PHENOTYPIC CHARACTERIZATION OF STRESS LEUKOCYTOSIS

By

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ABSTRACT

The present thesis describes research that characterizes the mobilization of cytotoxic T cell subsets and monocyte populations in response to acute psychological stress and β-agonist (isoproterenol) infusion. Chapter two showed that γδ T cells are mobilized in response to psychological stress and isoproterenol infusion, implicating β-adrenergic mechanisms in this response. Chapter three demonstrated that γδ T cells that were tissue migrating ($CD11a^{hi}$), of an effector memory phenotype ($CD27^{-}CD45RA^{+}$), and displaying NK-like features ($CD94^{+}$), were most sensitive to stress induced mobilization. Chapter four showed that a perforin $(pfn)^{+}$ $C27^{-}$ phenotype in $CD4^{+}$, $CD8^{+}$ and γδ T cells consistency identified cells most sensitive to stress and isoproterenol induced mobilization. However, although cytotoxicity (pfn^{+}) was important, differentiation ($CD27^{-}$) status better predicted mobilization. Chapter five revealed that of the three major monocyte populations; $CD14^{++}CD16^{-}$, $C14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$, the 'proinflammatory' $CD14^{+}CD16^{+}$ monocytes showed the largest mobilization response during stress and isoproterenol infusion. Thus, the selective mobilization of cells with a high effector ability applies to monocyte populations also. We speculate that mobilization of these leukocytes may represent an adaptive mechanism aimed at enhancing host immune defenses in times of threat. This response can have beneficial and detrimental effects depending on the inflammatory or infectious context.

For Mum and Papa

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DATA COLLECTION AND ANALYSIS

Chapters two and three

Data for the psychological stress study was collected by Leila Anane in 2005-7 at the University of Birmingham. The exercise study data was kindly provided by Dr. Jos Bosch at the University of Birmingham in 2005, and the infusion study data by Professor Paul Mills at the University of California San Diego in 2000.

Chapter four

Data for the stress study was designed and collected by Leila Anane at the University of Birmingham in 2006-2008, some samples were also collected by Natalie Riddell. Data for the infusion study was collected according to the flow cytometry panel designed in the stress study by Leila Anane. The latter data was collected by Natalie Riddell at the University of California San Diego in 2009.

Chapter five

Data for the stress study was kindly provided by Dr. Jos Bosch from a study carried out in the Ohio state University in 2003. Data for the infusion study was collected by Natalie Riddell in 2009 at the University of California Sand Diego.

Research questions were developed by Leila Anane and all analyses and interpretation of raw data was carried out by Leila Anane.

LIST OF PAPERS

This thesis incorporates four papers:

1) Anane L.H., Edwards K.E., Burns V.E., Drayson M.T., Van Zanten J.J., Wallace G.R. Mills P.J. Bosch J.A. (2009). Mobilization of $\gamma\delta$ T lymphocytes in response to psychological stress, exercise and β-agonist infusion. *Brain Behavior Immunity*. 23 (6) 823-9.
2) Anane L.H., Edwards K.E., Burns V.E., Van Zanten J.J., Drayson M.T., Bosch J.A. (2010) Phenotypic characterization of $\gamma\delta$ T cells mobilized in response to acute psychological stress. *Brain Behavior and Immunity*. 24(4) 608-14.
3) Anane L.H., Riddell N.E., Burns V.E., Edwards K.E., Drayson M.T., Van Zanten J.J., Mills P.J, Bosch J.A. Differentiated, perforin+ T cells are mobilized during psychological stress and isoproterenol infusion. *In preparation.*
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In addition, the following presentation arose from this thesis.

Anane L.H., Burns V.E., Edwards K.E., , Drayson M.T., Van Zanten J.J., Mills P.J. Bosch J.A. Characteristics of stress lymphocytosis. *New developments in psychoneuroimmunology*, April 2009.

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ABBREVIATIONS

Δ% - relative change in cell numbers

ACTH- adrenocorticotropic hormone

ANOVA- analysis of variance

APC- allophycocyanin

β-agonist- beta adrenergic receptor agonist

β-antagonist – beta adrenergic receptor antagonist

BMI- body mass index

BSA- body surface area

Bpm- beats per minute

CM- central memory

CRH – corticotropin releasing hormone

δ1- delta One T cells

δ2- delta Two T cells

DBP- diastolic Blood Pressure

EDTA - ethylenediaminetetraacetic acid

EM- effector memory

EMRA- $CD45RA^{+}$ effector memory

FITC- fluorescein isothiocyanate

γδ T cell – Gamma-Delta T cell

HPA- hypothalamic-pituitary-adrenal axis

HR - heart rate

IL- interleukin

IFN-γ- interferon gamma

MHC- major histocompatabilty complex

NA- naïve

NK cell – natural killer cell

PBS- phosphate buffered saline

PE- phycoerthryn

PEP- pre-ejection period

PERCP- peridinin chlorophyll protein

Pfn- perforin

PNI – psychoneuroimmunology

POMS – profile of mood states

RANTES - Regulated on Activation Normal T Cell Expressed and Secreted

RMSSD – root mean square of successive differences

RSA- respiratory sinnus arrhythmia

SAM- sympathetic-adrenal-medullary axis

SBP- systolic blood pressure

SD- standard deviation

SEM- standard error of the mean

SNS – sympathetic nervous system

TCR – T cell receptor

TNF-α- tumor necrosis factor alpha

CHAPTER ONE

GENERAL INTRODUCTION

The stress response

The human body exists in a state of equilibrium, maintained by a variety of physiological processes. This balance can be disturbed by numerous factors, including infection, injury, and the psychological threat of danger, which includes disturbances in social interaction (Tewes, 1999). When the body cannot maintain equilibrium in the face of such challenges a 'stress response', is initiated. Walter Cannon first coined the concept of stress in this context, describing it as an emergency mechanism that mobilizes energy for fight-or-flight responses (Cannon, 1929). Stress can be thought of as a constellation of events comprised of a stimulus 'stressor' that precipitates a reaction in the brain 'stress perception' that activates fight-flight mechanisms 'stress response' (Dhabhar, 2002). Richard Lazarus proposed that the initiation of this stress response, be it an emotional (i.e. anger, anxiety), behavioral (i.e. running from threat), or biological response (i.e. cardiovascular changes, immune alterations), is governed by a person's perception of their ability to cope with a particular stimulus. Therefore, psychological stress occurs when a person's perceived ability to 'cope' with a situation is exceeded (Lazarus and Folkman, 1984). One way of broadly categorizing psychological stress is into acute and chronic forms of stress; acute stressors are classically short lived, lasting minutes to hours, and typically elicit fight-flight responses. Chronic stressors, on the other hand, such as bereavement, or care-giving for an ill family member, often last months, or even years.

Stress, the immune system and health

It is now well established that psychological stress impacts health. Indeed, stress is implicated in the pathogenesis and exacerbation of a host of diseases and pathologies including cardiovascular disease, viral infection, inflammatory diseases, autoimmune diseases, cancer, allergy and wound healing (Antoni et al., 2006; Arck et al., 2006; Ashcraft et al., 2008; Burns et al., 2003; Chen and Miller, 2007; Cohen et al., 1991; Dimsdale, 2008; Glaser and Kiecolt-Glaser, 2005; Godbout and Glaser, 2006; Kemeny and Schedlowski, 2007; Thaker et al., 2006; Walburn et al., 2009; Wang et al., 2007; Wright, 2005). Although a multitude of factors are likely to underpin the effects of stress on health, stress-induced alterations in immune function are clearly an important mechanism. Stress impacts the immune system through multiple pathways, of which two endocrine pathways have received most attention: the hypothalamic-pituitary-adrenal axis (HPA) and the sympathetic-adrenal-medullary (SAM) axis (Elenkov et al., 2000; Miller et al., 2007; Segerstrom and Miller, 2004). In the HPA axis activation is initiated during stress at the paraventricular nucleus of the hypothalamus which secretes corticotropin releasing hormone (CRH), which acts upon the pituitary gland to release adrenocorticotropic hormone (ACTH), which in turn elicits the production of glucocorticoids, such as cortisol, from the adrenal glands.

In the SAM axis, activation involves preganglionic sympathetic nervous system (SNS) neurons that descend along the spinal cord from nuclei in the brain stem. The release of the neurotransmitter acetylcholine from these neurons in the adrenal medulla stimulates chromaffin cells to secrete the catecholamine epinephrine. Post-ganglionic SNS

fibres, however, predominantly secrete the catecholamine norepinephrine upon activation. Therefore, activation of the SAM mainly leads to the release of epinephrine and norepinephrine (Benschop et al., 1996b; Elenkov et al., 2000). These catecholamines can influence immune cells as all leukocytes express adrenergic surface receptors (Elenkov et al., 2000; Sanders and Kavelaars, 2007). In addition, post-ganglionic nerve fibers directly innervate lymphoid organs including the thymus, spleen, lymph nodes and bone marrow, and therefore come into close proximity with immune cells (Elenkov et al., 2000; Felton et al., 1985; Madden et al., 1995). It may be added that immune cells can also influence the SAM and HPA axis. For example, cytokines such as Interleukin (IL)-1 released by leukocytes can directly activate the HPA and SAM axes. This demonstrates that brain-immune communication is bidirectional (Besedovsky et al., 1986; Dunn, 1988). The field investigating the relationship between the brain and the endocrine and immune systems is termed psychoneuroimmunology (PNI).

Psychoneuroimmunology (PNI)

Over the last thirty years, PNI research has investigated multiple influences of stress on the immune system and revealed a general pattern whereby acute stress is immuno-enhancing, whilst chronic stress is immuno-suppressive (Segerstrom and Miller, 2004). For example, chronic stressors are associated with diminished antibody response to vaccination, slower wound healing and increased susceptibility to infection and cancer progression (Bosch et al., 2007; Gallagher et al., 2009; Glaser et al., 2000; Kiecolt-Glaser

et al., 1996; Marucha et al., 1998; Phillips et al., 2006; Thaker et al., 2006; Walburn et al., 2009). In contrast, acute stress has been demonstrated to enhance antibody response to vaccination, improve wound healing, enhance leukocyte migration into wounded tissue, increase the 'killing' (cytotoxic) capability of immune cells and increase cancer resistance (Dhabhar et al., 2010; Dhabhar and Viswanathan, 2005; Edwards et al., 2006; Kinsey et al., 2003; Saint-Mezard et al., 2003; Segerstrom and Miller, 2004; Viswanathan and Dhabhar, 2005). One of the most robust and widely documented PNI finding, however, is the rapid increase in the number of blood lymphocytes that occurs during acute psychological stress, known as stress lymphocytosis (Segerstrom and Miller, 2004).

Stress lymphocytosis

Stress lymphocytosis was first documented in the early 1900's and is also observed during exercise and physical trauma (Benschop et al., 1996b; Pedersen and Hoffman-Goetz, 2000). Stress lymphocytosis is driven to a large extent by an increase in Natural Killer (NK) cells (Bachen et al., 1992; Benschop et al., 1996b; Brosschot et al., 1992; Schedlowski et al., 1993), which form part of the innate immune system and, as such, are characterized by rapid, non-specific action. NK cells possess cytotoxic machinery and pattern recognition receptors that allow them to swiftly recognize and eliminate pathogens; consequently they provide a first line of defense against infection. Pharmacological β-agonist infusion and β- blockade studies showed that mobilization of NK cells was blocked by the administration of a β-blocker, such as propranolol, and mimicked by the

infusion of a β-adrenergic agonist, such as isoproterenol (Bachen et al., 1995; Benschop et al., 1996a; Schedlowski et al., 1996). This provided evidence that NK cell mobilization was β-adrenergically mediated. There are five main types of adrenergic receptor: α1; α2; β1; β2; and β3. Studies demonstrate that β2 adrenergic receptors in particular have been identified on lymphocyte populations, and mobilization of NK cells is inhibited by a β2, but not a β1-adrenergic blocker (Benschop et al., 1996b; Sanders and Straub, 2002; Schedlowski et al., 1996). Furthermore, in vitro studies demonstrated that treatment of NK cells bound to endothelium with catecholamines caused the detachment of these cells from endothelium (Benschop et al., 1993). Hence, NK cell mobilization is mediated by the action of catecholamines which stimulate β2-adrenergic receptors leading to the detachment of NK cells from endothelium. It is likely that these cells are mobilized from the marginal pool, where patrolling or 'waiting' leukocytes are loosely tethered to blood vessel endothelium in peripheral venules. There are many other potential reservoirs for the immune cells released during stress, including the spleen, lymphatic tissue and the bone marrow. The spleen was ruled out as a source, as splenectomised individuals still exhibit stress lymphocytosis (Benschop et al., 1996a; Schedlowski et al., 1996).

Another cytotoxic lymphocyte class, the $CD8^+$ T cell, is also mobilized during psychological stress by β-adrenergic mechanisms (Benschop et al., 1996b; Landmann. et al., 1984; Maisel et al., 1990; Mills et al., 1995; Murray et al., 1992). Like NK cells, $CD8^+$ T cells possess cytotoxic machinery that can act against virally infected and cancerous cells. Such cytotoxic weaponry includes the expression of perforin, a pore-forming protein that allows the entry of granzymes (enzymes that cleave caspases) into

target cells, which ultimately leads to programmed cell death (apoptosis) of the cell (Shresta et al., 1998). Unlike NK cells, however, $CD8^+$ T cells form part of the adaptive immune system and hence provide specific protection and display immunological memory, eliciting rapid responses to secondary infections.

Intriguingly, other adaptive immune lymphocytes, such as $CD4^+$ T cells and B cells, do not appear to be mobilized during acute stress (Segerstrom and Miller, 2004). $CD4^+$ T cells, also known as T helper cells, play an essential role in regulating the activity of other leukocytes and promoting various immune responses according to the infectious challenge. B cells are involved in the humoral response to infection and produce antibodies directed against specific antigens, neutralizing infectious agents and directing other cells of the immune system to pathogenic threat. Neither of these cells are characterized by cytotoxic capabilities[1] (Janeway et al., 2005).

Cellular adhesion molecules and leukocyte migration

Later work in NK cells and $CD8^+$ T cells separated these lymphocytes based on the expression of cellular adhesion molecules. The expression of cellular adhesion molecules on a leukocyte's surface provides a window into lymphocyte migration and circulation. Leukocytes continuously circulate from the blood into the tissues or lymph nodes and back into the blood. Correct functioning of this migration and circulation is critical to effective

[1] A small population of $CD4^+$ T cells have been shown to exhibit cytotoxicity (Appay et. al, 2002 and chapter 4)

immune defense (von Andrian and Mackay, 2000) and is a complex process involving cellular-adhesion molecules such as integrins, selectins and chemokines. Briefly, integrins and selectins are involved in tethering and rolling of leukocytes along endothelium. In the face of an inflammatory challenge, chemokines are released and lead to activation and integrin mediated ‘arrest’ of leukocytes on endothelium. A host of molecules are then involved in the migration of these cells through venular walls into the tissues or lymph nodes, in a process known as transendothelial migration (Ley et al., 2007). The adhesion molecules expressed on a cell’s surface reflect the destination of the cells. Thus, cells expressing high levels of the integrins CD11a and CD49d are tissue homing, whereas those cells expressing high levels of CD62L migrate into the lymph nodes, as CD62L is involved in the migration of lymphocytes across specialized high-endothelial venules leading to the lymph nodes (von Andrian and Mackay, 2000).

