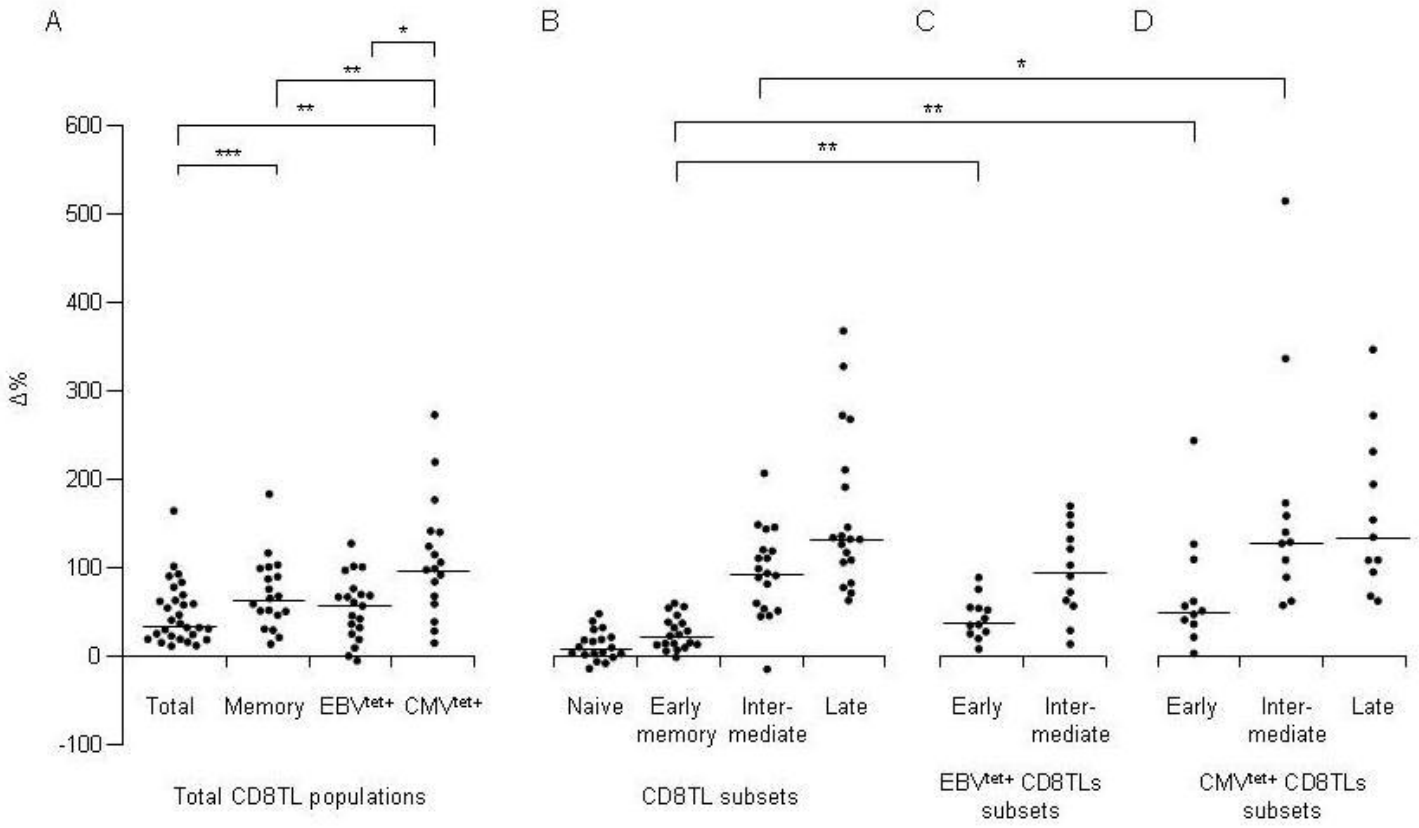

Figure 2. Individual effects of a psychological stress task on the mobilization of CD8TLs, EBV^{tet+} CD8TLs and CMV^{tet+} CD8TL populations. Median relative change in cell number (æ%) in response to stress (Wilcoxon signed ranks test). **p<.05, **p<.01 and ***p<.001* indicates the magnitude of mobilization is significantly different between subsets.

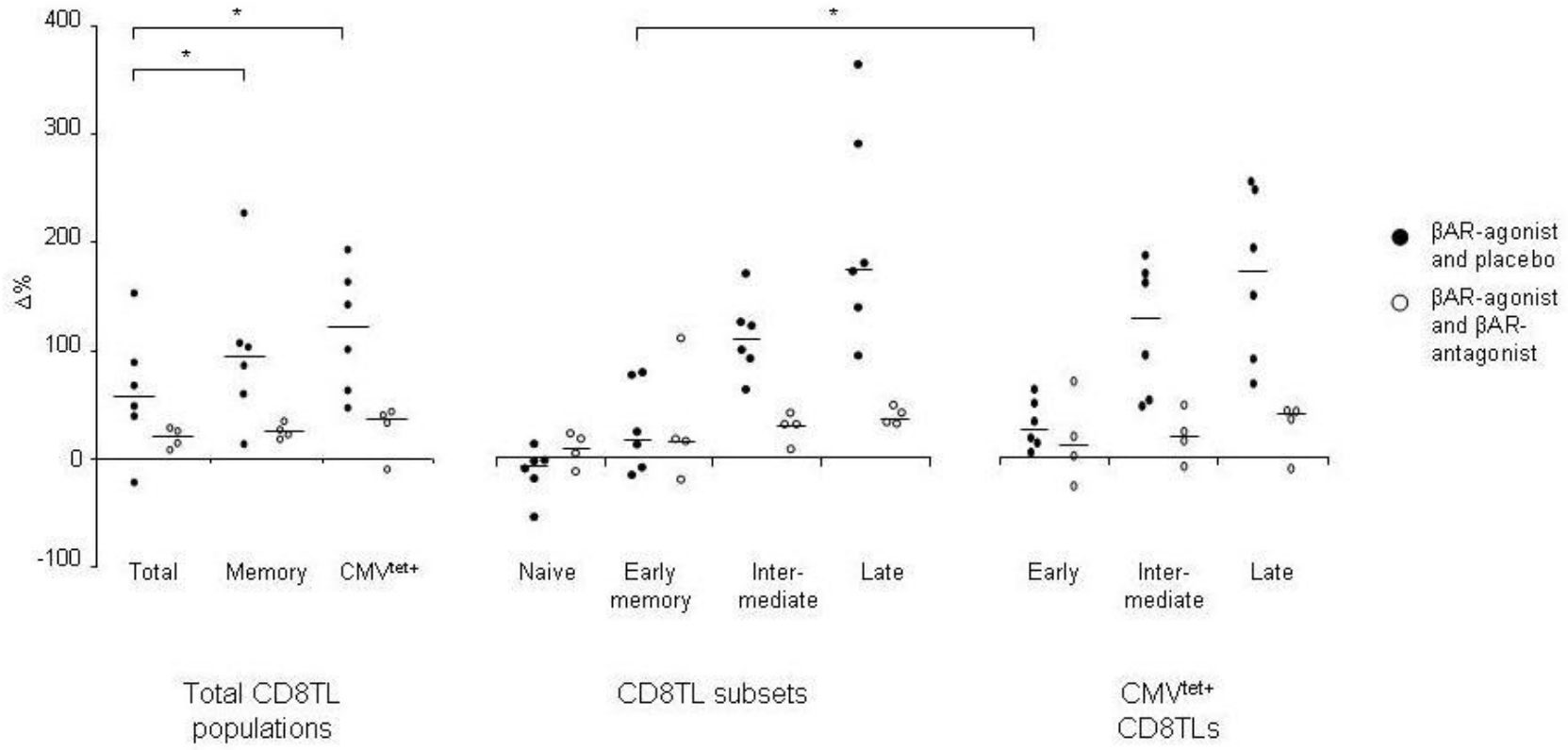

Figure 3. Individual effects of a ßAR-agonist infusion and ßAR-antagonist administration on the mobilization of CD8TL and CMV^{tet+} CD8TL populations. Relative change in cell number (æ%) in response to stress (Wilcoxon signed ranks test). *p<.05 indicates the magnitude of mobilization is significantly different between subsets.