The analysis of cellular adhesion molecules on $CD8^+$ T cells and NK cells showed that psychological stress, exercise and β-agonist infusion led to an increase in cells expressing high levels of CD11a ($CD11a^{hi}$), whilst $CD11a^{lo}$ cells were minimally affected. In contrast, stress increased $CD62L^{lo}$ cells, whilst $CD62L^{hi}$ cells remained largely unchanged (Bosch et al., 2005; Goebel and Mills, 2000; Mills et al., 2003; Mills et al., 2002; Mills et al., 2000; Mills et al., 1997). Thus, autonomic activation alters the proportion of lymphocytes in the blood, favoring tissue homing cells. Recent technical advances investigating T cell memory populations have shed new light on these observations. Indeed, rather than representing up or down regulation of adhesion molecules on leukocyte surfaces, or separate increases in $CD11a^{hi}$ cells and $CD62L^{lo}$

cells, this altered adhesion molecule profile likely represents an increase in a specific population of cells; one with a CD11a^{hi}CD62L^{lo} phenotype. T cell memory populations are generated in the course of an immune response.

Developing immunological memory

Every T cell expresses a T cell receptor (TCR) that is unique for a particular antigen. T cells only recognize antigen when it is presented by a Major Histocompatability Complex (MHC) molecule, of which there are two types; MHC I and MHC II. MHC I is expressed on the cell surface of all host cells as part of a constant process whereby body cells break down antigen from the cytosol and present it on the cell surface. This mechanism warns the immune system of any intracellular infections (i.e. viruses) or cell transformations, such as cancer. CD8$^+$ T cells recognize antigen presented by MHC 1 molecules. A number of viruses have learnt to down-regulate MHC I expression on host cells, to 'hide' from CD8$^+$ T cells. NK cells, though, express surface receptors like CD94/NKG2 that can recognize down-regulated MHC I expression and can initiate, or inhibit, NK cell mediated killing accordingly (Gunturi et al., 2004). MHC II is only found on the surface of professional antigen presenting cells (APC), such as dentritic cells and macrophages, which patrol the tissues and take up antigen, which they present to T cells in the peripheral lymphoid organs. MHC II bound antigens are recognized by CD4$^+$T cells. Recognition of MHC by CD4$^+$ T cells represents the first stage in generating an adaptive immune response.

When a naïve T cell's TCR recognizes an MHC-antigen peptide complex, provided necessary co-stimulatory signals are delivered, the T cell undergoes clonal expansion resulting in a large number of effector T cells specific for the antigen. These cells migrate to the site of infection and either directly destroy infected cells ($CD8^+$T cells) or regulate activities of other immune cells ($CD4^+$T cells), which, in turn, destroy pathogens. When an infection is eliminated, the majority of effector T cells die by apoptosis. A small minority of cells remain alive as memory cells, which have the ability to rapidly initiate an immune response upon re-infection (Janeway et al., 2005). The mechanisms of memory cell differentiation and maintenance are not fully elucidated. It is thought that progressive antigenic stimulation, and the influence of homeostatic cytokines (e.g., IL-15), are involved in the generation of functionally distinct memory populations (Appay et al., 2002a; Chiu et al., 2006; Hamann et al., 1999a; Sandau et al., 2010; van Lier et al., 2003). These can be broadly divided into four major subsets, naïve (NA), central memory (CM), effector memory (EM) and $CD45RA^+$ effector memory (EMRA) (Appay et al., 2008; Caccamo. et al., 2005; Hamann et al., 1999b; Sallusto et al., 2004).

NA, CM, EM and EMRA T cell populations

A number of methods have been used to identify memory T cell populations. The system used in the present thesis includes the cell surface expression of CD27, and CD45RA. CD27 is a co-stimulatory receptor involved in the stimulation of T cells and is progressively lost as a T cell becomes more differentiated. CD27 simulation plays an

important role in the generation of T cell memory and acquisition of effector function (Arens et al., 2001; Hendriks et al., 2000) and cells lacking CD27 have a less stringent requirement for activation (Hamann et al., 1999b). The leukocyte common antigen CD45RA is often used to identify memory populations. However, CD45RA alone cannot be used to distinguish between EMRA and NA T cells, as EMRA T cells re-express CD45RA. CD27 and CD45RA in combination, however, can be used to identify four memory populations; $CD27^{+}CD45RA^{+}$ (NA), $CD27^{+}CD45RA^{-}$ (CM), $CD27^{-}$ $CD45RA^{-}$ (EM) and finally $CD27^{-}$ $CD45RA^{+}$ (EMRA), that display distinct differences in function and vary in expression of adhesion molecules and other cell surface markers (see Table 1) (Angelini. et al., 2004; Appay et al., 2008; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003; Hamann et al., 1997; Hamann et al., 1999b; Moretta et al., 1997). To summarize, NA and CM populations express lymph node homing markers (e.g., $CD62L^{hi}$) and do not possess effector function. EM and EMRA, on the other hand, express higher levels of tissue homing markers (e.g., $CD11a^{hi}$), NK-like receptors (e.g., NKG2 (CD94)) and exhibit powerful effector function, including secretion of IFN-gamma (γ) and expression of cytotoxic machinery, such as perforin and granzymes. EMRA cells express the highest levels of perforin and, in conjunction, possess the greatest cytotoxicity of all populations.

Table 1. Phenotypic and functional characteristics of T cell memory populations[2]; naïve (NA), central memory (CM), effector memory (EM) and $CD45RA^+$ effector memory (EMRA).

Marker	NA $CD27^+CD45RA^+$	CM $CD27^+CD45RA^-$	EM $CD27^-CD45RA^-$	EMRA $CD27^-CD45RA^+$	References
Perforin	–	+	++	+++	(Angelini. et al., 2004; Appay et al., 2008; Appay et al., 2002b; Hamann et al., 1997; Romero et al., 2007)
Granzyme A	–	+++	+++	+++	(Appay et al., 2008; Takata and Takiguchi, 2006)
Granzyme B	–	+	++	+++	(Appay et al., 2008; Hamann et al., 1997; Romero et al., 2007; Takata and Takiguchi, 2006)
CD11a	+	++	++	+++	(De Rosa et al., 2004; Hamann et al., 1997)
CD62L	+++	+++	+/–	+/–	(Dieli et al., 2003; Hamann et al., 1997)
CD94	–	–	+	+++	(Angelini. et al., 2004; Gumá et al., 2005; Gunturi et al., 2004; Landmann. et al., 1984; Romero et al., 2007)
IFN-γ	–	++	+++	+++	(Angelini. et al., 2004; Appay et al., 2008)
TNF-α	–	++	+/++	+/++	(Angelini. et al., 2004; Appay et al., 2008)

– = negative, + = low expression, ++ = moderate expression, +++ = high expression

[2] This table provides a broad overview of marker expression in T cell subsets. Marker expression may vary slightly between populations of T cell classes, e.g., γδ T cells versus $CD8^+$ T cells.

Autonomic activation and cytotoxic lymphocyte sub-populations

Initial work by Mills and colleagues showed that memory type $CD8^+$ T cells appear in the blood during sympathetic activation, whilst naïve cells do not (Mills et al., 2000). More recent research has shown that this response involves EM and EMRA $CD8^+$ T cells; these cells become mobilized during exercise, epinephrine infusion and stress (Atanackovic et al., 2006; Campbell et al., 2009; Dimitrov et al., 2010; Riddell et al., 2009). Further, Bosch and colleagues demonstrated that within NK cells also it is the cytotoxic ($CD56^{lo}$) subset that is mobilized during stress (Bosch et al., 2005). It appears, therefore, that the cytotoxic potential of a lymphocyte predicts which NK and $CD8^+$ T cell subsets become mobilized.

In lymphocytes cytotoxic potential also coincides with elevated β-adrenergic receptor expression. Indeed lymphocytes with the strongest cytotoxic ability also show the highest expression of β2-adrenergic receptors; thus NK cells are known to possess higher levels of β-adrenergic receptors than $CD8^+$ T cells, which in turn show higher levels than $CD4^+$ T cells (Maisel et al., 1989; Maisel et al., 1990). Furthermore, within lymphocyte sub-populations β2-adrenergic receptor expression is higher in cells that show a higher cytotoxic potential, e.g., EMRA $CD8^+$ T cells have higher levels of β2-adrenergic receptors than NA and CM cells (Dimitrov et al., 2009; Holmes et al., 2005; Willinger et al., 2005). Thus, it is clear that cytotoxicity and β2-adrenergic receptor expression are closely linked.

γδ T cells

In addition to NK and CD8$^+$ T cells, humans have a third cytotoxic lymphocyte class known as the γδ T cell. γδ T cells are a small population of cells comprising approximately 5% of peripheral blood T cells, yet representing up to 50% of T cells in epithelial tissues (Carding and Egan, 2002). They play key roles in a number of pathologies (Askenase, 2001; Jameson et al., 2002; Nakasone et al., 2007; Wang et al., 2001), and are known to have immuno-regulatory properties and to be capable of presenting antigen to other immune cells (Brandes et al., 2005; Morita et al., 2007). Furthermore these cells play an important role in the immune surveillance of 'stressed' body cells (Hayday, 2009). γδ T cells are unique in that they display features of both adaptive and innate immune cells and are therefore sometimes referred to as a bridge between innate and adaptive immunity (Girardi, 2006). Similarly to CD8$^+$ T cells, γδ T cells can be divided into four memory populations (NA, CM, EM and EMRA) which differ in cytotoxic function and migratory capacity (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003).

Investigating the mobilization of γδ T cells will answer firstly whether the mobilization of cytotoxic lymphocyte classes during stress also extends to γδ T cells, and by which endocrine mechanisms they might be recruited. Further analysis of memory subpopulations and cellular adhesion molecule expression in these cells will characterize what 'types' of γδ T cells are mobilized. This will reveal whether stress selects for effector-like γδ T cells, similarly to what is known for CD8$^+$ T cells.

Cytotoxic CD4$^+$ T cells

The recent discovery of a subset of a cytotoxic CD4$^+$ T cells provides an opportunity to make a prediction based on the theory that cytotoxicity determines stress–induced mobilization. Cytotoxic CD4$^+$T cells comprise up to 2% of the total T cell pool and are believed to play a role in viral infection (Brown, 2010). These cells are unique in their possession of direct cytotoxic potential; all other forms of CD4$^+$ T cells e.g., TH1, TH2, TH17 and Treg serve instead to stimulate or dampen effector functions of other immune cells (Frevert et al., 2009; van de Berg et al., 2008; Zheng et al., 2009). If cytotoxicity is indeed the key to stress-induced mobilization, it can be predicted that cytotoxic CD4$^+$T cells will become mobilized, even though total CD4$^+$ T cells do not (Segerstrom and Miller, 2004).

Perforin expression versus a CD27$^-$ phenotype

Cytotoxic CD4$^+$ T cells are identified by the expression of perforin, in combination with CD4 (Appay et al., 2002b; van de Berg et al., 2008). Perforin is a marker of cytotoxicity and is carried in granules inside cytotoxic cells, such as EMRA CD8$^+$T cells and EMRA γδ T cells, which are both CD27$^-$(Angelini. et al., 2004; Chávez-Galán et al., 2009; De Rosa et al., 2004; Heintel et al., 2002; Sallusto et al., 2004). Studies have shown that during stress and adrenergic stimulation CD8$^+$ T cells that are more differentiated (CD27$^-$) are mobilized to a much greater extent than CD27$^+$ cells (Campbell et al., 2009;

Riddell et al., 2009; Riddell et al., In preparation) and it is likely that this is due to the highly cytotoxic nature of these cells (i.e. perforin and granzyme mediated target cell destruction). Perforin expression however has not been examined in $CD27^{-}$ T cells during psychological stress, and some $CD27^{+}$ T cells can also express perforin (Hamann et al., 1997; Romero et al., 2007; Takata and Takiguchi, 2006). Thus, in order to examine the importance of a cytotoxic marker (Pfn^{+}) versus differentiation status (CD27) perforin and CD27 were examined in $CD4^{+}$, $CD8^{+}$ and γδ T cells in chapter four.

Stress induced monocytosis

The first three experimental chapters of this thesis extensively characterize stress lymphocytosis. However, lymphocytes are not the only leukocyte type known to become mobilized during stress; monocytes are also mobilization responsive cells (Bosch et al., 2003; Goebel and Mills, 2000; Mills et al., 2003). The final chapter therefore characterizes the mobilization of monocyte subsets, with a view to answering what types of monocytes becomes mobilized, and what parallels may exist between the phenotype of lymphocytes and monocytes mobilized during stress.

Monocytes are innate immune cells and are the precursors of tissue macrophages and dentritic cells. Like lymphocytes, monocytes contain distinct subsets that differ in their migratory and functional capacity. They can be divided into three major populations based on the expression of CD14 and CD16: $CD14^{++}CD16^{-}$; $CD14^{++}CD16^{+}$; and

$CD14^{+}CD16^{+}$ (Ancuta et al., 2003; Crowe and Ziegler-Heitbrock, 2010; Skrzeczyńska-Moncznik et al., 2008; Ziegler-Heitbrock et al., 2010). $CD14^{++}CD16^{-}$, or 'classical' monocytes, comprise the majority of monocytes (80-90%) and express low levels of pro-inflammatory cytokines, such as IL-1 and TNF-α (Auffray et al., 2009). The intermediate $CD14^{++}CD16^{+}$ monocytes secrete large amounts of interleukin (IL)-10 and are hence purported to have an anti-inflammatory role (Skrzeczyńska-Moncznik et al., 2008). The $CD14^{+}CD16^{+}$, are dubbed 'pro-inflammatory' monocytes as they secrete TNF-α and express high levels of tissue homing markers, such as CD11a and CD49d (Ancuta et al., 2003; Belge et al., 2002; Steppich et al., 2000; Ziegler-Heitbrock, 1996). These cells are found in increased numbers in the blood in a range of pathologies, including atherosclerosis, and have been shown to exhibit a 'patrolling' behavior in blood vessels, possibly seeking out stressed or damaged cells (Auffray et al., 2007; Saha and Geissmann, 2011). Exercise and epinephrine infusion studies have shown that the pro-inflammatory $CD14^{+}CD16^{+}$ monocytes are selectively mobilized (Dimitrov et al., 2010; Gabriel et al., 1994; Hong and Mills, 2008; Simpson et al., 2009). However, the mobilization of these monocytes during psychological stress is yet to be investigated.

Summary and aims

The altered distribution of cells during stress demonstrates that stress impacts immune surveillance, a process essential to an effective immune defense network (von Andrian and Mackay, 2000). Characterizing the types of leukocytes involved in this

response will begin to pave the way in understanding its function; if we know what types of leukocyte subsets are mobilized we can begin to determine, and further investigate the purpose of their mobilization. The overarching aim of this thesis therefore, is to extensively characterize stress leukocytosis and to identify whether cytotoxicity is the key feature of mobilization responsive cells.

Chapter two investigates whether a cytotoxic lymphocyte; the γδ T cell, previously not studied within the context of PNI will become mobilized in response to adrenergic stimulation (psychological stress, exercise and β- agonist (isoproterenol) infusion).

Chapter three extensively characterizes the mobilization of γδ T cells, examining cellular adhesion molecule expression, differentiation status and NK like marker expression to answer whether, within total γδ T cells, cells known to possess the highest cytotoxic capability are preferentially mobilized.

Chapter four investigates whether the cytotoxicity hypothesis could be used to predict mobilization of a small subset of cytotoxic cells; cytotoxic $CD4^+$ T cells, within a population not mobilized during stress; total $CD4^+$ T cells. Further, this chapter aims to identify whether a cytotoxic marker (pfn^+) predicts mobilization during stress and isoproterenol infusion in $CD4^+$, $CD8^+$ and γδ T cells, and to what extent differentiation status ($CD27^+$ vs $CD27^-$) plays a role.

Chapter five investigates the mobilization of monocyte populations during acute psychological stress and isoproterenol infusion.

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CHAPTER TWO

MOBILIZATION OF γδ T LYMPHOCYTES IN RESPONSE TO PSYCHOLOGICAL STRESS, EXERCISE, AND β-AGONIST INFUSION

Abstract

The mobilization of cytotoxic lymphocytes, such Natural Killer (NK) cells and $CD8^+$ T cells, during stress and exercise is well documented in humans. However, humans have another cytotoxic lymphocyte subset that has not been studied in this context: the Gamma Delta (γδ) T lymphocyte. These cells play key roles in immune processes including the elimination of bacterial infection, wound repair and delayed-type hypersensitivity reactions. The current study investigated the effects of stress, exercise, and β-agonist infusion on the mobilization of γδ T lymphocytes. Three separate studies compared lymphocytosis in response to an acute speech stress task (n = 29), high (85%W_{max}) and low (35%W_{max}) intensity concentric exercise (n = 11), and isoproterenol infusion at 20ng/min/kg and 40ng/min/kg (n = 12). Flow cytometric analysis was used to examine lymphocyte subsets. γδ T lymphocytes were mobilized in response to all three tasks in a dose-dependent manner; the extent of mobilization during the speech task correlated with concomitant cardiac activation, and was greater during higher intensity exercise and increased dose of β-agonist infusion. The mobilization of γδ T lymphocytes was greater (in terms of % change from baseline) than that of $CD8^+$ T lymphocytes and

less than NK cells. This study is the first to demonstrate that γδ T cells are stress-responsive lymphocytes which are mobilised during psychological stress, exercise, and β-agonist infusion. The mobilization of these versatile cytotoxic cells may provide protection in the context of situations in which antigen exposure is more likely to occur.

Introduction

Lymphocytes engage in continuous trafficking, migrating from the blood, through various organs, and back into the blood. This migration is essential to the maintenance of an effective immune defense network (von Andrian and Mackay, 2000). Characterizing how stress affects this continuous redeployment may therefore clarify how stressors influence the capacity of the immune system to protect its host (Bosch et al., 2005). The rapid deployment of lymphocytes into the blood, known as lymphocytosis, is probably the best documented stress- and exercise-induced effect on the immune system (Benschop et al., 1996b; Pedersen and Hoffman-Goetz, 2000; Segerstrom and Miller, 2004). It has been proposed that this response reflects an adaptive mechanism, protecting the organism in contexts where wounding and infection are more likely. Indeed, animal studies have shown that acute stress-induced leukocyte redistribution may be associated with an enhanced immune defense, such as an increased delayed type hypersensitivity response (Dhabhar and McEwen, 1996), an increased leukocyte migration into wounded tissue (Viswanathan and Dhabhar, 2005), and an enhanced response to immunization (Dhabhar and Viswanathan, 2005). Recent studies have replicated some of these observations in humans (Edwards et al., 2007), although it remains to be determined how lymphocytosis specifically contributes to these effects.

A key mechanism underlying this lymphocytosis is beta (β)-adrenergic receptor activation (Benschop et al., 1996b; Pedersen and Hoffman-Goetz, 2000; Sanders and Straub, 2002; Schedlowski et al., 1996; Van Tits et al., 1990). However, while all

lymphocyte subsets appear to express functional β-adrenergic receptors (Sanders and Kavelaars, 2007), lymphocytosis during acute stress and exercise is largely driven by a selective increase in cytotoxic lymphocytes, such as Natural Killer (NK) cells and cytotoxic $CD8^+$ T cells (Benschop et al., 1996b; Pedersen and Hoffman-Goetz, 2000; Segerstrom and Miller, 2004). Other, non-cytotoxic, lymphocyte subsets such as certain T helper[3] ($CD4^+$) and B ($CD19^+$) lymphocytes, although expressing functional β2-adrenergic receptors (Sanders and Kavelaars, 2007), contribute little to stress-lymphocytosis (Maisel et al., 1990b; Segerstrom and Miller, 2004). Thus, it emerges that the cytotoxic potential of lymphocytes predicts which lymphocyte subsets are mobilized during adrenergic stimulation (i.e. psychological stress, exercise, β-agonist infusion). In addition to NK cells and cytotoxic $CD8^+$ T cells, humans have another cytotoxic lymphocyte subset, known as the Gamma Delta (γδ) T lymphocyte. In light of the preceding discussion, it would be predicted that these γδ T cells would become mobilized in response to psychological stress, exercise, and β-agonist infusion. Further, it can be postulated that the magnitude of γδ T cell mobilization will be proportional to the level of sympathetic activation.

γδ T lymphocytes constitute around 5% of total T cells in the blood, yet comprise up to 50% of T cells in epithelial tissues, such as the skin, and linings of the gastro-intestinal, respiratory, and genito-urinary tract (Carding and Egan, 2002) . These cells display characteristics of both innate and adaptive immunity. For example, γδ T cells can

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[3] It has been shown that a small fraction of $CD4^+$ T cells have cytotoxic properties (Appay et al., 2002).

develop antigen-specific memory, similarly to conventional cytotoxic T cells, but also use pattern recognition receptors to respond to a broad range of antigens, akin to cells of the innate immune system (Girardi, 2006). For this reason, γδ T cells are sometimes referred to as 'a bridge between the innate and adaptive immune system' (Carding and Egan, 2002; Girardi, 2006). γδ T cells also display immunoregulatory and antigen-presenting capabilities (Girardi, 2006; Morita et al., 2007), and have been demonstrated to play key roles in a variety of processes including the elimination of bacterial infection (Nakasone et al., 2007; Wang et al., 2001), wound repair (Girardi, 2006; Jameson et al., 2002) and delayed-type hypersensitivity reactions (Askenase, 2001).

Considering the many protective functions of γδ T cells, and the fact that the mobilization of cytotoxic lymphocytes in response to acute exercise and/or psychological stress represents one of the most reliable stress responses in humans (Benschop et al., 1998; Segerstrom and Miller, 2004), it is surprising that no attempts have been made to characterize the mobilization of γδ T cells. The aim of the three studies presented in the current report was to investigate the effects of acute psychological stress, exercise, and β-agonist infusion on the mobilization of γδ T lymphocytes.

Methods

Participants

Participants in the stress study were 29 university students (Mean age = 21.8 (*SD* = 2.2) years, 14 women). Participants in the exercise study were 11 non-sedentary male university students (Mean age = 20.9 (*SD* = 1.6) years). Participants in the isoproterenol infusion were 12 healthy community volunteer students (Mean age = 42.4 (*SD* = 4.9) years, 10 women). All participants reported to be in good health and were non-medicated with 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. Demographic, health and lifestyle information was obtained by self-report questionnaire. Blood was taken from an 18 -gauge intravenous cannula (Becton-Dickinson, UK/US) inserted into a palpable vein in the antecubital fossa. In all three studies cannulation was performed before the 'baseline' periods were initiated and the same catheter was used for subsequent blood withdrawals. Participants provided informed consent and study protocols were approved by the relevant institutional review/ethics boards (University of Birmingham; University of California, San Diego).

Procedures

Psychological stress study protocol

Participants were tested between 9am and 1pm. Following instruction and instrumentation, participants completed a 20 min seated baseline, during which questionnaires were completed. At the end of this period, a 'baseline' blood sample was obtained and the laboratory stressor was initiated. A second ('task') blood sample was obtained during the final two minutes of the social stress task , and a third blood sample ('Recovery') was taken 15 minutes post-task. The social stress task involved participants delivering two consecutive speeches, each with two minutes preparation and four minutes of delivery (Bosch et al., 2003a). To enhance social stress, speech tasks were performed in the presence of an audience (comprised of one male and two females) and videotaped. Recorded task instructions were presented on a computer screen in order to standardise instruction and timing. Affective responses were assessed with Profile of Mood States (POMS)(McNair et al., 1992), which was given at baseline, immediately post task, and at the end of the recovery period. To assess autonomic nervous system activation, cardiovascular activity was recorded throughout.

Cardiovascular analysis

Assessment of the cardiovascular response focused on cardiac autonomic control and blood pressure. Indices of sympathetic and parasympathetic drive were obtained by analysis of electrocardiogram (ECG) and thoracic impedance (ICG) signals (Berntson et al., 1997; Sherwood et al., 1990). The ICG and ECG signals were recorded from six Ag/AgCl spot-electrodes (Conmed corporation, UTICA, NY, USA) using a VU-AMD

device (Vrije Universiteit, Amsterdam, Holland). Reliability and validity of the VU-AMD device have been reported elsewhere (de Geus et al., 1995; De Geus and van Doornen, 1996; Willemsen et al., 1996). The ICG complexes were ensemble averaged with reference to the ECG R-wave across 1-minute periods. From these 1-minute ensembles, averages were computed for heart rate (HR), heart rate variability (RMSSD) and the pre-ejection period (PEP). These minute-by-minute means were averaged over the last 6-minutes of baseline, each 6-minute speech, and the last 6-minutes of recovery. Changes in PEP were used to index changes in cardiac sympathetic drive (Sherwood et al., 1990), whereas RMSSD (Root mean Square of Successive Differences) was used to index changes in cardiac vagal tone (Bosch et al., 2003b; Goedhart et al., 2007). The RMSSD was log transformed to restore normality.

Measurements of systolic and diastolic blood pressure (SBP and DBP respectively) were taken at two minute intervals using an Omron M5 blood pressure monitor (Omron Healthcare UK Ltd., Milton Keynes, UK). Baseline, task and recovery values (10-min post-task onwards) were averaged and used in subsequent analyses.

Exercise study protocol

Participants visited the exercise laboratory at 2pm on three separate days, each one week apart. The first visit involved an incremental submaximal test (Astrand et al., 2003), to determine the appropriate individualised workloads for the standardized exercise tasks. Following a 4-min warm-up, participants completed the 12-minute test, which started at a

workload of 60 W, increasing by 30 W every 3 min, to a maximum of 180 W. Workload for the low (35% W_{max}) and high (85% W_{max}) intensities were estimated by correlating individual HR with age-corrected (220 bpm – age) maximal HR with workload (W), as described by Astrand et al (2003), in a regression analysis (Minitab version 13.0, Minitab Inc, USA).

During the second and third visits, the 20 min resting baselines were performed while seated on the cycle ergometer to avoid confounding by postural changes. A 'baseline' blood sample was taken at the end of this period. Participants then completed a four minute warm-up (60 rpm at 60 Watts) followed by 16 minutes of cycling (60 rpm at 35% (low intensity) or 85% (high intensity) predicted W_{max}). During the final two minutes of exercise, a 'task' blood sample was obtained. Following exercise cessation, participants were asked to warm-down for three mins on the cycle-ergometer at an intensity of their choice. Each participant remained seated on the cycle ergometer until a 'recovery' blood sample was taken 15-min post-exercise. The order of the two exercise sessions was counterbalanced across participants. Perceived exertion was assessed with a 10-point category-ratio scale ranging from 0 (nothing) to 10 (extremely strong) (Borg, 1998) and was obtained at the end of the baseline and exercise periods. HR was recorded throughout all three sessions using a Polar S810 heart rate monitor (Polar Electro, Kempele, Finland). During the exercise task, HR was recorded at minutes six, nine, 14 and 19 of exercise. These readings were averaged to give a 'task' HR value for each of the low intensity and high intensity conditions. During the exercise sessions, two 30-second gas collections were

taken at minutes six and 11 of exercise, using Douglas Bags (Cranleigh, Birmingham, UK).

Gas analysis

Participants breathed through a Salford respiratory valve (Hydraulic Transmission Services, Salford, UK) and Falconia tubing (Cranleigh, Birmingham, UK). Expired air was analyzed for O_2 and CO_2 concentrations by an infrared analyzer (Servomex 1400B, Crowborough, UK). The O_2 and CO_2 analyzers were calibrated before and after each test using certified gases (British Oxygen Company, London, UK). Volumes were determined by a dry gas meter (Harvard Ltd., Edenbridge, UK) and, following calibration, were checked regularly against a precision 3 L gas syringe (Hans Rudolph Inc., Kansas City, USA). Oxygen consumption was then calculated as an average ($ml \cdot Kg^{-1} \cdot min^{-1}$) for each exercise session.

β-agonist infusion study protocol

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 laboratory at 1pm, subjects lay supine for 5–10 min following placement of ECG electrodes and an i.v. cannula into an antecubital vein. Isoproterenol was then infused in two sequential doses; 20 ng/kg/min for 15 min and then at 40 ng/kg/min for 15 minutes. The half-life of isoproterenol in the body is approximately 2–3 min (Goebel et al., 2000). Blood was sampled prior to initiation of isoproterenol infusion and then immediately after

each dose. For safety purposes, ECG was monitored throughout the infusion. The infusion is generally well-tolerated and all participants successfully completed the protocol.