DISCUSSION

The present study investigated the effect of acute psychological stress and ß-adrenergic receptor (ßAR) stimulation on the mobilization of CMV and EBV-specific CD8TLs. As anticipated, the results showed a strong heterogeneity in responses; CMV tetramer positive (CMV^{tet+}) CD8TLs (+94 œ%) showed a larger mobilization response than EBV tetramer positive (EBV^{tet+}) CD8TLs (+54 œ%, $p<.05$) and the total memory CD8TL populations ($CD45RA^{-}CD28^{+}CD27^{+}$; +61 œ%, $p<.01$). CMV^{tet+} and EBV^{tet+} subset mobilization increased in parallel with greater differentiation (late > intermediate > early) similar to total CD8TLs and consistent with previous findings (Chapter 2). The mobilization of CMV^{tet+} CD8TLs was replicated by ßAR-agonist infusion and blocked by ßAR-antagonist administration, suggesting involvement of beta-adrenergic mechanisms.

Differences in the stress responsiveness of antigen-specific CD8TLs may be explained by their varying levels of differentiation. As demonstrated in chapter 2, and consistent with the literature, CD8TL mobilization increases in parallel with increasing cell differentiation towards an effector-like phenotype (Atanackovic et al., 2006; Campbell et al., 2009; Dimitrov et al., 2010). Indeed, CMV^{tet+} and EBV^{tet+} subsets demonstrated similar mobilization response pattern to total CD8TLs (late > intermediate > early). As shown in Figure 1, CMV^{tet+} CD8TLs were mainly of an intermediate or late phenotype and thus, are highly stress responsive. In contrast, nearly all (~96%) of EBV^{tet+} CD8TLs were of a less stress sensitive early- or intermediate-differentiated phenotype. However, differentiation status may not fully explain the larger mobilization of CMV^{tet+} CD8TLs, as the early and intermediate CMV^{tet+} subsets showed an enhanced responsivity compared to the same subsets within the total CD8TL population. This suggests that CMV-specific cells may be intrinsically more responsive, even at the same stage of differentiation. As such, greater mobilization of CD8TLs within CMV seropositive individuals, as demonstrated in Chapter 3, may not be solely explained by an

increase in the number of stress-responsive late-differentiated cells. The mechanisms underlying such enhanced sensitivity would require further investigation.

Although there was a clear difference between the mobilization of total memory CD8TL and CMV^{tet+} CD8TLs, this difference is likely larger than demonstrated here. The tetramers only identify CMV-specific cells that specifically recognize the CMV peptide fragments used, and it is likely many other CMV-specific cells remained undetected (Betts et al., 2000; Khan et al., 2002b; Sylwester et al., 2005). It is estimated that a large proportion (~10%) of memory CD8TLs are indeed CMV-specific in healthy CMV^{pos} adults and this number may rise to >20% in almost a third of the population (Sylwester et al., 2005). The unidentified and CMV^{tet+} cells were included within the total CD8TL population and thereby may have increased the total memory CD8TL stress response. This aspect may explain why during infusion the response of CMV^{tet+} CD8TLs and total memory CD8TLs did not significantly differ from each other, although the relatively small samples size may also have contributed. The theory can be extended to CD8TL subsets and may have prevented further differences between CMV^{tet+} subsets and CD8TLs subsets from being demonstrated i.e., late CMV^{tet+} compared to late CD8TLs during the stress task.

The current study used a ß-AR-agonist and a ß-AR-antagonist. Therefore, it was only possible to infer that ß-AR, rather than Ŭ-AR, mechanisms, were involved in the mobilization of CMV^{tet+} CD8TLs; this methodology did not allow us to identify the specific ß-AR subtype involved. However, evidence suggests that increased expression of the $ß_2$-AR on stress responsive CD8TLs may explain the specificity of stress mobilization (Dimitrov et al., 2010). This would follow results found for natural killer cells (Benschop et al., 1994; Schedlowski et al., 1996). Indeed, CMV infection up-regulates $ß_2$-AR gene (ADRB2) expression, which may infer increase protein expression and, in turn, raise the stress sensitivity of CD8TLs (Bosch, In preparation). Inclusion of a $ß_1$-AR- and $ß_3$-AR-antagonist would clarify $ß_2$-AR receptor subtype

involvement in future studies (Benschop et al., 1994; Benschop et al., 1996b; Schedlowski et al., 1996).

Increased ß-adrenergic sensitivity may not merely change a cellôs mobilization response; it could also alter other functions. Indeed, adrenergic stimulation of T cells has been shown to alter a variety of effector functions including proliferation, lytic activity, and cytokine production (Bartik et al., 1993; Borger et al., 1998; Hatfield et al., 1986; Kalinichenko et al., 1999; Leo and Bonneau, 2000; Sekut et al., 1995). Considering normal cell mediated immunity is essential for controlling latent viruses, extrapolation of enhanced ß-adrenergic sensitivity to other latent-virus specific cell functions may provide a mechanism for the observed loss of viral control during acute stress (Glaser and Kiecolt-Glaser, 2005; Gratama et al., 2008; Lilleri et al., 2008; Morita-Hoshi et al., 2008; Ozdemir et al., 2002; Sarid et al., 2001, 2004). Supporting evidence for this notion can be drawn from the murine model; stress-induced reactivation of Herpes Simple Virus type-1 (HSV1) is associated with reduced CD8TL IFN-ɔ production and cell proliferation, and further, stress alters HSV1-specific CD8TL surveillance of latently infected neurons (Freeman et al., 2007; Padgett et al., 1998). The classic explanation for the link between stress and loss of latent viral control is that stress hormones directly stimulate viral gene expression via activation of promoter regions within the virus genome (Glaser et al., 1995; Prosch et al., 2000). However, altered CD8TL immunity by ßAR-stimulation may provide another possible mechanism.

It has been speculated previously that mobilization may enhance inflammation (Bosch et al., 2003a; Bosch et al., 2003b; Marsland et al., 2002; Nyklicek et al., 2005). Here, we will focus specifically on how CMV infection may amplify stress-exacerbated inflammation. CMV infection of endothelial cells induces expression of surface molecules that promote leukocyte recruitment and migration, such as ICAM-1 (Groh et al., 2001; Kloover et al., 2000; Knight et al., 1999; Waldman and Knight, 1996; Waldman et al., 1998). Further, CMV-infected

endothelial cells can activate T cells, possibly through the transfer of CMV antigens by exosome-like particles to antigen presenting cell (Walker et al., 2009). Moreover, recruited T cells can proliferate and cause endothelial cell damage (Bolovan-Fritts et al., 2004; Waldman and Knight, 1996; Waldman et al., 1998). Migration of leukocytes into tissue is a major aspect in various inflammatory conditions, such as atherosclerosis, and enhanced mobilization of CMV-specific cells may promote initial cell recruitment (Libby et al., 2002; Ross, 1999). Heightened cell recruitment may also enhance autoimmune conditions such as rheumatoid arthritis (RA). Indeed, CD8TLs found in synovial fluid are activated late-differentiated cells and a large proportion (up to 15.5%) are EBV^{tet+} or CMV^{tet+} (Tan et al., 2000). As infection of synovial tissue by EBV and CMV is increased in RA patients, it is possible that recruited T cells will elicit an immune response in the inflamed joint (Mehraein et al., 2004).

It has frequently been noted that stress appears to have a larger impact on immunity with increasing age (Gouin et al., 2008; Graham et al., 2006; Segerstrom et al., 2008). For example, older adults tend to have poorer vaccine responses than younger individuals and chronic stress further reduces the poor immunisation response, particularly in older individuals (Goodwin et al., 2006; Kiecolt-Glaser et al., 1996; Pedersen et al., 2009). Interestingly, cells that accumulate in immunosenescence and are likely, therefore, to contribute to the age-related decline in immune function, are the same cells that exhibited an increased sensitivity to acute stress and ßAR-stimulation: late-differentiated and CMV-specific CD8TLs (Gillespie et al., 2000; Khan, 2007; Khan et al., 2002b; Sansoni et al., 2008). For example, increased numbers and proportions of late-differentiated cells and CMV infection predicts weaker vaccination responses, faster cognitive decline, and increased incidences of morbidity and mortality in older adults (Pawelec et al., 2005; Trzonkowski et al., 2003; Wikby et al., 2005). That the differentiated, or áagedô, CD8TL exhibits altered neuro-

endocrine sensitivity may thus, provide a mechanistic explanation for why stress could have a larger impact in older adults.