Flow cytometry

In all studies, blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (BD, BD Biosciences), kept at room temperature and prepared within 2 hours. Lymphocyte subsets were identified by immunoflourescent antibody staining of whole blood using four-colour flow cytometry (FACS-Calibur, BD Biosciences). A range of antibodies were used; IgG1 FITC, IgG1 PE, CD4 APC, CD8 PE , CD19 FITC, γδ TCR APC (Pharmingen), CD3 PERCP, CD56 PE (BD Biosciences), all antibodies were of the IgG1 isotype. Briefly, whole blood samples were incubated with antibody for 30 minutes in the dark at room temperature (RT), and red blood cells were subsequently lysed using FACS Lysing Solution (Becton Dickenson). Following lysis, samples were centrifuged (217 x G, 6 minutes**,** at room temperature), and fixed in 250μl 1% paraformaldehyde/PBS (without $Ca^{2+}Mg^{2+}$) (w/v). Fixed preparations were stored in the dark at 4°C and read within 24 hours, collecting a minimum of 10,000 gated lymphocytes from each sample. Matched isotype controls were used to set negative staining criteria. The PERCP and APC labelled antibodies provide very discrete staining on these cells and it is easy to distinguish between positive and negative events; for these antibodies, the obviously positive events were gated. The FACS-Calibur machine (BD Biosciences) was routinely calibrated using Calibrite beads (BD Biosciences), and further fine tuning of compensation was adjusted

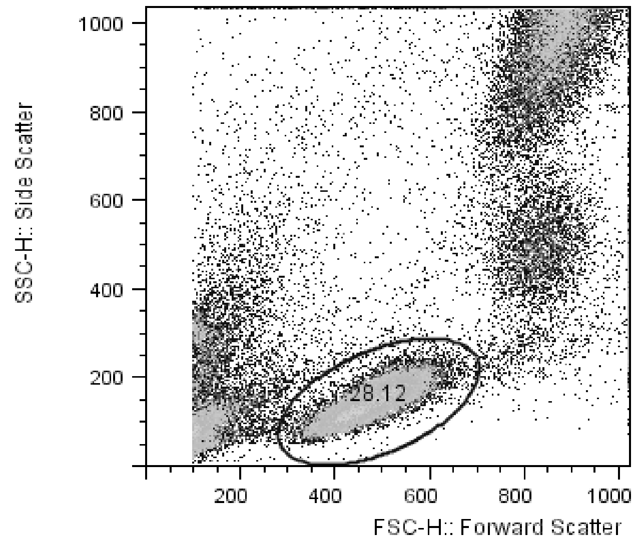
using single labeled antibody tubes. Data was analysed using FlowJo software v5.2 (Tree Star, Inc., Ashland, OR). Lymphocyte numbers were determined using a Coulter ACT^{diff} (Beckman Coulter, High Wycombe, UK) or a Coulter GEN-S haematology analyser (Beckman-Coulter, Miami, USA).

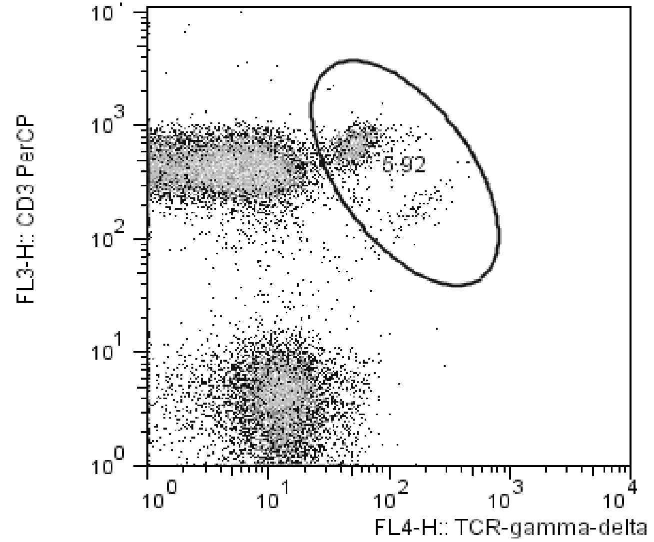
As displayed in Figure 1, total lymphocytes were gated using forward versus side scatter. γδ T cells were then gated as the $CD3^{+}$ γδ TCR^{+} population. NK cells were gated as $CD3^{-}CD56^{+}$, $CD8^{+}$ T cells were gated as $CD3^{+}CD8^{+}$, $CD4^{+}$T cells were gated as $CD3^{+}CD4^{+}$ and B cells were gated as $CD3^{-}CD19^{+}$. Lymphocyte subset numbers were quantified using the percentage values obtained from each lymphocyte subset gate and the absolute lymphocyte count (Coulter ACT^{diff}).

Data analysis

Repeated measures analysis of variance (ANOVA) was used to assess the effects of each condition on immunological, cardiovascular and psychological measures. The association between cardiovascular and immune cell responses was explored using Pearson's correlation coefficients. Paired t-tests were used to analyse the difference in % change between subsets and between conditions. Occasional data was missing; degrees of freedom were adjusted accordingly. Where means are presented, standard deviation is given in brackets. In the psychological stress study, γδ T cells and total lymphocytes were assessed in all 29 participants; other lymphocyte subsets were assessed in a subgroup of 19 participants. Data was analyzed using SPSS 15 (SPSS, Chicago, IL.).

Figure 1. Dot plots representing the analytical strategy used to identify γδ T cells ($CD3^{+}$ $\gamma\delta TCR^{+}$).





Results

Psychological stress study

Anxiety and cardiovascular responses

Analysis of the tension-anxiety POMS subscale confirmed that the speech tasks were perceived as stressful (+5.8 (*SD* = 5.4); $F_{(2, 56)} = 53.5$, $p<.001$). There were significant increases in SBP (+22.0 (*SD* = 8.5) mmHg; $F_{(2, 56)} = 103.7$, $p<.001$), DBP (+16.6 (*SD* = 8.1) mmHg; $F_{(2, 56)} = 74.7$, $p<.001$) and HR (+ 14.7 (*SD* = 9.2) bpm; $F_{(2, 56)} = 70.7$, $p<.001$) and a significant decrease in PEP (-14.0 (*SD* = 9.4) ms; $F_{(2, 52)} = 17.8$, $p<.001$); these findings demonstrate that the speech task caused sympathetic activation. Additionally, there was a significant decrease in RMSSD (-0.6 (*SD* = 0.5) ms; $F_{(2, 52)} = 40.5$, $p<.001$), reflecting vagal withdrawal (Goedhart et al., 2007). At 15-min recovery, all cardiovascular and autonomic measures had returned to baseline values.

γδ T cells and lymphocyte subsets

As displayed in Table 1, the speech task induced a marked increase in γδ T cell ($\gamma\delta TCR^{+}CD3^{+}$), NK ($CD56^{+}CD3^{-}$) cell, and $CD8^{+}$ T cell ($CD8^{+}CD3^{+}$) numbers. All cell numbers returned to baseline values following a 15 minute recovery period. Consistent with a published meta-analysis, there were no significant increases in B cells or $CD4^{+}$ T cells (Segerstrom and Miller, 2004).

Figure 2(a) displays the percentage change in γδ T cells and other lymphocyte subsets between baseline and stress. The NK cell response (Δ %) was found to be significantly greater than that of γδ T cells ($t_{(18)} = 5.57$, $p < .001$) and, in turn, γδ T cells were shown to be significantly more responsive to the stress task than $CD8^+$ T cells ($t_{(18)} = -5.2$, $p < .001$).

Supporting a role of the sympathetic nervous system in these responses, changes in NK cell numbers correlated with changes in PEP ($r = -.42$), SBP ($r = .55$) and HR ($r = .40$), and changes in γδ T cell numbers correlated with changes in PEP ($r = -.45$) and HR ($r = .48$). Finally, changes in total lymphocyte numbers correlated with changes in PEP ($r = -.37$) and HR ($r = .46$) (all $p<.05$).

Exercise Study

Perceived exertion, oxygen consumption, and HR responses

Data confirmed that the two exercise conditions were of clearly distinct intensities. As expected, a significantly greater increase in HR was observed in the 85%W_{max} high intensity (+94.8 (*SD* = 7.1) bpm) condition compared to the 35%W_{max} low intensity (+42.8 (*SD* = 7.6) bpm) condition ($t_{(10)} = -13.7$, $p<.001$). Likewise, the mean oxygen consumption was significantly higher for the high intensity (42.6 (*SD* = 9.7) $ml \cdot Kg^{-1} \cdot min^{-1}$) exercise condition than for the low intensity (20.5 (*SD* = 4.2) $ml \cdot Kg^{-1} \cdot min^{-1}$) condition ($t_{(10)} = -9.4$, $p<.001$). Finally, perceived exertion was also higher in the high intensity (8.3

(*SD* = 1.4)) than the low intensity (2.5 (*SD* = 0.8)) exercise condition ($t_{(10)} = -17.2$, $p<.001$).

γδ T cells and lymphocyte subsets

Table 2 demonstrates that both exercise conditions led to an increase in the number of γδ T cells and all other lymphocyte subsets. Figure 2b displays the percentage change of lymphocyte subsets during the low and high intensity exercise conditions. During the high intensity session, all lymphocyte subsets were mobilised to a significantly greater extent than during the low intensity session.

Replicating findings in the stress study, γδ T cell mobilization was found to be less (in terms of % change from baseline) than that of NK cells and greater than that of $CD8^+$ T cells . NK cells responded more robustly than γδ T cells (Δ %) in both the low intensity ($t_{(9)} = -4.9$, $p < .001$) and high intensity ($t_{(9)} = -8.2$, $p <.001$) conditions. In turn, γδ T cells increased to a greater extent than $CD8^+$ T cells (Δ %) in both the low intensity ($t_{(8)} = 5.2$, $p <.001$) and high intensity conditions ($t_{(8)} = 4.0$, $p =.004$). There was no significant difference between the mobilization of $CD8^+$ T cells, B cells or $CD4^+$ T cells for either the low or high intensity exercise conditions.

β-agonist Infusion study

γδ T cells and lymphocyte subsets

Table 3 demonstrates that both β-agonist infusion conditions led to an increase in γδ T cells, a pronounced lymphocytosis, an increase in circulating NK and $CD8^+$ T cells, and a decrease in circulating B cells and $CD4^+$ T cells.

As illustrated in Figure 2c, during the 40 ng/kg/min condition, NK cells, $CD8^+$ T cells and γδ T cells were mobilized to a significantly greater extent than during the 20 ng/kg/min condition. There were no significant differences between the two conditions for total lymphocytes, $CD4^+$ T cells and B cells.

In agreement with findings from the psychological stress and exercise studies, NK cells (Δ%) showed the greatest mobilization, followed by γδ T cells, and then $CD8^+$ T cells, in both infusion conditions (in pairwise comparisons, all *p*s $< .01$).

Table 1. Mean (SEM) cell numbers at each time point in the psychological stress study, with results from repeated measures ANOVA

Cell type (cells/µl)	Baseline	Task	Recovery	*F*(*df*)	*p*
Lymphocytes	1705 (71)	2128 (91)	1755 (81)	44.8 (2,56)	<.001
NK cells	122.7 (12.3)	347.9 (38.1)	144.4 (12.3)	45.1 (2,36)	<.001
γδ T cells	80.2 (11.8)	122.6 (17.1)	91.5 (14.1)	20.5 (2, 56)	<.001
$CD8^+$ T cells	401.9 (25.1)	478.9 (35.0)	404.9 (29.6)	14.9 (2,54)	<.001
$CD4^+$ T cells	753.0 (57.9)	786.0 (62.1)	786.0 (52.2)	0.8 (2,36)	.47
B cells	195.9 (16.4)	198.4 (19.5)	214.5 (17.3)	1.9 (2,36)	.16

Table 2. Mean (SEM) cell numbers at each time point in the exercise study, with results from repeated measures ANOVA

a) Low intensity (35% W_{max}) exercise

Cell type (cells/ µl)	Baseline	Exercise	Recovery	$F(df)$	p
Lymphocytes	1627 (134)	2264 (187)	1727 (158)	73.5 (2,20)	<.001
NK cells	164.0 (17.0)	420.5 (41.5)	180.2 (26.0)	72.2 (2,20)	<.001
γδ T cells	48.3 (6.6)	75.2 (12.2)	50.1 (8.4)	18.5 (2,18)	<.001
$CD8^+$ T cells	322.4 (28.1)	400.0 (38.7)	320.1 (32.9)	18.7 (2,18)	<.001
$CD4^+$ T cells	624.0 (39.0)	737.4 (60.9)	635.2 (52.3)	14.2 (2,20)	<.001
B cells	212.8 (33.0)	276.3 (38.0)	215.6 (37.1)	4.3 (2,14)	.034

b) High intensity (85% W_{max}) exercise

Cell type (cells/ µl)	Baseline	Exercise	Recovery	$F(df)$	p
Lymphocytes	1609 (112)	4473 (317)	2082 (199)	95.8 (2,20)	<.001
NK cells	168.8 (19.5)	1567.6 (129.4)	327.0 (67.5)	109.7(2,20)	<.001
γδ T cells	52.0 (8.84)	166.0 (30.9)	75.9 (14.3)	18.8 (2,18)	<.001
$CD8^+$ T cells	335.4 (27.5)	665.1 (76.4)	406.2 (42.8)	19.5 (2,18)	<.001
$CD4^+$ T cells	592.8 (44.8)	1070.2 (102.1)	687.6 (47.4)	24.5 (2, 18)	<.001
B cells	232.4 (51.9)	424.4 (97.7)	266.1 (53.0)	9.1 (2, 12)	.004

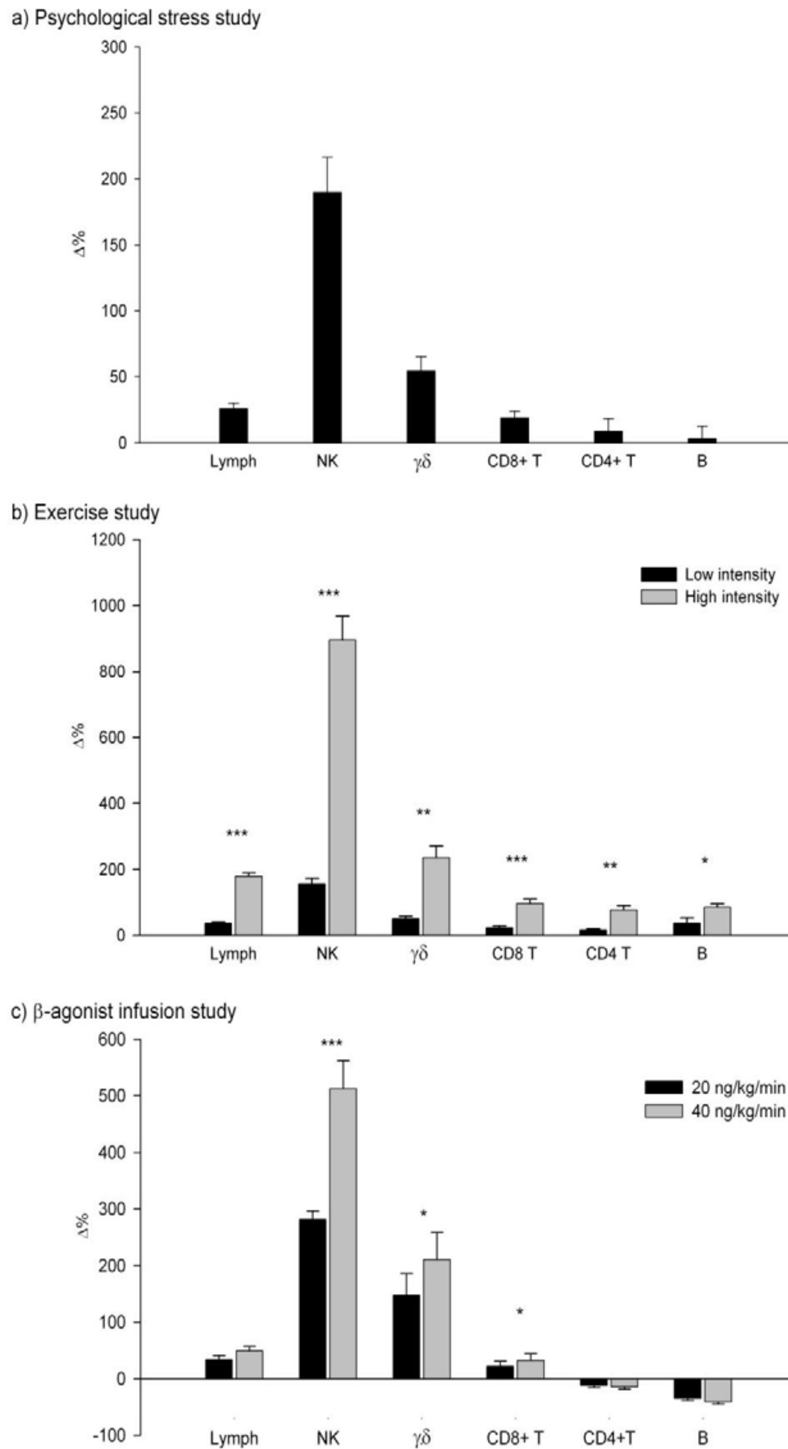
Table 3. Mean (SEM) cell numbers at each time point and dose in the β-agonist infusion study, with results from repeated measures ANOVA

Lymphocyte subset	Baseline	20ng/Kg/min	40ng/Kg/min	*F(df)*	*p*
Lymphocytes	1981.6 (197.4)	2605.9 (263.8) **	2747.1 (246.6) ***	21.89 (2,20)	<.001
NK cells	138.1 (16.0)	507.3 (47.5)***	783.3 (77.6)*** †††	76.2 (2, 20)	<.001
γδ T cells	56.7 (6.5)	138.7 (22.3) **	168.9 (24.4) **†	20.0 (2,20)	<.001
$CD8^+$ T cells	475.5 (72.8)	560.4 (87.7) **	859.4 (139.7) ***†††	25.7 (2,20)	<.001
$CD4^+$ T cells	1005.0 (105.6)	902.8 (119.4) ***	818.3 (99.2) ***	7.4 (2,20)	.004
B cells	264.7 (35.0)	174.7 (27.2) ***	152.2 (26.6) ***	33.3 (2,20)	<.001

* p<.05, ** p<.01***, p<.001; indicates value is significantly different from baseline (Paired t-test)

†p<.05, ††p<.01, †††p<.001; Results of Paired t-test comparing subset numbers at 20ng/Kg/min with numbers at 40ng/Kg/min.

Figure 2. Relative change (Δ%; SEM) in lymphocyte subset numbers during psychological stress, high and low intensity exercise, and β-agonist infusion.



* $p<.05$, ** $p<.01$***, $p<.001$; indicates mobilization of individual lymphocyte subsets is significantly different between high and low intensity exercise or between 20 ng/kg/min and 40ng/kg/min β-agonist infusion conditions.

Discussion

To the best of our knowledge, the present study is the first to demonstrate that γδ T cells are mobilized in response to acute psychological stress, exercise, and β-agonist infusion. The extent of γδ T cell mobilization was greater than observed in $CD8^+$ T cells and less than that of NK cells. In contrast, consistent with existing literature (Benschop et al., 1996b; Segerstrom and Miller, 2004), the numbers of $CD4^+$ T cells and B cells, which generally lack cytotoxic potential, were essentially unchanged. These findings lend further credence to our proposal that stress-induced mobilization is a characteristic of cytotoxic lymphocytes. With regard to potential mechanisms, it seems plausible that γδ T cell mobilization is mediated by β-adrenergic mechanisms. The current studies provide evidence to support this contention. For example, the increase in γδ T lymphocytes during psychological stress correlated with PEP and HR, which are both indices of sympathetic activation (Sherwood et al., 1990). Moreover, γδ T lymphocyte numbers increased in a dose-dependent manner during exercise and β-agonist infusion. Such a role for β-adrenergic mechanisms has already been demonstrated with NK and $CD8^+$ T cell mobilization (Benschop et al., 1996a; Benschop et al., 1993; Benschop et al., 1996b; Benschop et al., 1997; Carlson et al., 1996; Mills et al., 1995; Murray et al., 1992; Sanders and Straub, 2002; Schedlowski et al., 1996; Van Tits et al., 1990). Finally, the expression and sensitivity of β-adrenergic receptors varies across lymphocyte populations (Maisel and Michel, 1990), in a way that parallels the extent of their mobilization during psychological

stress (Mills et al., 1995) and exercise (Maisel et al., 1990a). As such, we would predict that γδ T lymphocytes would also express high levels of β-adrenergic receptors.

Discussion of the possible clinical implications of these changes is warranted. Mobilization of lymphocytes into the blood has been proposed to be the first step in a stress-induced response that ultimately facilitates targeted migration. Dhabhar has used a military metaphor to describe this phenomenon; he proposes that an acute stress response directs the body's "soldiers" (leukocytes), to exit their "barracks" (spleen and bone marrow), travel the "boulevards" (blood vessels), and take position at potential "battle stations" (skin and lymph nodes) (Dhabhar, 2002). Consistent with this theory, acute stress in animal models has been shown to elicit a lymphocytosis, followed by a lymphopenia, which is accompanied by increased cellularity in various tissues (Engler et al., 2004; Krüger et al., 2008; Stefanski et al., 2003). Although lymphopenia is not found in response to acute stress in humans (possibly due to the short duration and moderate intensity stressors typically used), high intensity, long duration exercise is known to cause lymphopenia (Pedersen and Hoffman-Goetz, 2000; Shephard, 2003; Simpson et al., 2007b); this suggests that Dhabhar's (Dhabhar, 2002) model may also be relevant in humans. Based on this model, it is possible to speculate on the potential implications of stress induced γδ T cell mobilization. γδ T cells have a strong ability to attract and stimulate other immune cells (Tikhonov et al., 2006). Additionally, they can promote wound healing (Jameson et al., 2002), tumor surveillance (Girardi, 2006), and activated γδ T cells are able to act as professional antigen presenting cells (Brandes et al., 2005). Thus, the rapid deployment of γδ T cells during psychological stress, exercise, and β-agonist

infusion may help attract immune cells to any potential pathogenic threat, enhance wound healing and provide more opportunity for antigen encounter. It may likewise be argued that cellular redistribution during acute stress may have detrimental effects. Firstly, a selective mobilization of γδ T cells may accelerate atherosclerotic plaque formation by facilitating the recruitment of circulating immune cells into the inflamed sub endothelia (Bosch et al., 2003a; Dyugovskaya et al., 2003) as enhanced migration of T cells, a significant proportion of which are γδ T cells, into the vascular sub-endothelium signifies the first phase in the atherogenic process (Kleindienst et al., 1993; Wick and Xu, 1999). Secondly, stress lymphocytosis may not necessarily enhance tumour surveillance; treatment of rats with Metaproterenol (a β2-adrenergic receptor agonist) is associated with enhanced metastasis of an NK-sensitive tumour (Shakhar and Ben-Eliyahu, 1998). In sum, it seems reasonable to speculate that stress-induced lymphocyte redistribution could affect clinically relevant outcomes, although the specific health implications may depend on the particular disease context (Dhabhar and McEwen, 2001).

One of the limitations of the current study was the lack of a control group. However, the experimental paradigms used in this study have been extensively used in the literature and run alongside relevant controls previously (Benschop et al., 1998; Bosch et al., 2003a; Bosch et al., 2005; Goebel and Mills, 2000; Goebel et al., 2000; Mills et al., 1995). Moreover, the mobilization of γδ T cell during stress, exercise and infusion was observed in the context of consistently replicated findings, such as NK and $CD8^+$ T cell mobilization (Benschop et al., 1996b; Marsland et al., 1995; Pedersen and Hoffman-Goetz, 2000; Sanders and Straub, 2002; Segerstrom and Miller, 2004; Simpson et al., 2007a;

Willemsen et al., 2002). Finally, γδ T cell mobilization increased in parallel with autonomic activation, such as exercise intensity, cardiac activity during stress, and β-agonist dose. A second limitation is the fact that isoproterenol infusion was not accompanied by a parallel β-adrenergic blockade condition, and we cannot fully exclude the possibility of some alpha (α)-adrenergic involvement. However, isoproterenol has a high selectivity for the β-adrenergic receptor, and α-adrenergic receptor expression on lymphocytes is infrequent and low-level (Elenkov et al., 2007). Further, using a similar study design, we have shown previously that β-blockade completely abrogates lymphocyte trafficking during infusion and psychological stress, again indicating that a residual effect of α-adrenergic activation is unlikely (Mills et al., 2000; Mills et al., 1999). It is also worthwhile to note that although there were age and gender differences between studies, γδ T cell results remained consistent throughout, indicating a high generalizability of the findings.

Future research targets might include further elucidation of the mechanisms involved in the mobilization of γδ T cells and the characterization of surface receptors, such as adhesion molecules, on stress responsive γδ T cells. Research has revealed that the lymphocytes mobilized during stress/exercise/β-agonist infusion express certain types of adhesion molecule (Bosch et al., 2005; Mills et al., 2003; Mills et al., 2000; Simpson et al., 2006). It is important to determine whether γδ T cells exhibit a similar phenomenon. Finally, it is clear from the extensive literature that different types of stress (e.g., chronic *v* acute; social *v* immobilization; passive coping *v* active coping) can produce different immune effects and may operate under more than one mechanism (Bosch et al., 2001 ;

Bosch et al., 2003b; Engler et al., 2004; Krüger et al., 2008; Stefanski et al., 2003). The further elucidation of such mechanisms in humans, particularly in the context of lymphocyte trafficking, remains an important future target.

In summary, this study is the first to demonstrate that γδ T cells are stress-responsive lymphocytes, evidenced by their mobilization during psychological stress, exercise, and β-agonist infusion. These observations are consistent with the notion that cytotoxic lymphocytes are preferentially mobilized during stress. The mobilization of these powerful effector cells may provide protection in the context of situations in which antigen exposure is more likely to occur.

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CHAPTER THREE
PHENOTYPIC CHARACHTERIZATION OF γδ T CELLS MOBILIZED DURING ACUTE PSYCHOLOGICAL STRESS.

Abstract

Gamma Delta (γδ) T lymphocytes are versatile cells that play key roles in a number of functions, including bacterial clearance, wound repair, and delayed-type hypersensitivity reactions. Recently we showed that these cells are mobilized into the blood during acute psychological stress. γδ T lymphocytes are a heterogeneous population of cells, and the current study aimed to characterize the effects of stress on distinct γδ T cell populations. Twenty nine healthy participants completed a 12 minute speech task. Blood samples were taken after a resting baseline, during the last two minutes of the task, and after a 15 minute recovery period. Flow cytometry was used to investigate the response of memory phenotypes (i.e. Naïve, Central memory, Effector Memory, and $CD45RA^{+}$ Effector Memory (EMRA)) within the δ1 and δ2 γδ T cell populations. Cells were further analysed on expression of adhesion molecules (CD11a, CD62L) and the NK receptor CD94. Both the δ1 and δ2 subsets were mobilized during stress, and for both subsets, EMRA cells were mobilized to a much greater extent than the other memory phenotypes. Analysis of migration markers revealed that mobilized cells had a predominantly tissue migrating phenotype ($CD11a^{hi}CD62L^{lo/neg}$) and expressed high levels of the NK receptor CD94.

The current findings indicate that stress primarily mobilizes γδ memory cells that have high cytotoxic capability, tissue homing potential, and the capacity for rapid, innate-like target recognition. This selective mobilization possibly provides protection in contexts when tissue damage and antigen exposure are more likely to occur.

Introduction

The rapid mobilization of lymphocytes into the blood, known as lymphocytosis, is one of the best documented effects of stress on the immune system (Benschop et al., 1996; Segerstrom and Miller, 2004). This response is largely mediated by the effects of catecholamines on β-adrenergic receptors expressed on lymphocytes (Benschop et al., 1996; Elenkov et al., 2000). Accordingly, stress lymphocytosis is considered to be integral to the proverbial 'fight-flight' response, potentially preparing the immune system for an impending assault (e.g. wounding, infection) (Benschop et al., 1996; Dhabhar, 2002). Supporting this notion, animal studies have shown that stress-induced lymphocyte redistribution is associated with an enhanced immune defense, such as an increased delayed-type hypersensitivity reaction (Dhabhar and McEwen, 1996), an increased leukocyte migration into wounded tissue (Viswanathan and Dhabhar, 2005), and an enhanced response to immunization (Dhabhar and Viswanathan, 2005). These observations have been partly replicated in humans (Edwards et al., 2007), although how lymphocytosis may contribute to these beneficial effects remains to be determined. A more detailed phenotypic characterization of this stress response may help elucidate its possible role.

Meta-analysis indicates that stress preferentially mobilizes lymphocytes with cytotoxic functions, such as NK cells and $CD8^+$ T cells (Segerstrom and Miller, 2004). More recently, we found a third cytotoxic lymphocyte population, the gamma-delta (γδ) T cell, that is mobilized during psychological stress, as well as during exercise and beta (β)-

agonist infusion (Anane et al., 2009). γδ T cells constitute approximately 5% of total T cells in the blood, yet comprise up to 50% of T cells in epithelial tissues, such as the skin and lining of the gastro-intestinal tract. Here, they form sentinel cytotoxic cells involved in a variety of immune processes (Carding and Egan, 2002; Girardi, 2006). These include the elimination of bacterial infection (Nakasone et al., 2007; Wang et al., 2001), delayed-type hypersensitivity reactions (Askenase, 2001), and wound repair (Girardi, 2006; Jameson et al., 2002), as well as having immunoregulatory and antigen presenting capabilities (Brandes et al., 2005; Morita et al., 2007).