In summary, this study is the first to demonstrate that stress-induced mobilization of CD8TLs is, in part, determined by antigen-specificity. Larger responses were seen for CMV-specific cells compared to EBV-specific and total CD8TLs, and appeared driven by a greater differentiation and concomitant enhanced adrenergic sensitivity of CMV-specific CD8TLs. As our understanding of the impact of CMV infection upon adaptive immunity grows, future research on the potential clinical outcomes of this phenomenon will be increasingly warranted.

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CHAPTER 5

PROGENITOR CELLS ARE MOBILIZED BY ACUTE PSYCHOLOGICAL STRESS BUT NOT BETA-ADRENERGIC RECEPTOR AGONIST INFUSION

ABSTRACT

Objectives: Stimuli that activate the sympathetic nervous system, such as acute psychological stress and exercise, rapidly invoke a robust mobilization of lymphocytes into the circulation. Recent evidence suggests that bone marrow-derived progenitor cells (PCs) also mobilize in response to sympathetic stimulation such as exercise and trauma. **Methods:** In two studies, we investigated PC mobilization in response to an acute psychological stressor (speech task; *n*=26) and ßAR-agonist infusion (isoproterenol; *n*=20). A subset of 8 participants completed the infusion protocol twice, with or without administration of the ßAR-antagonist propranolol. Haematopoietic stem cell (HSC) subsets (common lymphoid PCs, common myeloid PCs, granulocyte/ macrophage PCs and megakaryocyte/ erythrocyte PCs) and endothelial PCs (EPCs) were enumerated by flow cytometry. **Results:** Psychological stress induced a significant mobilisation of total PCs, HSCs, and EPCs into the peripheral circulation. However, these findings were not replicated by infusion of a ßAR-agonist. **Conclusion:** PCs are mobilized by psychological stress via mechanisms independent of ßAR-stimulation. The increase in peripheral HSC and EPC numbers during stress may be part of an adaptive mechanism to promote repair in situations of increased risk for wounding and immune activation.

INTRODUCTION

Progenitor cells (PCs) are clonogenic cells capable of both self renewal and multi-lineage differentiation (Till and Mc, 1961; Weissman, 2000). They act as a repair system for the host, replenishing specialized somatic cells and maintaining the normal turnover of regenerative organs, such as the blood or skin. Haematopoietic stem cells (HSCs) are a subset of PCs that give rise to all blood cell types, including leukocyte populations. They can be used clinically to reconstitute the obliterated haematopoietic system in patients undergoing chemotherapeutic or radiation treatments (Akashi et al., 2000; Kondo et al., 1997; Stewart et al., 1999; Thomas, 1991). Endothelial PCs (EPCs), another PC subset, also have angiogenic and vascular regenerative properties (Asahara et al., 1997; Khakoo and Finkel, 2005; Takakura et al., 2000).

The majority of PCs reside in the bone marrow, with a small number continually migrating into the circulation and tissue and then re-homing back to the bone marrow (Abkowitz et al., 2003; Wright et al., 2001). This process of PC egress into the peripheral circulation is utilized in modern clinical stem cell transplantation, whereby HSCs can be harvested from the blood rather than from bone marrow grafting. Enhanced mobilization of EPCs into the blood has been associated with improved endothelial function and repair and thus may promote cardiovascular health (Foresta et al.; Hill et al., 2003; Werner et al., 2005; Werner and Nickenig, 2006). Indeed, low PC number and reduced PC function is associated with atherosclerosis, hypertension, myocardial infarction and, increased risk of death due to cardiovascular causes (Hill et al., 2003; Vasa et al., 2001; Werner et al., 2005).Thus understanding the mechanisms governing PC mobilization from the bone marrow are of great clinical interest.

Stimuli that activate the sympathetic nervous system and the concurrent release of epinephrine and norepinephrine, such as acute psychological stress and exercise, rapidly invoke a robust mobilization of lymphocytes into the circulation (Benschop et al., 1996b; Pedersen and Hoffman-Goetz, 2000; Segerstrom and Miller, 2004). The mechanism of stress lymphocytosis has been well characterized; stimulation of beta-2-adrenergic receptors (β_2ARs) on the lymphocyte surface facilitates endothelial detachment and release into the peripheral blood (Benschop et al., 1996a; Benschop et al., 1994a; Benschop et al., 1994b; Benschop et al., 1996b; Dimitrov et al., 2010; Schedlowski et al., 1996). Exercise studies have shown that in addition to lymphocytes, PCs are also mobilized (Barrett et al., 1978; Bonsignore et al., 2002; Goussetis et al., 2009; Morici et al., 2005; Rehman et al., 2004; Schmidt et al., 2007). It is possible that this mobilization is similarly regulated by βAR-mechanisms. Firstly, both murine and human PCs express functional adrenergic receptor subtypes, including the β_2AR-subtype (Muthu et al., 2007; Spiegel et al., 2007). Secondly, the bone marrow is highly innervated by sympathetic nerve fibres (Elenkov et al., 2000). Thirdly, there is experimental evidence that HSC and EPC numbers in peripheral blood are increased in response to stimuli associated with sympathetic stimulation, such as acute myocardial infarction and circadian sympathetic oscillations (Mendez-Ferrer et al., 2008; Shintani et al., 2001). Finally, sympathetic stimulation regulates murine PC mobilization from bone marrow into the peripheral blood, and this finding can be replicated by administration of β_2AR-agonist (Katayama et al., 2006; Spiegel et al., 2007). Thus there is strong evidence to suggest that heightened SNS-activity and elevated levels of plasma epinephrine may promote HSC and EPC mobilization (Klein et al., 1968; Little et al., 1985; McDonald et al., 1969; Turan et al., 2007; Wojakowski et al., 2006).

PCs are a highly heterogeneous population and subsets can be identified from gated lymphocytes and monocytes by cell surface protein expression using flow cytometry. HSCs are a type of PC that give rise to the common myeloid progenitor (CMP) subset and the

common lymphoid progenitor subset (CLP) (Akashi et al., 2000; Barnett et al., 1999; Gajkowska et al., 2006; Manz et al., 2002). CMP cells can further commit to either megakaryocyte/ erythrocyte progenitor (MEPs) or granulocyte/ macrophage progenitors (GMPs) lineages (Akashi et al., 2000; Manz et al., 2002). HSCs are characterised by low granularity, high expression of the CD34 antigen, and low levels of the common leukocyte antigen CD45; the subsets are further delineated using the markers CD38, CD123, CD45RA, and CD7 (Table 1 and Figure 1). A less extensive way to identify HSCs which have multi-lineage potential (early HSCs) or lineage committed HSCs (late HSCs) is by lack of CD38 expression and positive CD38 expression, respectively (Terstappen et al., 1991). Although there is currently no known specific maker to identify EPCs, it was recently demonstrated that isolated $CD34^{+}CD45^{\bar{I}}$ cells can form endothelial cell colonies, which is a key characteristic of EPCs (Case et al., 2007; Timmermans et al., 2007). These $CD34^{+}CD45^{-}$ cells often expressed KDR but were negative for CD133 expression (Timmermans et al., 2007). Thus, EPCs may be identified as $CD34^{+}CD45^{\bar{I}}$ cells that may also express KDR but not CD133 (Fadini et al., 2008; Hristov et al., 2009; Timmermans et al., 2009).

The current study tested the hypothesis that acute stress and ß-adrenergic stimulation (isoproterenol infusion) may promote the mobilization of HSC and EPC into the blood. Immune phenotyping was used to characterize mobilization of EPCs and different HSC subtypes.

METHODS

Participants

Participants were recruited from community volunteers and staff and students attending the University of Birmingham (UoB), UK or University of California San Diego (UCSD), USA. All participants reported to be in good health and were non-medicated with exception of the contraceptive pill. Volunteers were instructed not to engage in strenuous physical exercise and to refrain from consuming alcohol or non-prescription drugs 24 hours before their experimental session, and to abstain from smoking and caffeine on the day of the experiment. Student volunteers performing the psychological stress task and all volunteers undertaking the infusion procedure received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the appropriate institutional review board (UoB or UCSD).

Psychological stress study

Procedure

26 volunteers took part (age 31.5 years, SD ±8.0; 12 female). Upon arrival at the Exercise and Behavioural Immunology Laboratory: (1) informed consent was obtained; (2) electrodes for electrocardiography (ECG) and impedance cardiography (ICG) were attached; (3) a 20-gauge intravenous cannula (Becton-Dickinson) was placed into a palpable vein in the lower arm; and, (4) an occluding cuff was placed over the brachial artery of the other arm for systolic (SBP) and diastolic (DBP) blood pressure measurements. Subsequently, while seated in a comfortable upright position, participants filled out questionnaires and engaged in leisure reading. After 20 minutes, a baseline blood sample was obtained and the procedure for the laboratory stressor was initiated.