The γδ T cells are divided into two major subsets: the delta 1 (δ1) and delta 2 (δ2) cells (Girardi, 2006). Both δ1 and δ2 T cells can be further separated into four memory phenotypes using the markers CD45RA and CD27 (Angelini. et al., 2004; Caccamo. et al., 2005; Dieli et al., 2003); this is comparable to the characterization of $CD8^+$ T cell memory classification (Hamann et al., 1997; Sallusto et al., 2004). The first phenotype comprises Naïve (NA) cells ($CD45RA^+$, $CD27^+$) which have never encountered their cognate antigen and lack cytotoxic effector functions. These cells express high levels of the adhesion molecule CD62L which enables migration to the lymph nodes. The remaining three phenotypes are antigen experienced (i.e. memory cells), and are divided into Central Memory cells (CM; $CD45RA^-$, $CD27^+$); Effector Memory cells (EM: $CD45RA^-$, $CD27^-$); and $CD45RA^+$ Effector Memory cells (EMRA: $CD45RA^+$, $CD27^-$) (Angelini. et al., 2004; Caccamo. et al., 2005; Dieli et al., 2003; Sallusto et al., 2004). CM cells, like NA cells, express CD62L, indicating a lymph node homing potential, and lack immediate cytotoxic capability. In contrast, EM and EMRA cells express the adhesion molecule CD11a, which

aids migration into sites of inflammation, and have downregulated CD62L (Angelini. et al., 2004; De Rosa et al., 2004; Dieli et al., 2003; Sallusto et al., 2004). These effector memory phenotypes also exhibit NK-like features (e.g. expression of CD94, a surface molecule involved in the innate recognition of aberrant MHC expression on infected and cancerous cells) (Angelini. et al., 2004; Moretta et al., 1997), and have a greater cytotoxic capability than the NA and CM subsets (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003; Hamann et al., 1997; Sallusto et al., 2004).

Recent research has demonstrated that EM and EMRA $CD8^+$ T cells are preferentially mobilized during adrenergic stimulation (Campbell et al., 2009; Dimitrov et al., 2009a; Dimitrov et al., 2009b; Riddell et al., 2009), when compared to NA and CM phenotypes. These results are consistent with earlier findings that $CD11a^{hi}$ and $CD62L^{lo/neg}$ $CD8^+$ T cells are mobilized during stress and exercise (Goebel and Mills, 2000; Mills et al., 2003) and a similar pattern of adhesion molecule expression is seen on mobilized NK cells (Bosch et al., 2005; Goebel and Mills, 2000; Mills et al., 2003). The aim of the current study is therefore to perform a phenotypic characterization of γδ T cell subsets mobilized in response to psychological stress. Based on the above findings, it was hypothesized that γδ T cells expressing rapid response ability (i.e. an effector-memory phenotype), the capacity to migrate to inflamed tissue (e.g. $CD11a^{high}$), and an NK-like ability for target recognition (e.g. expression of CD94) will be preferentially mobilized. The two main γδ T cell subsets, δ1 and δ2, are known to exhibit distinct tissue preferences (Girardi, 2006), and therefore the effects on these subsets, and their memory phenotypes, were separately analyzed.

Methods

Participants

Twenty nine university undergraduates (Mean age = 21.8 (*SD* = 2.2) years, 14 women) took part in this study. All participants reported being 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. Health and lifestyle information was obtained by self-report questionnaire. Blood was taken from an 18-gauge intravenous cannula (Becton-Dickinson) inserted into a palpable vein in the antecubital fossa. Cannulation was performed 20 minutes before the first blood draw ('baseline') and the same cannula was used for subsequent blood withdrawals. Participants provided informed consent and study protocols were approved by the University of Birmingham ethical review board. A small section of the data from this study has been presented elsewhere (Anane et al., 2009).

Procedures

Participants were tested between 9am and 1pm. Following instruction and instrumentation, participants completed a 20 min seated baseline during which questionnaires were completed. At the end of this period, a 'baseline' blood sample was obtained and the laboratory stressor was initiated. A second 'task' blood sample was

obtained during the final two minutes of the social stress task, and a third 'Recovery' blood sample was taken 15 minutes post-task. The social stress task involved participants delivering two consecutive speeches, each with two minutes preparation and four minutes of delivery (Bosch et al., 2003a). To enhance social stress, speech tasks were performed in the presence of an audience of three confederates and videotaped. Recorded task instructions were presented on a computer screen in order to standardise instruction and timing. Affective responses were assessed with Profile of Mood States (POMS)(McNair et al., 1992), which was given at baseline, immediately post task, and at the end of the recovery period. Instructions of the post-task questionnaire were adapted to reflect how the participants felt during the task. To assess autonomic nervous system activation, cardiovascular activity was recorded throughout.

Cardiovascular analysis

Indices of sympathetic and parasympathetic drive were obtained by analysis of electrocardiogram (ECG) and thoracic impedance (ICG) signals (Berntson et al., 1997; Sherwood et al., 1990). The ICG and ECG signals were recorded from six Ag/AgCl spot-electrodes (Conmed corporation, UTICA, NY, USA) using a VU-AMD device (Vrije Universiteit, Amsterdam, Holland). Reliability and validity of the VU-AMD device have been reported elsewhere (de Geus et al., 1995; De Geus and van Doornen, 1996; Willemsen et al., 1996). The ICG complexes were ensemble averaged with reference to the ECG R-wave across 1-minute periods. From these 1-minute ensembles, averages were

computed for heart rate (HR), heart rate variability (RMSSD) and the pre-ejection period (PEP). These minute-by-minute means were averaged over the last 6-minutes of baseline, each 6-minute speech, and the last 6-minutes of recovery. Changes in PEP were used to index changes in cardiac sympathetic drive (Sherwood et al., 1990), whereas RMSSD (Root mean Square of Successive Differences) was used to index changes in cardiac vagal tone (Bosch et al., 2003b; Goedhart et al., 2007). The RMSSD was log transformed to restore normality. Measurements of systolic and diastolic blood pressure (SBP and DBP, respectively) were taken at two minute intervals using an Omron M5 blood pressure monitor (Omron Healthcare UK Ltd., Milton Keynes, UK). Baseline, task and recovery values (10-min post-task onwards) were averaged and used in subsequent analyses.

Flow cytometry

Blood was collected in ethylene-diamine-tetraacetic acid (EDTA) coated vacutainer tubes (BD, BD Biosciences). Blood was kept at room temperature and prepared within 2 hours. Lymphocyte subsets were identified by immunofluorescent antibody staining of whole blood using four-colour flow cytometry (FACS-Calibur, BD Biosciences). The antibodies used were IgG1 FITC, IgG1 PE, CD11a FITC , CD27 FITC, CD27 PE, CD45RA APC, CD62L PE, CD94 FITC, γδ TCR APC, Vδ-2 TCR PE (Pharmingen), Vδ1 TCR FITC (Endogen, Pierce), CD3 PERCP (BD Biosciences, Oxford, UK). Briefly, 50μl whole blood samples were incubated with appropriate amounts of antibody (as determined by titration) for 30 minutes in the dark at room temperature (RT),

and erythrocytes were subsequently lysed using FACS Lysing Solution (Becton Dickinson). Following lysis, samples were centrifuged (217 x G, 6 minutes, at RT), and fixed in 1% paraformaldehyde/PBS (without $Ca^{2+}Mg^{2+}$) (w/v). Fixed preparations were stored in the dark at 4°C and read within 24 hours, collecting a minimum of 30,000 gated lymphocytes from each sample. Where appropriate, matched isotype controls were used to set negative staining criteria. The FACS-Calibur machine (BD Biosciences) was calibrated weekly using Calibrite beads (BD Biosciences), and further compensation was performed before each experiment using single-labeled cells. Data was analysed using FlowJo software v5.2 (Tree Star, Inc., Ashland, OR). Lymphocyte numbers were determined using a Coulter ACT^{diff} (Beckman Coulter, High Wycombe, UK).

Total lymphocytes were gated using forward versus side scatter. γδ T cells were then gated as the $CD3^{+}$ γδ TCR^{+} population, δ1 T cell were gated as $CD3^{+}V\delta1^{+}$ and δ2 T cells were gated as $CD3^{+}$ $V\delta2^{+}$. Lymphocyte subset numbers were quantified using the percentage values obtained from each lymphocyte subset gate and the absolute lymphocyte count (Coulter ACT^{diff}).

Data analysis

Repeated-measures analysis of variance (ANOVA) was used to assess the effects of each condition on immunological, cardiovascular and psychological measures, and to explore subset × time interactions. The association between autonomic, cardiovascular and immune cell responses was explored using Pearson's correlation coefficients. Paired t-

tests were used to compare response differences (% change, Δ %) between subsets. For occasional missing data degrees of freedom were adjusted. Where means are presented, standard deviation is given in brackets. Data was analyzed using SPSS 15 (SPSS, Chicago, IL).

Results

Anxiety and cardiac autonomic parameters

Analysis of the POMS tension-anxiety subscale confirmed that the speech tasks were perceived as stressful (+5.8 (*SD* = 5.4); $F_{(2, 56)} = 53.5$, $p<.001$). Confirming sympathetic activation, there was a significant decrease in PEP (–14.0 ms (*SD* = 9.4); $F_{(2, 52)} = 17.8$, $p<.001$), accompanied by significant increases in SBP (+22.0 mmHg (*SD* = 8.5); $F_{(2, 56)} =103.7$, $p<.001$), DBP (+16.6 mmHg (*SD* = 8.1); $F_{(2, 56)} = 74.7$, $p<.001$) and HR (+ 14.7 bpm (*SD* = 9.2); $F_{(2, 56)} = 70.7$, $p<.001$). Additionally, there was vagal withdrawal, as reflected by a decrease in RMSSD (–0.6 ms (*SD* = 0.5); $F_{(2, 52)} = 40.5$, $p<.001$). At 15-min post-task all cardiovascular and autonomic measures had returned to baseline values.

Mobilization of γδ T cell subsets

γδ T cells and δ1and δ2 subsets

The speech task induced a pronounced lymphocytosis and an increase in the number of circulating γδ T cells (see Table 1). Both δ1 and δ2 γδ T cell subsets were mobilized during the stress task (see Table 1 and Figure 1). There was an overall subset × time interaction ($F_{(2, 54)} = 15.4$, $p<.001$); paired t-test analyses revealed a trend for mobilization of δ1 compared to δ2 cells (Δ%: $t_{(28)} = -1.85$, $p<.075$). All effects survived correction for baseline measures. There were no gender differences in the mobilization of any γδ T cell subset. Post-hoc analyses also did not demonstrate an association with caffeine, alcohol consumption, or smoking (only one participant was a smoker).

δ1 cell memory phenotypes

Using the markers CD45RA and CD27, δ1 cells were divided into four memory phenotypes; Naïve (NA), Central Memory (CM), Effector Memory (EM), and $CD45RA^+$ Effector Memory (EMRA) cells. As illustrated in Figure 1a and Table 1, δ1 EMRA and NA cells increased significantly during the speech task. There were no significant increases in the number of circulating δ1 CM and EM cells. A comparison of the relative mobilization of these subsets revealed a significant phenotype × time interaction ($F_{(6, 156)} = 4.8$, $p<.001$,); as indicated in Figure 1a, EMRA cells were mobilized to a significantly greater extent than all other phenotypes during the speech task, and NA cells were

mobilized more robustly than CM cells. There was no significant difference between the mobilization of CM and EM cells.

δ2 cell memory phenotypes

The δ2 cells were also divided into four memory phenotypes. As indicated in Figure 1b and Table 1, all δ2 memory phenotypes increased during the stress task. Again, there was a phenotype × time interaction ($F_{(6, 156)} = 6.6$, $p<.001$); as shown in Figure 1b, EMRA cells were mobilized most robustly during the speech task, followed by NA cells and then CM and EM cells. There was no significant difference between the mobilization of CM and EM cells. All reported statistical analyses for δ1 and δ2 subsets gave similar results after controlling for baseline values.

CD11a and CD62L

CD11a is a marker of tissue-migratory potential, and γδ T cells can be separated into either $CD11a^{hi}$ or $CD11a^{lo}$ subsets. As illustrated in Figure 2a and Table 2, acute stress mobilized γδ T cells with a $CD11a^{hi}$ phenotype, whereas $CD11a^{lo}$ γδ T cells were not mobilized.

CD62L expression reflects the potential to migrate to the lymph nodes, and γδ T cells can be divided into $CD62L^{hi}$, $CD62L^{lo}$ and $CD62L^{neg}$ subsets. As shown in Figure 2b and Table 2, $CD62L^{hi}$, $CD62L^{lo}$ and $CD62L^{neg}$ subsets were all mobilized during the stress

task. There was an overall subset × time interaction ($F_{(4, 109)} = 8.9$, $p<.001$), which was driven by the fact that $CD62L^{lo}$ and $CD62L^{neg}$ γδ T cells were mobilized more robustly than $CD62L^{hi}$ γδ T cells (see Figure 2b). There was no significant difference between the mobilization of $CD62L^{lo}$ and $CD62L^{neg}$ γδ T cells. All analyses yielded similar results after controlling for baseline values.

<u>CD94</u>

The surface molecule CD94 is involved in the innate recognition of aberrant MHC expression on infected and cancerous cells. As shown in Figure 2c and Table 2, both $CD94^{+}$ and $CD94^{-}$ γδ T cells were mobilized during the task. After controlling for baseline values, a subset × time interaction appears ($F_{(2, 50)} = 4.1$, $p<.03$); $CD94^{+}$ γδ T cells were mobilized more robustly than $CD94^{-}$γδ T cells (see Figure 2c).

Correlation between γδ T cell subset mobilization and cardiac autonomic responses

As displayed in Table 3, γδ T cell subset mobilization (% change from baseline) was found to correlate with markers of sympathetic drive, i.e. PEP, SBP, and HR. No consistent associations were observed with RMSSD and DBP.

Table1. Mean (SEM) cell number (cells/μl), with results of repeated measures ANOVA for total lymphocytes and γδ T subsets during baseline, task and recovery.