Public Speaking Task

To induce stress, participants performed two back-to-back speeches, each with 2 minutes of preparation and 4 minutes of speech delivery (Bosch et al., 2005; Bosch et al., 2003b).

Social stress was enhanced by recording the speeches on videotape and by the attendance of an audience of three. For the first speech, the participant had to defend him/herself imagining being falsely accused of shoplifting (Saab, Matthews, Stoney, & McDonald,1989), and for the second speech the participant gave a presentation about his or her best and worst personal characteristics (van Eck, Nicolson, Berkhof, & Sulon, 1996). Instructions for the task were presented via a DVD recording, which ensured standardization of instructions and timing of the tasks. Including instructions the task lasted 15 minutes. A blood sample was obtained during the second presentation, 13 minutes after initiation of the task. Following the task the participants again engaged in leisure reading, and a final blood sample was obtained after 15 minutes of recovery.

Cardiovascular assessment

Assessment of cardiovascular responses focused on cardiac sympathetic and vagal control as previously described (Berntson et al., 1993; Bosch et al., 2003a). Indices of sympathetic and parasympathetic drive were obtained by analysis of ECG and ICG signals. The thoracic ICG and ECG signals were recorded from six Ag-AgClspot-electrodes (AMI type 1650-005, Medtronic) using the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) device (Berntson et al., 1997). The ECG and ICG complexes were ensemble averaged with reference to the ECG R-wave across 30-sec periods. From these 30-sec ensembles, average levels were computed for heart rate (HR) and pre-ejection period (PEP). These means were further averaged over a 6-min pre-task baseline, each 6-min task and a 6 minute post task recovery. Changes in PEP were used to index changes in cardiac sympathetic drive, whereas hear rate variability, or Root Mean Square of Successive Difference (RMSSD), was used to index changes in cardiac vagal tone.

βAR-agonist infusion study

Procedures

20 volunteers took part (age 35.9 years, SD±9.3; 8 female). βAR-agonist (isoproterenol) infusion was performed according to a standardized protocol (Mills et al., 2000; Mills et al., 1997). Upon arrival at the laboratory, participant height and weight were taken to ensure correct calculation of body surface area (BSA) used to calculate the βAR-agonist infusion rate. Subsequently, participants were in a semi-supine position for 15 minutes following placement of: 1) two 22-gauge intravenous cannulas (Becton-Dickinson) of which one was for drawing blood samples and one was for βAR-agonist infusion, inserted into a palpable veins in opposite lower arms; 2) three spot ECG electrodes; and, 3) an occluding cuff on the non-infusion arm.

βAR-agonist was then infused at incremental rates (0.1µg, 0.5µg and 1µg /min/1.73 m^2 BSA for 5 minutes each) for 15 minutes until the participants heart rate had increased by ~20 beats per minute (bpm) compared to resting heart rate. The final infusion rate was maintained for 10 minutes. On average, the maximal dose reached 1µg/min/1.73 m^2 BSA. Blood was taken prior to initiation of isoproterenol infusion (ìbaselineî) and in the final minutes of the infusion. ECG, heart rate and blood pressure were monitored throughout the infusion. The half-life of isoproterenol is approximately 2ï 3 min (Goebel et al., 2000). The infusion was generally well-tolerated and all participants successfully completed the protocol.

βAR-antagonist (blockade) procedure

A sub group of 8 participants (age 34.6 ±11.5 years, 2 female) underwent the infusion twice following a 5-day course of either of 80mg of the βAR- antagonist propranolol or placebo. Condition was counter balanced and single blinded.

Questionnaires

In both studies, health and lifestyle variables were assessed by self-report questionnaire. Variables included recent symptoms of illness, exercise behaviour, alcohol consumption, caffeine consumption, smoking, and use of recreational drugs. Affective responses to the psychological stress task were assessed using the short-form of the Profile of Mood States (POMS) (McNair et al., 1992). Participants completed the POMS at baseline, immediately post-task and at 15-minutes recovery.

Flow cytometry

Blood was collected into EDTA tubes, maintained at room temperature and processed within 4 hours after collection. In brief, 9 ml of ammonium chloride lysis solution (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) was added to 1 ml of whole blood to remove red blood cells. After 10 minutes of gentle rotation on ice, 5 ml of phosphate-buffered saline (PBS) was added to stop the reaction, and the samples were centrifuged (283G, 7-min at RT). After supernatant removal, the sample was incubated for 20 minutes at room temperature with a cocktail of fluorescent-labelled monoclonal antibodies to allow identification of progenitor cell subsets: CD34-FITC, CD45RA-FITC, CD7-PE, CD34-PE, CD133-PE, IgG Isotype-PE, CD45-PerCP and CD123-APC (Becton-Dickinson, Oxford, UK) and CD38-PE-Cy7 (eBioscience, Insight Biotechnology Ltd, Middlesex, UK). Cell suspensions were then washed and re-suspended in 500ε l PBS containing 1-2% paraformaldehyde, and stored in the dark at 4°C until analysis. Preparations were read within 18 hours. At least $1x10^6$ gated lymphocytes and monocytes (which forms the ógateô from which PCs are identified), were acquired from each preparation using a dual-laser flow cytometer (FACS-Canto II, Becton Dickinson, Oxfordshire UK). For certain markers, matched isotype controls were used to set negative staining criteria. Data were analyzed using Flowjo 7.4 (Treestar Inc, Ashland, OR, USA). A complete white blood cell count was obtained for each blood sample using a Haematology analyzer (Coulter ACT^{diff}, Beckman Coulter, High Wycombe, UK or Coulter

GEN-S haematology analyser, Beckman-Coulter, Miami, USA). Numbers of PCs were then calculated using standard dual platform methods.

Total PC, HSC, early HSC and late HSC numbers were examined in both studies. Endothelial progenitor cell (EPC) markers were assessed in the psychological stress study and common myeloid precursor (CMP) and common lymphoid precursor (CLP) markers were analysed in the infusion study.

Statistical analysis

The Kolmogorovï Smirnov test indicated that the immunological data was not normally distributed. Therefore, non-parametric analyses (Friedman test) were used to examine the effects of the psychological stress task on cardiovascular, psychological and immunological parameters, and the effect of ß-agonist infusion and ßAR-antagonist procedure on cell numbers. For pairwise comparisons, Wilcoxon signed rank tests were used. Where medians are presented, interquartile range is given in brackets. Variations in degrees of freedom reflect occasional missing data. Data were analyzed using SPSS 16 for windows (SPSS Inc, Chicago, Illinois).

RESULTS

Psychological stress study

Anxiety and cardiovascular responses

Increases in the tension-anxiety POMS subscale confirmed that the speech tasks were perceived as stressful (+9.5 (6.0); $\chi^2_{(23)} = 42.0$, $p<.001$). A physiological stress response was confirmed by significant increases in SBP (+25.3 (13.2) mmHg; $\chi^2_{(23)} = 44.7$, $p<.001$), DBP (+17.8 (9.8) mmHg; $\chi^2_{(23)} = 43.1$, $p<.001$), and HR (+ 21.8 (11.5) bpm; $\chi^2_{(23)} = 37.7$,

p<.001). These cardiovascular changes appeared driven by an increase in sympathetic cardiac drive, as reflected by a decrease in PEP (ï 8.3 (9.0) ms; ɢ$^2_{(12)}$ = 23.2, *p*<.001), and a vagal withdrawal, as evidenced by a decrease in RMSSD (ï 16.3 (30.8) ms; ɢ$^2_{(12)}$ = 19.0, *p*<.001). At 15-min post-stress, all cardiovascular and autonomic measures had returned to baseline values.

Psychological stress induced mobilization of PC subsets

As can be seen in Table 2, the Friedman test yielded a significant effect of time for the added total of lymphocytes and monocytes, PCs, HSCs, late HSCs and EPC2s. Post hoc analyses, using Wilcoxon signed-rank test, confirmed that the former effects were largely driven by an increase in cell number from baseline to stress, and that all PC numbers returned to baseline value at post-15 minute recovery. The added total of lymphocytes and monocytes remained modestly increased at recovery.

Haemoconcentration significantly increased from baseline to stress (+1.7%; ɢ$^2_{(23)}$ =11.6, *p*=.001) and blood volume decreased (ī 4 æ%; $t_{(25)}$= -6.2, *p*<.001). Cell numbers during task were adjusted and analyses was repeated to confirm that any significant effects were not explained by the change in blood volume; the results were essentially unaltered (*p*ŝ <.05).

βAR-agonist infusion study

βAR-agonist infusion did not induce PC mobilization

Table 3 demonstrates that the results of the infusion study did not replicate the stress study data; an effect of time was only seen for the total of lymphocytes and monocytes. The only HSC population that appeared to become mobilized was the GMP subset.

Table 1. Progenitor cell subsets and identification methods

Cell type	*Abbreviation*	*Protocol/ Description* À	*Reference*
Total progenitor cells	PCs	Milian/ Mulhouse protocol used to identify and enumerate mobilized progenitor cells	(Gajkowska et al., 2006; Sutherland et al., 1993)
Haematopoietic stem cell	HSC	International Society of Haemtotherapy and Graft Engineering (ISHAGE) protocol for progenitor cell identification	(Barnett et al., 1999; Gajkowska et al., 2006; Sutherland et al., 1994)
Early Haematopoietic stem cell	Early HSC	Non lineage committed (early) HSC	(Terstappen et al., 1991)
Late Haematopoietic stem cell	Late HSC	Lineage committed (late) HSC	(Terstappen et al., 1991)
Endothelial progenitor cell 1	EPC1	EPC, identification method 1	(Case et al., 2007; Timmermans et al., 2007)
Endothelial progenitor cell 2	EPC2	EPC, identification method 2	(Timmermans et al., 2007)
Common lymphoid progenitor cell	CLP	HSC committed to the lymphoid lineage ÀÀ	(Galy et al., 1995; Hao et al., 2001; Hoebeke et al., 2007; Terstappen et al., 1991)
Common myeloid progenitor cell	CMP	HSC committed to the myeloid lineage but are still capable of committing to either GMP or MEP lineages	(Akashi et al., 2000; Manz et al., 2002)
Granulocyte/ macrophage progenitor cell	GMP	CMP cell committed to granulocyte/macrophage lineage	(Akashi et al., 2000; Manz et al., 2002)
Megakaryocyte/ erythrocyte progenitor cell	MEP	CMP cell committed to megakaryocyte/ erythrocyte lineage	(Akashi et al., 2000; Manz et al., 2002)

À Light scatter and marker identification methods were obtained from the protocols/ references provided. However, due to the extensive PC subtypes assessed in the current study, and the two-platform enumeration method used, some gating and enumeration strategies have been adapted.

ÀÀ As the number of $CD38^{\bar{}}$ $CD7^{+}$ CLP was extremely low, CLP were taken to be $CD38^{+/\bar{}}$. This decision is supported by findings of Galy et al, and Terstappen et al, that found $CD38^{+}$cells can form all lineages of lymphocytes and that lineage committed CLP can express CD38, respectively.

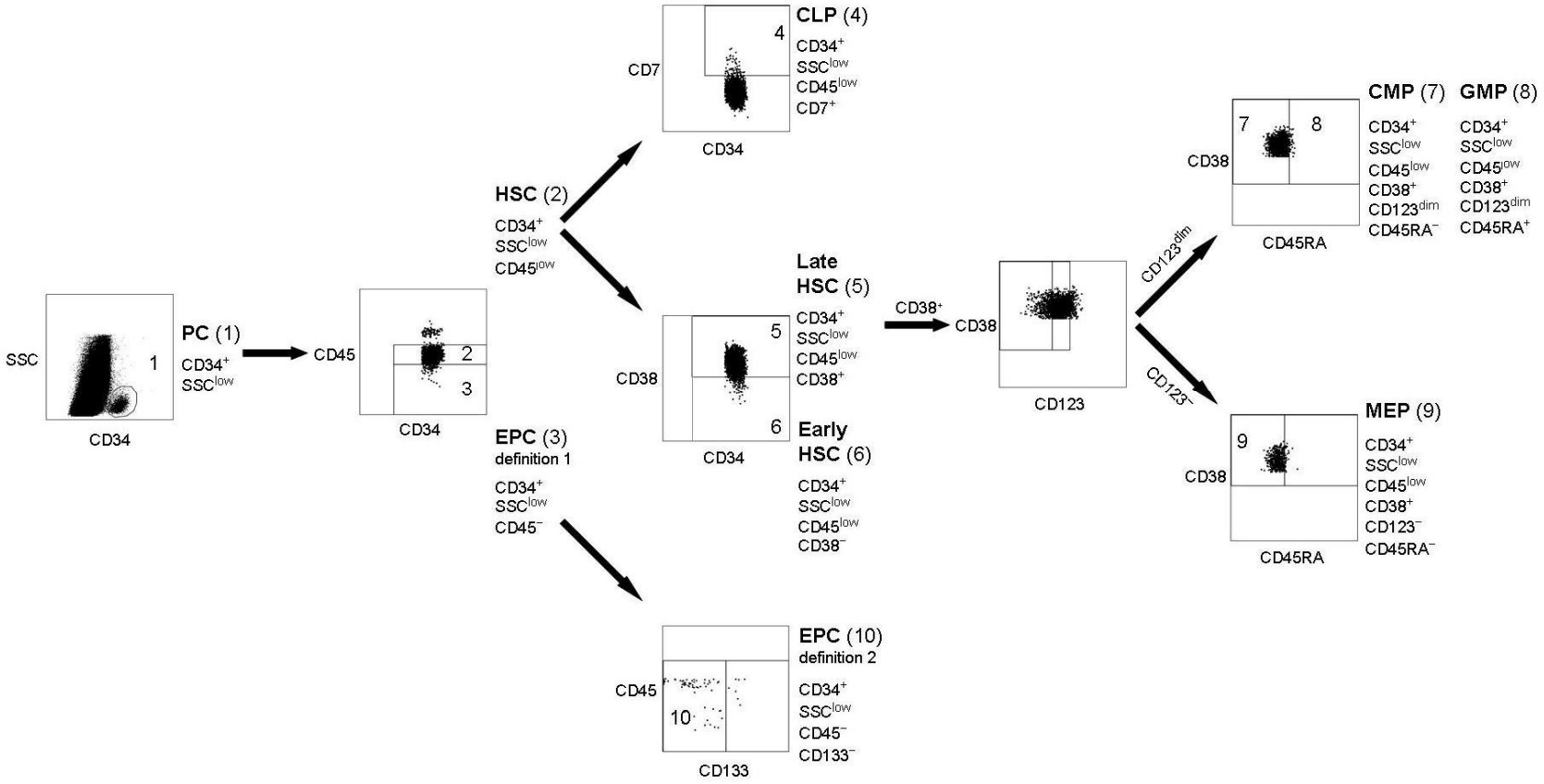

Figure 1. Cell surface markers and flow cytometry gating strategy used in the current study to identify PC subsets.

Table 2. Median (interquartile range) of lymphocyte and monocytes and progenitor cell subsets at each time point during the psychological stress study, with results from Friedman test (non-parametric analyses).

Cell type (cells/ µl)	*Baseline*	*Task*	*Recovery*	χ^2 *(df)*	*p*
Lymphocytes and monocytes	2000 (600)	2700 (1200)***	2500 (900)*	$\chi^2_{(23)} = 29.65$	<.001
Total PCs	1.960 (1.14)	2.200 (1.44)***	1.900 (1.52)	$\chi^2_{(23)} = 17.77$	<.001
HSCs	1.770 (1.14)	1.991 (1.43)**	1.727 (1.46)	$\chi^2_{(23)} = 13.52$	=.001
Early HSCs	0.210 (0.92)	0.209 (1.39)	0.215 (1.27)	$\chi^2_{(21)} = 2.70$	=.260
Late HSCs	1.602 (0.26)	1.735 (0.32)**	1.663 (0.24)	$\chi^2_{(21)} = 11.04$	=.004
EPC1	0.055 (0.04)	0.064 (0.05)	0.065 (0.05)	$\chi^2_{(23)} = 3.92$	=.141
EPC2	0.047 (0.04)	0.059 (0.05)*	0.049 (0.04)	$\chi^2_{(15)} = 7.18$	=.028

Results from post hoc analyses; *p<.05, **p<.01 and ***p<.001 indicates a significant increase in cell number from baseline value (Wilcoxon Signed Rank Test).