Subset (cells/μl)	Baseline	Task	Recovery	*F(df)*	*p*
Lymphocytes	1705 (71.0)	2128 (91.0)	1755 (81.0)	44.8 (2, 56)	<.001
γδ T cells	80.2 (11.8)	122.6 (17.1)	91.5 (14.1)	20.5 (2, 56)	<.001
Total δ1 cells	13.0 (1.4)	17.8 (2.2)	13.5 (1.6)	15.7 (2, 54)	<.001
δ1 NA	7.1 (0.7)	9.1 (0.8)	7.5 (0.7)	14.6 (2, 52)	<.001
δ1 CM	2.8 (0.3)	3.2 (0.6)	2.9 (0.4)	0.9 (2, 52)	0.40
δ1 EM	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.02 (2, 52)	0.98
δ1 EMRA	2.6 (0.9)	5.2 (1.7)	2.6 (0.9)	7.5 (2, 52)	.001
Total δ2 cells	72.3 (11.1)	109.8 (16.1)	81.8 (13.5)	18.2 (2, 56)	<.001
δ2 NA	18.2 (2.6)	30.3 (5.1)	17.6 (2.5)	14.5 (2, 52)	<.001
δ2 CM	41.3 (7.1)	56.3 (9.3)	47.6 (8.7)	10.4 (2, 52)	<.001
δ2 EM	7.2 (1.9)	10.3 (2.4)	8.2 (2.2)	8.1 (2, 52)	<.001
δ2 EMRA	5.0 (1.4)	10.2 (2.9)	5.6 (1.6)	7.9 (2, 52)	<.001

Table 2.Mean (SEM) cell number (cells/µl), with results of repeated measures ANOVA for total γδ T cell subsets differentiated on receptor expression (CD62L, CD11a, CD94) during baseline, task, and recovery.

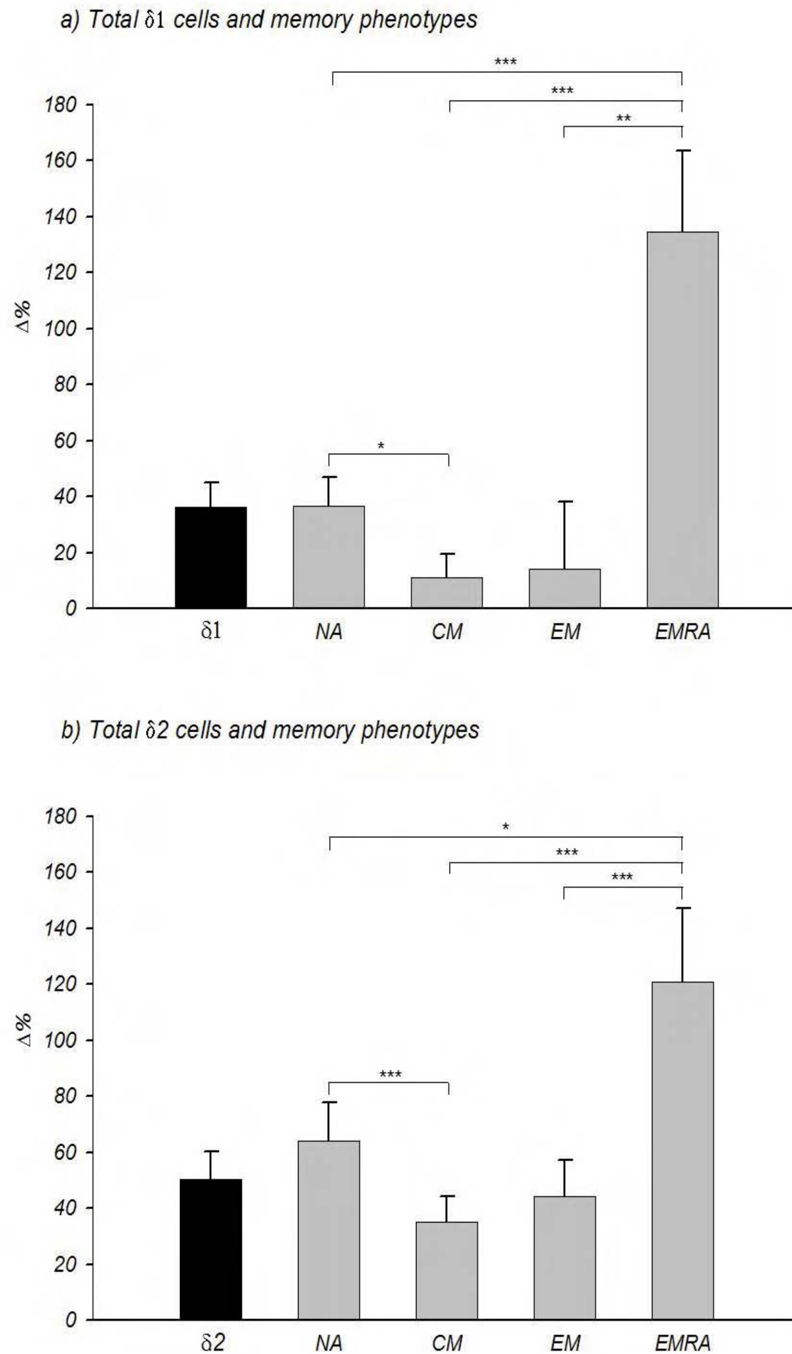
Subset (cells/µl)	Baseline	Task	Recovery	*F(df)*	*p*
CD11a^{hi}	78.3 (13.9)	117.9 (18.5)	81.7 (14.6)	22.4 (2,52)	<.001
CD11a^{lo}	11.6 (1.1)	12.5 (1.4)	12.2 (1.3)	.80 (2,52)	.49
CD62l^{hi}	39.2 (6.0)	50.6 (7.3)	39.3 (5.4)	11.2 (2,52)	<.001
CD62l^{lo}	29.9 (6.7)	46.1 (9.0)	32.0 (7.6)	18.2 (2,52)	<.001
CD62l^{neg}	20.1 (3.1)	33.7 (5.4)	22.6 (3.7)	15.0 (2,52)	<.001
CD94^{+}	47.7 (9.2)	71.8 (12.7)	49.3 (10.0)	13.1 (2, 54)	<.001
CD94^{-}	44.5 (5.4)	61.3 (7.8)	46.8 (6.9)	14.8 (2, 54)	<.001

Table 3. Associations between cardiac autonomic responses (absolute Δ) and increases in γδ T cell subset numbers (Δ%).

γδ T cell subsets	HR	PEP	SBP
Total γδ	.49**	–.44*	.21
Total δ1	.37$^{\alpha}$	–.43*	.08
Total δ2	.46*	–.37$^{\alpha}$	.20
δ1 EMRA	.08	–.20	.40*
δ2 NA	.43*	–.40*	.20
δ2 CM	.41*	.46*	.13
CD11a^{hi} γδ	.20	–.20	.50**
CD62L^{hi} γδ	.19	–.15	.42*
CD62L^{lo} γδ	.39*	–.10	.45*
CD94^{-} γδ	.23	.09	.37*

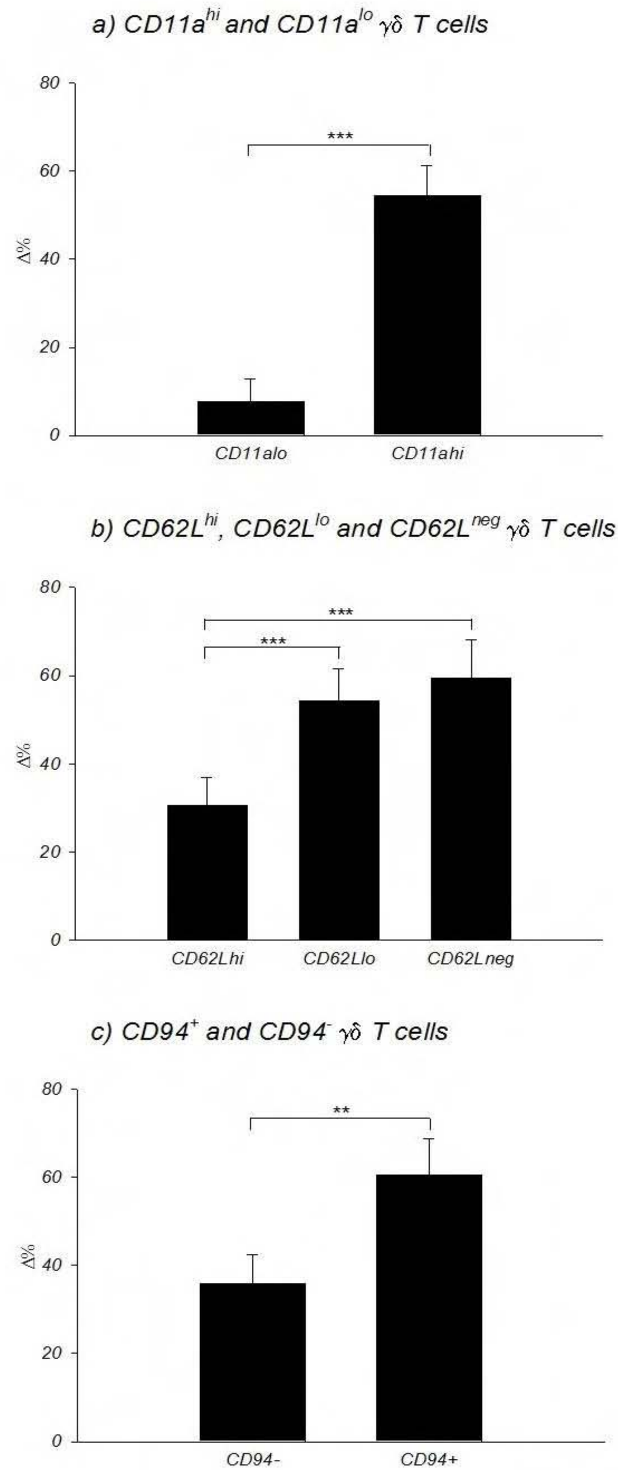
*p<.05, **p<.01, $^{\alpha}$p<.07.

Figure 1. Mean (SEM) change in cell number (Δ%) for (a) δ1 and (b) δ2 cells and memory phenoypes; naïve (NA), central memory (CM), effector memory (EM) and CD45RA+ effector memory (EMRA).



* indicates p<.05, **p<.01***p<.001 (Paired t-test analysis).

Figure 2. Mean (SEM) change in cell number (Δ%) for γδ T cell subsets.



*indicates p<.05, **p<.01***p<.001 (Paired t-test analysis).

Discussion

The present study performed a detailed phenotypic analysis of γδ T cell subset mobilization during acute stress. The results revealed that within each γδ T cell population, EMRA cells were mobilized to a greater extent than each of the other memory phenotypes. EMRA cells have well-defined effector characteristics that include a high cytotoxic ability and a strong tissue migrating potential (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003; Hamann et al., 1997). The strong tissue migrating capability of these stress-sensitive cells was further confirmed by the selective increase in γδ T cells expressing high levels of the tissue-homing marker CD11a. This observation essentially replicates what has been found for other cytotoxic lymphocytes such as NK and $CD8^{+}$ T cells (Bosch et al., 2003a; Bosch et al., 2005; Dimitrov et al., 2010; Goebel and Mills, 2000; Mills et al., 2003).

Only small differences were observed between the mobilization of $CD62L^{hi}$, $CD62L^{lo}$ and $CD62L^{neg}$ subsets, which is in contrast to previous findings in NK and CD8 T cells (Bosch et al., 2005; Kimura et al., 2008; Mills et al., 2003). It is possible that the unexpected mobilization of $CD62L^{hi}$ cells reflected a recruitment of recently activated γδ T cells, which have been shown to re-express lymph node homing markers (Moser and Eberl, 2007). Further analyses demonstrated that expression of the NK-receptor CD94 ($CD94^{+}$) was also associated with greater propensity for mobilization. In sum, the current findings indicate that stress predominantly mobilizes γδ memory cells known to have high cytotoxic capability, tissue homing potential, and the capacity for rapid, innate-like target

recognition (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003).

The mobilization of γδ T cells displaying a naïve phenotype ($CD27^{+}CD45RA^{+}$) is seemingly at odds with the idea that cells with immediate effector potential are preferentially mobilized. However, unlike naïve $CD8^{+}$ T cells, naïve γδ T cells possess the ability for immediate effector responses, such as rapid secretion of the pro-inflammatory cytokine IL-17, which helps orchestrate early immune responses (Jensen et al., 2008; Roark et al., 2008). Further, it is likely that the $CD27^{+}CD45RA^{+}$ population is not solely comprised of NA cells. For example, it is known that some EMRA T cells can re-express CD27 (Sallusto et al., 2004), which gives these effector cells the phenotypic appearance of NA cells. These explanations could be further explored in future studies.

It has frequently been suggested that the rapid redeployment of immune cells during acute stress reflects an adaptive mechanism designed to place cells 'at the ready' in anticipation of pathogenic attack (Dhabhar, 2002). This concept appears supported by the integrative specificity of stress-lymphocytosis (Denson et al., 2009), i.e. the present study reveals that mobilized cells appear to share the ability to act rapidly, possess powerful effector functions, and have the capacity to migrate to sites of tissue damage where infection is likely to occur. Thus it appears that stress specifically recruits those cells ideally equipped to deal with pathogenic attack. Animal models support an adaptive role for stress lymphocytosis (Dhabhar and Viswanathan, 2005; Viswanathan and Dhabhar, 2005). However this idea is not uncontroversial. For example, it can be argued that peripheral blood contains only 2% of total leukocytes, and that experimental studies have

yet to demonstrate a survival advantage for rapid changes in the cellular composition of peripheral blood. Clearly, more evidence is needed to conclude that stress-lymphocytosis has implications for the way in which infection and inflammation are dealt with. We may add, however, that apart from its possible implications for the immune response, stress lymphocytosis itself is an immune response. In fact, it is represents the most robust phenomenon in human psychoneuroimmunology, and yet remains poorly understood. From that perspective this aspect of the stress response warrants further investigation.

Assuming an adaptive function for lymphocytosis, the mobilization of cells with a high propensity to migrate into the tissues may enhance immune protection at sites of tissue damage and infection. For example, γδ T cells have a strong ability to attract and stimulate other immune cells (Tikhonov et al., 2006), play a role in the promotion of wound healing (Jameson et al., 2002), and finally, possess professional antigen presenting capabilities (Brandes et al., 2005). However, the stress induced mobilization of γδ T cells may also have deleterious consequences, particularly in a context of inflammatory diseases, such as rheumatoid arthritis (Holoshitz, 1999; Straub et al., 2005) and atherosclerosis (Bosch et al., 2003a; Dyugovskaya et al., 2003). Further elucidation of the clinical consequences of stress induced γδ T cell mobilization is an important future research target.

In order to better understand the function of stress induced γδ T cell mobilization it is important that the mechanisms driving this response are identified. γδ T cells were recently shown to be mobilized during exercise and β-agonist (isoproterenol) infusion in a dose-dependent manner (Anane et al., 2009), and the increase in these cells during

psychological stress correlated with indices of sympathetic activation, such as heart rate and pre-ejection period. Further support for an adrenergic mechanism is provided by recent evidence demonstrating γδ T cell mobilization in response to epinephrine infusion. In addition, in vitro data suggests that mobilization is likely mediated by an epinephrine induced detachment of γδ T cells from endothelia (Dimitrov et al., 2009b). The present study revealed that this adrenergic-dependent response is specific to γδ T cells of an effector memory phenotype. Further research should identify the basis for the apparently higher adrenergic sensitivity of these cells (e.g. receptor density, receptor sensitivity), and the possible implications for their unique effector functions such as, rapid target killing, production of inflammatory cytokines and tissue migration (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003).