Table 3. Median (interquartile range) of lymphocyte and monocytes and progenitor cell subsets at each time point during the βAR-agonist infusion stress study, with results from Friedman test (non-parametric analyses).

	βAR-agonist infusion				*βAR-agonist infusion and βAR-antagonist*			
Cell type (cells/ µl)	*Baseline*	*Infusion*	$\chi^2(df)$	*p*	*Baseline*	*Infusion*	$\chi^2(df)$	*p*
Lymphocytes and monocytes	2226 (1140)	2688 (770)	$G^2_{(17)} = 8.90$	=.003	2127 (570)	2122 (320)	$G^2_{(6)} = .000$	=1.00
PCs	2.517 (2.21)	2.455 (2.34)	$G^2_{(17)} = .474$	=.491	2.350 (2.30)	2.552 (2.28)	$G^2_{(6)} = .500$	=.480
HSCs	2.211 (2.15)	2.135 (1.91)	$G^2_{(17)} = .053$	=.819	2.122 (2.32)	2.310 (2.33)	$G^2_{(6)} = .500$	=.480
Early HSCs	0.169 (0.24)	0.194 (0.23)	$G^2_{(17)} = .474$	=.491	0.136 (0.25)	0.195 (0.23)	$G^2_{(6)} = .000$	=1.00
Late HSCs	1.985 (1.98)	1.814 (1.76)	$G^2_{(17)} = .053$	=.819	1.926 (2.04)	2.096 (2.05)	$G^2_{(6)} = .000$	=1.00
CLP	0.214 (0.17)	0.193 (0.14)	$G^2_{(17)} = .474$	=.491	0.190 (0.20)	0.174 (0.24)	$G^2_{(6)} = .500$	=.480
CMP	1.220 (1.08)	1.188 (1.58)	$G^2_{(16)} = .000$	=1.00	1.392 (1.86)	1.460 (1.82)	$G^2_{(6)} = 2.00$	=.157
GMP	0.057 (0.07)	0.100 (0.14)	$G^2_{(16)} = 5.56$	=.018	0.110 (0.11)	0.094 (0.11)	$G^2_{(6)} = .000$	=1.00
MEP	0.284 (0.30)	0.264 (0.37)	$G^2_{(16)} = .222$	=.637	0.313 (0.40)	0.312 (0.40)	$G^2_{(6)} = 2.00$	=.157

DISCUSSION

Physical stressors, such as exercise and trauma rapidly mobilize progenitor cell (PC) subsets into peripheral blood (Barrett et al., 1978; Bonsignore et al., 2002; Goussetis et al., 2009; Morici et al., 2005; Rehman et al., 2004; Schmidt et al., 2007; Shintani et al., 2001). The present study investigated whether PC mobilization could also be induced by psychological stress and ϐAR-agonist infusion. Results demonstrated that total PCs, hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs), were indeed mobilized during a mild acute stressor. However, mobilization was not induced by infusion of the ϐAR-agonist, isoproterenol, indicating that non-ϐ-adrenergic mechanisms are likely to play a role in PC mobilization during acute psychological stress.

It has been proposed that stress lymphocytosis enhances immune surveillance during perceived threat when the risk of injury and concurrent infection are heightened (Benschop et al., 1996b; Dhabhar and McEwen, 1997; Segerstrom and Miller, 2004). Concurrent mobilization of PCs capable of tissue regeneration and repair may further benefit the host. Such a protective and regenerative effect of PC mobilization has been demonstrated with other stressors, such as ischemia and infectious stimuli. For example, mobilized PCs home to sites of tissue damage, such as areas of ischemia, where they promote vasculogenesis and improvement of endothelial function (Asahara et al., 1997; Rafii and Lyden, 2003; Takahashi et al., 1999). Likewise, infection and injection of endotoxin can also induce PC mobilization (Cline and Golde, 1977; Yamada et al., 2005). Some PC subsets express toll-like receptors (TLR) which, when activated, induce vigorous PC proliferation (Nagai et al., 2006). This may boost the local supply of immune cells, such as dendritic cells and monocytes, to clear infection (Massberg et al., 2007). Thus, PC mobilization is produced by various stressors, and may directly contribute to tissue repair and clearance of microbes.

At present it is unclear what mechanism may play a role in stress-induced mobilization. The finding that ßAR-agonist infusion did not induce PC mobilization, does not exclude the involvement of adrenergic mechanisms; HSC also express alpha-adrenergic receptors (ŬARs) (Muthu et al., 2007), In support of ŬAR-involvement, the impairment of PC mobilization was more profound in mice chemically sympathectomised than it was in mice injected with ßAR-antagonist (Katayama et al., 2006). Further, administration of a $ß_2$AR-agonist to mice unable to synthesis norepinephrine only partially restores the PC mobilization defect. Finally, $ß_2$AR-agonist did not elicit circulating PCs in mice without co-stimulation with G-CSF. Taken together, this data suggests the contribution of other adrenergic signals or non-adrenergic signals in the mobilization of PCs during acute stress (Mendez-Ferrer and Frenette, 2007).

In addition to norepinephrine and epinephrine, several other factors released during exercise and psychological stressors have demonstrated the ability to induce PC mobilization. These include the adrenocorticotropic hormone (ACTH), granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), IL-1, IL-8, macrophage inflammatory protein-1 alpha (MIP-1Ŭ), and the enzyme nitric oxide synthase 3 (NOS3) (Aicher et al., 2004; Barrett et al., 1978; Cacioppo et al., 1998; Elenkov et al., 2005; Goshen and Yirmiya, 2009; Lapidot et al., 2005; Morici et al., 2005; Ostrowski et al., 2001; Pedersen et al., 2001; Steptoe et al., 2007; Suzuki et al., 2000; Winkler and Levesque, 2006; Yang et al., 2007). Limiting the focus on possible mediators to those that affect PC mobilization within minutes, as seen in the present study, IL-1, IL-8, MIPŬ, and NOS3 or nitric oxide are possible candidates (Fibbe et al., 1992; Laterveer et al., 1995; Laterveer et al., 1996; Lord et al., 1995; Yang et al., 2007). Moreover, of these only NOS3 and nitric oxide are upregulated within minutes of stressor onset, which, on the basis of current knowledge, makes these factors and ŬAR-agonist relevant targets for future research.

A limitation of the current study is that CLPs, CMPs and MEPs were not assessed in the stress study, and that EPCs were not identified in the infusion study. Comparisons on the PC mobilizing effect of psychological stress and ßAR-stimulation were possible though, as total PC, HSCs, early HSCs and late HSCs were assessed in both study. In addition, progenitor subsets were only identified using cell-surface markers; future studies may complement the current findings by including culture assays that ultimately determine the differentiating potential of mobilized cells. However, the identification methods used in the present study have been validated in several studies (see Table 1 for references) and we are confident, therefore, that the phenotypic pattern of PC mobilization would largely be replicated using culturing methods. One exception may perhaps be the EPC responses, as there is currently much debate regarding the validity of identifying EPCs by surface marker expression (Timmermans et al., 2009). Various phenotyping methods for EPC identification have been reported in the literature and most involve expression of CD34 and/ or CD133 in combination with KDR (VEGF receptor), with or without low expression of CD45 (Hirschi et al., 2008). Preliminary tests for the current study showed that the number of $CD34^{+}KDR^{+}$ cells was too low for reliable assessment, and thus KDR was not used to identify EPCs in the current study.

Understanding the mechanisms by which PCs are mobilized during acute stress may have great clinical utility. To successful reconstitute the haematopoietic system of the recipient, at least $2x10^{6}$ HSCs per kg body mass need to be transplanted (Villalon et al., 2000). In approximately 1-5% of donors, current pharmacological treatments fails to reach this threshold (Winkler and Levesque, 2006). As approximately 45,000 patients a year worldwide receive HSC treatment, improved mobilization methods are imperative. Methods to increase circulating progenitor cells numbers may also benefit patients with cardiovascular disease (CVD), as low PC number and reduced PC function are associated with atherosclerosis, hypertension, myocardial infarction, and increased risk of death due to cardiovascular

causes (Hill et al., 2003; Vasa et al., 2001; Werner et al., 2005). Sufferers of CVD may therefore benefit from exercise induced PC mobilization. However, in situations where regular exercise cannot be undertaken, alternative pharmacological methods of increasing the circulating number of PCs may improve vascular function.

In conclusion, the present study demonstrated that acute psychological stress, but not βAR-stimulation, can induce PC mobilization. These findings are in contrast to the clearly βAR-mediated stress lymphocytosis, and suggest that stress-induced mobilization of PC and lymphocytes are governed by different mechanisms. Increased PC circulation may serve a protective function by being conducive to vascular repair and promoting innate immunity.

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CHAPTER 6

SUMMARY AND DISCUSSION

In the preface of this thesis three aims were set for the research in the subsequent chapters:

1) To characterise the CD8TL response to stress and beta-adrenergic stimulation
2) To determine the influence of CMV infection on CD8TL mobilization
3) To determine whether PCs can similarly be mobilized by acute psychological stress and ϐAR-stimuli

The subsequent sections summarise and discuss the main findings for each of these aims. We also address the possible implications of the observed results and suggest avenues for future exploration.

THE $CD8^+$ T LYMPHOCYTE RESPONSE TO STRESS AND BETA-ADRENERGIC STIMULATION

The data collected in Chapter 2 extended earlier observations by showing that stress-induced mobilization of CD8TL is correlated to cell differentiation (Atanackovic et al., 2006; Campbell et al., 2009). Specifically, highly differentiated cells with a $CCR7^-CD28^-CD27^-$ phenotype showed the strongest mobilization during stress. These cells are known to have strong effector capabilities, such as high tissue migratory potential, cytotoxicity, and production of effector cytokines such as IFN-ɔ (Monteiro et al., 2007; Romero et al., 2007). These results were exactly replicated by ɓAR-agonist infusion, clearly suggesting ɓ-adrenergic receptor stimulation mediated the observed effects of stress.

The above findings show a strong parallel with the literature on NK mobilization, which is similarly found to be mediated by ɓ-adrenergic mechanisms (Benschop et al., 1996; Benschop et al., 1994; Schedlowski et al., 1996). It seems likely that the main ɓ-adrenergic receptor involved is the $ɓ_2$-subtype, although this was not specifically studied here; this receptor was found to be upregulated on late-differentiated CD8TLs as determined by radio-ligand studies and micro-array studies (Dimitrov et al., 2009; Holmes et al., 2005). It has recently been shown that epinephrine causes a rapid detachment of differentiated CD8TLs from cultured endothelium, clearly suggesting that this mechanism is also involved in the current findings (Dimitrov et al., 2010).

The selective mobilization of differentiated CD8TLs provides a unifying explanation for a number of reported observations, such as the change in adhesion molecule expression on mobilized cells (i.e, decrease in CD62L, increase in CD11a), their apparently increased replicative senescence, and increased expression of cytotoxic granules and inflammatory mediators (Atanackovic et al., 2006; Bosch et al., 2003; Mills et al., 2000; Simpson et al.,

2007). Rather than describing actual changes in cell features, these independent findings appear better explained by a selective mobilization of cells that share these phenotypic and functional characteristics, i.e., the late-differentiated CD8TLs.

It is significant that lymphocyte mobilization, in particular of cytotoxic subsets, is generalized over various stressors including psychological stress, exercise, trauma, heat, and cold shock (Bouchama et al., 1992; Bruunsgaard et al., 1999; Karandikar et al., 2002; Simpson et al., 2007). This generalizability is consistent with the notion that mobilization is part of an adaptive response to threat. Using a military metaphor, Dhabhar et al, describe the immunoenhancing nature of this phenomenon as the bodyôs ósoldiersò(leukocytes) exiting their óbarracksò(spleen and bone marrow) and traveling over the óboulevardsò(blood vessels) in order to take position at potential óbattle stationsò(skin and other peripheral organs) (Dhabhar, 2002). In support of this theory, the cells that were found mobilized during stress would indeed qualify as ósoldiersôthat have a rapid killing activity without the need for conventional co-stimulatory signals and a propensity to migrate into inflamed, óbattle-groundô tissues. Chapters 2, 3 and 4 discuss the potential protective (e.g., during infection) and damaging (e.g., during inflammatory diseases) implication of this selective stress response.

Immune dysregulation by stress is enhanced in older individuals (Gouin et al., 2008; Graham et al., 2006; Segerstrom et al., 2008). It is therefore intriguing that the cells that were found to be most stress and adrenergic sensitive are also denoted ñagedò T cells as they accumulate in the older adult (Gillespie et al., 2000). Indeed, the accumulation of late-differentiated CD8TLs is considered a hallmark of immunosenescence and predicts increased morbidity and mortality in older individuals (Sansoni et al., 2008; Wikby et al., 2005). Thus, the altered neuro-endocrine sensitivity of these late-differentiated cells might provide a mechanistic explanation for why stress has a larger impact in older adults (Segerstrom and Miller, 2004).

THE INFLUENCE OF CMV INFECTION ON $CD8^+$ T LYMPHOCYTE MOBILIZATION

Latent CMV infection causes a dramatic expansion of late-differentiated CD8TLs (Gillespie et al., 2000; Khan et al., 2002). As these are the same cells that were found to be stress- and ßAR- responsive, it was speculated that CMV infection might lead to an amplified CD8TL stress response. The results in Chapter 3 confirm this prediction, showing that CMV^{pos} individuals, as compared to CMV^{neg}, have a ~3-fold higher CD8TL mobilization during stress and an even larger increase (~17-fold) CD8TL mobilization during ßAR-agonist infusion. As hypothesized, greater mobilization of CD8TL numbers was consistently correlated (r between ~.46 ï .86) with greater resting numbers of late-differentiated CD8TL subsets, accounting for the observed CMV effect. This data provides the first evidence that host-viral interactions can modify immunological responses to stress and neuro-endocrine stimulation.

It could be excluded that the enhanced mobilization associated with latent CMV infection was due to a higher sensitivity to stress, which might, for example reflect neuropsychological effects of the virus (Lafferty, 2005; Nau and Schmidt, 2007; Perry et al., 2008; Phillips et al., 2008). No differences were observed in psychological, autonomic, or cardiac responses between the two groups. Also, the enhanced effect was replicated by ßAR-agonist infusion, excluding a mere enhanced psychological responsivity. Finally, the effect of CMV serostatus appeared not generalize, but was selective to CD8TLs and other T cell populations known to be affected by CMV infection.

The expansion of late-differentiated cells in CMV-infected individuals can, at least in part, be attributed to an accumulation of CMV-specific CD8TLs, which are known to have a largely late-differentiated phenotype. This raised the question whether the enhanced response to stress and ßAR-stimulation, described in Chapter 3, is due to an enhanced mobilization of CMV-specific cells, or whether phenotype (i.e., irrespective of antigen specificity) is a

determinant of mobilization. In other words, is the enhanced CD8TL responsivity due to an expansion of late-differentiated cells, or due to an expansion of CMV-specific CD8TLs? The data presented in Chapter 4 showed that differentiation, and not antigen-specificity, is the major predictor of mobilization. With the exception of the early subsets, the mobilization of memory phenotypes within CMV^{tet+} CD8TLs and total CD8TLs were similar; in both populations $CD28^{i}$ cells (i.e., intermediate- and late-differentiated) displayed at least a 2-fold larger mobilization than $CD28^{+}$ cells (i.e., early-differentiated). Significantly, the same pattern was also replicated for EBV-specific CD8TLs; again confirming that phenotype and not antigen specificity is the main predictor of mobilization to stress.

CMV infection is known to impact a number of immunological parameters frequently studied in psycho-neuro-immunology (PNI), such as vaccine responses, mitogen-induced proliferation, low grade inflammation, and telomere length (Bruunsgaard et al., 1999; Effros, 1997; Epel et al., 2006; Saurwein-Teissl et al., 2002; Trzonkowski et al., 2003; van de Berg et al., 2010). This raises the possibility that CMV infection may be a confounding factor in PNI research. The possibility that CMV infection is a confounder in PNI seems even more likely because characteristics such as socio-economic status and ethnicity are associated with CMV infection, which, in turn, correlate with psychological and physical health outcomes (Aiello et al., 2009; Czernochowski et al., 2008; Lupien et al., 2007; Pawelec et al., 2005; Trzonkowski et al., 2003; Wikby et al., 2005; Zajacova et al., 2009). Thus, on the basis of our findings we suggest that CMV serostatus should be considered as a standard control measure in studies examining the relation between psychosocial factors and immune function.

In humans, CMV is known to reactivate in periods of enhanced stress (e.g., end of year examination periods) and increased sympathetic activation (e.g., myocardial infraction) (Prosch et al., 2000; Sarid et al., 2004). In Chapter 4 we discussed the possibility that

increased ß-adrenergic sensitivity of late-differentiated CD8TLs may also alter other cell functions such as, cell proliferation, lytic activity, and cytokine production, which, in turn, could contribute to a reduced control of latent CMV (Bartik et al., 1993; Borger et al., 1998; Gratama et al., 2008; Hatfield et al., 1986; Kalinichenko et al., 1999; Leo and Bonneau, 2000; Ozdemir et al., 2002; Sekut et al., 1995). Alternatively, increased adrenergic sensitivity and enhanced immune surveillance during stress may have evolved in coexistence with CMV infection, as an immunological counter measure to heighten defences during periods when CMV reactivation is more likely to occur.

ACUTE STRESS, BETA-ADRENERGIC RECEPTOR STIMULATION, AND PROGENITOR CELL MOBILIZATION

To the best of our knowledge, the study presented in Chapter 5 is the first to demonstrate that progenitor cells (PCs) mobilize in response to psychological stress. This effect was observed for both the haematopoietic stem cells (HSC) and endothelial progenitor cells (EPC). Within the HSC and EPC population, only cells that showed late-differentiation (e.g., $CD38^{+}$ and $CD133^{-}$ respectively), and are thus committed to a particular lineage, were mobilized. The effect of stress on PC mobilization was hypothesized because experimental human and animal data demonstrated adrenergic receptor expression on PCs (Muthu et al., 2007; Spiegel et al., 2007). Moreover, mouse experiments showed that ablation of adrenergic neurotransmission prevented PC egress from the bone-marrow into the blood and that administration of a $ß_2$AR-agonist could partially reverse this affect (Katayama et al., 2006). Hence it was unexpected that infusion of the ßAR-agonist was unable to replicate the effects of stress. This suggests that the stress induced response is governed by ŭAR or non-adrenergic mechanisms.

The findings in Chapter 5, can be seen as complementing a general theme, observed throughout the various chapters, that the effects of acute stress on blood cellularity appear to have adaptive utility. Indeed, based on extant data, it seems reasonable to assume that increased mobilization of PC subsets is conducive to restorative process including tissue repair, angiogenesis, and immune reconstitution (Ballard and Edelberg, 2007; Dimmeler and Zeiher, 2004; Foresta et al., 2010; Kocher et al., 2001; To et al., 1997; Yamada et al., 2005).

REMAINING ISSUES

Whereas the research in this thesis has brought to light a number of new facts, it also raised questions that require further research. One of the most relevant issues is perhaps the clinical relevance of leukocyte mobilization during stress. While it is possible to propose a number of beneficial functions for this response, at this point, such suggestions remain largely speculative. Studies in humans and animals do appear to provide preliminary support for immune-enhancement during acute stress, and extending this work is an important next step (Dhabhar, 2002; Gosain et al., 2006; Rosenberger et al., 2009; Shimaoka et al., 1998; Viswanathan et al., 2005; Viswanathan and Dhabhar, 2005).

The studies presented in this thesis have provided extensive phenotyping of mobilized cells, in particular CD8TLs and PCs. It was proposed that future studies should address how these changes in blood composition affect the results of functional tests. For example, does the increased mobilization of late-differentiated cells, known to exhibit a reduced ability to respond to mitogens and an enhanced ability to produce INF-ɔ, lead to parallel changes in proliferation and INF-ɔ production using functional assays? Moreover, at this point it is unclear which, or if, cellular functions are affected in parallel with cell mobilization.

Another important next step would be to determine the exact mechanisms for stress-induced mobilization of cytotoxic lymphocytes and PCs. For example, while the research presented here was able to demonstrate an effect of ßAR-stimulation on CD8TL mobilization, it is unclear which cellular events subsequently lead to the release of these cells into the blood. Similarly, the mechanisms governing stem cells release during acute stressors are currently unknown. Although the current work demonstrated that ßAR-stimulation alone does not induce PC mobilization, we can not rule out the involvement of adrenergic or non-adrenergic receptor mechanisms.

A final issue pertains to the role of host-microbe interactions in human behaviour and stress responses. It has been estimated that the number of microbial cells that infest the human body is at least 10-fold higher than the number of actual bodily cells (Todar, 2004). This number is even more impressive when considering that the calculation excludes non-cellular microbes such as viruses. Many of these micro-organisms have coevolved with human evolution, and there is ample evidence that much of our biology is shaped through this microbial co-existence, with perhaps the primeval incorporation of mitochondria in eukaryotes as a prime example (Forterre, 2006; Hunter, 2010; Raven, 1970). If microbes can shape our biology then that surely would not exclude our psychobiology. The fact that CMV infection can substantially amplify the immune response to stress and neuro-endocrine stimuli is one of only a few examples that have been identified thus far, and this theme clearly warrants further exploration.

SUMMARY

The work in this thesis will contribute to the literature with several novel findings, and provided explanations for previous observations. It was found that;

- Stress and βAR-sensitivity of CD8TLs increased in parallel with cell differentiation (progressive loss of CCR7, CD28 and CD27) which has been linked with accumulation of greater effector functions.
- Infection with CMV enhanced the stress- and/ or βAR- reactivity of CD8TLs, CD4TLs and NKT-like cells. Enhanced CD8TL mobilization was associated with larger numbers of late-differentiated cells in CMV^{pos} individuals.
- CMV^{tet+} CD8TLs responded greater to stress and βAR-stimulation when compared to total CD8TLs or the EBV^{tet+} population. The mobilization of CMV^{tet+} subsets demonstrated the same mobilization pattern as total CD8TLs ($CD28^{-} > CD28^{+}$), suggesting that cell differentiation is the major determinant of stress responsiveness, and not antigen specificity; the total CMV^{tet+} population showed greater mobilization during stress and βAR-stimulation as a larger proportion of the cells were of the late-differentiated phenotype.
- PCs were mobilized by acute psychological stress but βAR-mechanisms did not govern the phenomenon.

In sum, the current findings further support the notion that stress induced cell mobilization is an adaptive response geared to protect the host by increasing immune surveillance and tissue repair mechanisms, in the face of immediate threat. However, the actual impact on health outcomes may not always be beneficial depending on the particular situation, i.e., infection verses inflammation.

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APPENDIX

VOLUNTEER PARTICIPATION IN PSYCHOLOGICAL STRESS STUDIES

Table 1. Volunteer participation in each of the four stress studies

Participant	*Chapter 2*	*Chapter 3*	*Chapter 4*	*Chapter 5*
102	X	X	X	
103	X	X	X	
106			X	X
107		X	X	X
120		X		X
123	X		X	X
130	X	X	X	
131	X	X		X
139	X	X	X	X
142	X	X	X	
143	X		X	X
144			X	X
145	X	X		X
146	X	X	X	X
147	X	X	X	X
148	X	X		
151	X		X	
160	X	X	X	
166	X	X	X	
167	X			
177	X	X	X	X
178	X	X		
184	X			X
193	X	X	X	
195	X	X	X	
198	X	X	X	
201		X		
202		X	X	
203	X	X	X	X
210	X		X	X
235	X	X	X	
236			X	X
239	X	X		X
240	X	X		X
241	X	X		X
245		X		X
246		X	X	X
247		X	X	X
248		X	X	X
249			X	X
250		X	X	X
251		X		X

βAR-AGONIST INFUSION STUDY- DATA COLLECTION AND VOLUNTEERS

The βAR-agonist infusion study was performed at the UCSD as part of an on going collaboration between The School of Sport and Exercise Sciences, UoB, UK and laboratory of Professor Paul J Mills, UCSD, USA. The author of this thesis gained two travel awards which helped fund the seven week laboratory visit. The protocol was performed by nurses in the General Clinical Research Centre at UCSD under the supervision of Miss Natalie Riddell. The samples were then prepared and analysed by Miss Riddell.

Table 2. Volunteer participation in each of the four infusion studies

Participant	*Chapter 2*	*Chapter 3*	*Chapter 4*	*Chapter 5*	*βAR-antagonist procedure*
1	X	X		X	X
2		X		X	X
3		X		X	
4	X	X	X	X	
5	X	X		X	
6	X	X		X	X
7	X	X	X	X	X
8	X	X		X	
9	X	X		X	
10	X	X		X	
11	X	X	X	X	
12	X	X	X	X	X
13		X		X	
14		X		X	
15	X	X	X	X	X
16	X	X		X	
17	X	X		X	X
18	X	X	X	X	X
19	X	X		X	
20	X	X		X	