In sum, the present study was the first to provide a phenotypic characterization of γδ T cell subset mobilization in response to psychological stress. Preferentially mobilized, were memory cells known to possess the highest cytotoxic capability (Angelini. et al., 2004; Caccamo. et al., 2005; De Rosa et al., 2004; Dieli et al., 2003), displaying a tissue migrating phenotype, and expressing NK-like features.

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CHAPTER FOUR

DIFFERENTIATED, PERFORIN $^{+}$ T CELLS ARE MOBILIZED DURING PSYCHOLOGICAL STRESS AND ISOPROTERENOL INFUSION.

Abstract

The mobilization of cytotoxic lymphocytes, such as NK cells, $CD8^{+}$ T cells, and γδ T cells during psychological stress suggests that cytotoxic potential may predict which T cells become mobilized. One way of identifying lymphocytes that have cytotoxic capability is by analyzing the intracellular expression of perforin (pfn). The current study investigated the effects of acute psychological stress (speech task) and isoproterenol infusion on the mobilization of pfn^{+} and pfn^{-} $CD4^{+}$, $CD8^{+}$ and γδ T cells. CD27 expression was analyzed in conjunction to assess the differentiation status of mobilized T cells. The results show that while total $CD4^{+}$ T cells remained unchanged, pfn^{+} $CD4^{+}$ T cells became mobilized during stress. Also, among $CD8^{+}$ and γδ T cells, pnf^{+} cells were mobilized more robustly than pfn^{-} subsets. Analysis of CD27 expression in T cell subsets revealed that $Pfn^{+}CD27^{-}$ T cells were consistently mobilized robustly, but $pfn^{+}CD27^{+}$ T cells were not. These effects were largely replicated by infusion of a β-agonist (isoproterenol), suggesting β-adrenergic mechanisms of mobilization. These results suggest that although cytotoxic potential (pfn^{+}) is an important feature of mobilization

responsive cells, it is not the only determinant of stress lymphocytosis as a differentiated phenotype ($CD27^{-}$) more consistently identified mobilized T cells.

Introduction

The mobilization of lymphocytes into the blood during acute psychological stress is one of the best established phenomena in psychoneuroimmunology (Benschop et al., 1996; Segerstrom and Miller, 2004). In vitro and in vivo pharmacological studies have revealed that this lymphocytosis is mediated by the actions of catecholamines on lymphocyte beta (β)-adrenergic receptors (Benschop et al., 1996; Elenkov et al., 2000). Evidence suggests that this stress-lymphocytosis is confined to cytotoxic lymphocytes, whereas little change is seen for non-cytotoxic subsets, such as $CD4^+$ T cells and B cells (Anane et al., 2009; Segerstrom and Miller, 2004). Cytotoxic lymphocytes are a heterogeneous group of cells that include $CD8^+$ T cells, γδ T cells and NK cells, and which share the ability to kill infected cells via apoptosis. Within each of these subsets, phenotypes that are known to have a higher cytotoxic ability, such as effector-memory $CD8^+$ T and γδ T cells, or $CD56^{low}$ NK cells, mobilize more readily in response to stress or catecholamines than less cytotoxic subsets. This further supports the idea that stress-mobilization is unique to cytotoxic cells (Anane et al., 2010; Atanackovic et al., 2006; Bosch et al., 2005; Campbell et al., 2009; Dimitrov et al., 2010; Riddell et al., 2009).

To further test this hypothesis of cytotoxic specificity, the current study investigated the effects of acute psychological stress and β-adrenergic stimulation on cytotoxic $CD4^+$T cells. Cytotoxic $CD4^+$T cells are unique from other functional $CD4^+$ T cell subsets (e.g. TH1, TH2, TH17, Treg) in that they are capable of killing infected target cells via cytotoxic mechanisms (Brown, 2010; Frevert et al., 2009; van de Berg et al.,

2008; Zheng et al., 2009). These $CD4^+$ T cells can be identified by the expression of the cytotoxic marker perforin (pfn) (Appay et al., 2002), a pore forming protein involved in the destruction of target cells (Chávez-Galán et al., 2009; Shresta et al., 1998). In healthy individuals cytotoxic $CD4^+$ T cells comprise no more than 2% of the total $CD4^+$T cell population, yet can constitute up to 50% of total $CD4^+$T cells in certain pathologies (Appay, 2004).

In the current experiments the hypothesis that cytotoxic potential is the main predictor of which lymphocytes become mobilized during stress was investigated. If cytotoxicity is indeed the key determinant of stress-induced mobilization, then it would be predicted that cytotoxic $CD4^+$ T cells would be mobilized during psychological stress *despite* evidence that total $CD4^+$ T cells are minimally altered (Segerstrom and Miller, 2004). To test this assumption, it is not sufficient to merely demonstrate that perforin-positive (pfn^+) $CD4^+$ T cells are mobilized during acute psychological stress (Atanackovic et al., 2006). For example, there is a proportion of $CD8^+$ T cells that co-express CD4 ($CD4^+CD8^+$ T cells) (Appay et al., 2002), and it could thus be that an increase in pfn^+CD4^+ T cells merely reflects a mobilization of $CD8^+$ T cells. Moreover, in cytotoxic T lymphocytes the ability to kill target cells is also related to differentiation status, which involves the co-expression of other effector functions, a less stringent activation requirement, and a tissue-migrating signature (Angelini. et al., 2004; Appay et al., 2002; De Rosa et al., 2004; Guidotti and Chisari, 1996; Hamann et al., 1997; Shresta et al., 1998). Such effector features are correlated with the expression of the surface marker CD27 (Appay et al., 2008; Hamann et al., 1997). Consequently, it could not be ruled out

that any mobilization of pfn^+ T cells actually reflected a broader phenomenon; the mobilization of differentiated tissue-migrating effector cells. The above issues were investigated in the present study which analysed the mobilization of perforin and CD27 expressing $CD4^+$, $CD8^+$ and $\gamma\delta$ T cell subsets during stress and β-adrenergic receptor agonist (isoproterenol) infusion.

Methods

Participants

Thirty one healthy student and staff volunteers from the University of Birmingham (Mean age = 25.6 years (*SD* = 10.6), 22 women) took part in the stress study. Nineteen participants (Mean age = 36.2 years (*SD* = 9.5), 8 women) were recruited from community volunteers and student and staff from the University of California San Diego (UCSD) to take part in the infusion study. Among these, a subgroup of seven participants (Mean age = 35.3 years (*SD* = 12.2), 2 women) underwent the infusion twice (see procedures section below). All participants 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. Health and lifestyle information was obtained by self-report questionnaire. Blood was taken from a 19 gauge intravenous cannula (Becton-Dickinson)

inserted into a palpable vein in the antecubital fossa. Cannulation was performed at least 20 minutes before the ‘baseline’ measurement was initiated and the same catheter was used for subsequent blood withdrawals. Volunteers received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the University of Birmingham, and University of California San Diego ethical review boards.

Psychological stress study

Participants were tested between 10 am and 2 pm. Following instruction and instrumentation, participants completed a 20 min seated baseline, during which questionnaires were completed. At the end of this period, a (‘baseline’) blood sample was obtained and the laboratory stressor was initiated. A second (‘task’) blood sample was obtained during the final two minutes of the stress task , and a third blood sample (‘recovery’) was taken 15 minutes post-task. The stress task involved participants delivering two consecutive speeches, each with two minutes preparation and four minutes of delivery (Bosch et al., 2003a). To enhance social stress, speech tasks were performed in the presence of an audience of three confederates and videotaped. Recorded task instructions were presented on a computer screen in order to standardise instruction and timing. Affective responses were assessed with Profile of Mood States (POMS)(McNair et al., 1992), which was given at baseline, immediately post task, and at the end of the

recovery period. Instructions of the post-task questionnaire were adapted to reflect how the participants felt during the task. Cardiovascular activity was recorded throughout.

Cardiovascular analysis

Assessment of the cardiovascular response focused on cardiac autonomic control and blood pressure. Indices of sympathetic and parasympathetic drive were obtained by analysis of electrocardiogram (ECG) and thoracic impedance (ICG) signals (Berntson et al., 1997; Sherwood et al., 1990). The ICG and ECG signals were recorded from six Ag/AgCl spot-electrodes (Conmed corporation, UTICA, NY, USA) using a VU-AMD device (Vrije Universiteit, Amsterdam, Holland (de Geus et al., 1995; De Geus and van Doornen, 1996; Willemsen et al., 1996). The ICG complexes were ensemble averaged across 1-minute periods as described by Bosch et al. 2003, 2005 and Anane et al. 2009 (Anane et al., 2009; Bosch et al., 2003a; Bosch et al., 2005). Changes in PEP were used to index changes in cardiac sympathetic drive (Sherwood et al., 1990), whereas RMSSD (Root mean Square of Successive Differences) was used to index changes in cardiac vagal tone (Bosch et al., 2003b; Goedhart et al., 2007). The RMSSD was log transformed to restore normality. Measurements of systolic (SBP) and diastolic (DBP) blood pressure were taken at two minute intervals using an Omron M5 blood pressure monitor (Omron Healthcare UK Ltd., Milton Keynes, UK).

Infusion study

Isoproterenol infusion was performed using a standardized protocol described previously (Goebel et al., 2000; Mills et al., 2002; Mills et al., 2000; Mills et al., 1997). In brief, upon arrival at the laboratory height and weight measurement were taken and body surface area (BSA) was used to calculate the isoproterenol infusion rate. Participants lay supine for 15 min following placement of ECG electrodes and two intravenous cannulas were inserted into an antecubital vein in the arm. The 'baseline' blood sample was collected at this point. Isoproterenol was then infused at incremental steps for 15 minutes until an approximate 20 bpm heart rate (HR) increase had been reached from baseline. On average this equated to a dose of 1μg/min/1.73 m^2. An 'infusion' blood sample was acquired in the final minutes of isoproterenol infusion. The half-life of isoproterenol in the body is approximately 2–3 min (Goebel et al., 2000). ECG was monitored throughout the infusion. The infusion is generally well-tolerated and all participants successfully completed the protocol.

<u>β-blockade</u>

A sub group of 7 participants performed the isoproterenol infusion procedure under two conditions, using a single-blind counterbalanced design. In the blockade condition participants took a capsule containing 80mg of the β-adrenergic receptor antagonist propranolol (Inderal®) for five consecutive days while in the placebo condition participants were administered a placebo in similar capsules.

Flow cytometry

Blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes (BD, BD Biosciences), kept at room temperature and prepared within 2 hours. Flow cytometry analysis involved both extracellular and intracellular (perforin) staining protocols. Lymphocyte subsets were identified by immunofluorescent antibody staining of cell surface markers using whole blood (FACS-Calibur, FACS-Canto II, BD Biosciences). The antibodies used were CD3 PERCP, CD4 APC, CD8 APC-Cy7, CD8 FITC, CD27 FITC, perforin PE, IgG2b PE (BD Biosciences, Oxford, UK). Briefly, 50μl whole blood samples were incubated with appropriate amounts of antibody (as determined by titration) for 30 minutes in the dark at room temperature (RT), and erythrocytes were subsequently lysed using FACS Lysing Solution (Becton Dickinson). Following lysis, samples were centrifuged (217 x G, 6 minutes, at RT) and samples only requiring extracellular staining were fixed in 1% paraformaldehyde/PBS (without $Ca^{2+}Mg^{2+}$) (w/v). Samples also requiring intracellular staining, were instead re-suspended in FACSPERM2 (BD Biosciences), and incubated in the dark for 10 minutes. Samples were then washed (PBS/0.5% BSA (w/v) (217 x G, 6 minutes, at RT)), re-suspended in perforin PE, or IgG2bPE, and incubated in the dark for a further thirty minutes. Finally, samples were washed (PBS/0.5% BSA (w/v)) (217 x G, 6 minutes, at RT) and fixed in 2% paraformaldehyde/PBS (without $Ca^{2+}Mg^{2+}$ (w/v)). All fixed preparations were stored in the dark at 4°C and read within 24 hours, collecting a minimum of 20,000 gated lymphocytes from each sample. Data was analysed using FlowJo software v7.5 (Tree Star,

Inc., Ashland, OR). Lymphocyte numbers were determined using a Coulter ACT^{diff} (Beckman Coulter, High Wycombe, UK).

Gating procedure

Lymphocytes were gated using forward versus side scatter. Using histogram analysis perforin positive (pfn^{+}) populations were gated using isotype controls to set negative staining criteria. γδ T cells were identified as the CD4^{-}CD8^{-} T cell population (Carding and Egan, 2002; Dimitrov et al., 2010; Girardi, 2006). The leukocyte and differential count was obtained using a cell counter (Coulter ACT^{diff}).

Data analysis

Repeated-measures analysis of variance (ANOVA) was used to assess the effects of each condition on immunological, cardiovascular and psychological measures. Paired t-tests were used to compare response differences (Δ %) between subsets. Occasional data was missing due to incomplete lysis, which is reflected in the degrees of freedom. Where means are presented, standard deviation is given in brackets. Standard errors were provided in the Figures. Data was analyzed using SPSS 16 (SPSS, Chicago, IL).

Results

Psychological stress study

Anxiety and cardiac autonomic parameters

Analysis of the POMS tension-anxiety subscale confirmed that the speech tasks were perceived as stressful (+7.6; *SD* = 5.4; $F_{(2, 60)} = 62.78$, $p<.001$). Confirming sympathetic activation, there was a significant decrease in PEP (–8.7 ms; *SD* = 8.2; $F_{(2, 38)} = 13.68$, $p<.001$), accompanied by significant increases in SBP (+22.1 mmHg ; *SD* = 8.0; $F_{(2, 60)} = 106.66$, $p<.001$), DBP (+16.4 mmHg; *SD* = 7.3; $F_{(2, 60)} = 70.57$, $p<.001$) and HR (+ 17.1 bpm; *SD* = 8.0; $F_{(2, 42)} = 82.14$, $p<.001$). Additionally, there was vagal withdrawal, as reflected by a decrease in RMSSD (–0.5 ms; *SD* = 0.35; $F_{(2, 42)} = 20.10$, $p<.001$).

Immune parameters

T cell mobilization and perforin expression

Table 1 presents the summary data for T cell subsets, comparing perforin (pfn^+) and pfn^- subsets, during baseline, stress and recovery. The stressor increased the number of pfn^+CD4^+T cells, whereas the number of pfn^-CD4^+ T cells, or total $CD4^+$T cells (Δ% = 9.7 cells/ul; *SD* = 18.4) did not significantly increase (Figure 1a and Table 1).

Essentially replicating the findings for $CD4^+$T cells , Table 1 and Figure 1a show that stress-induced mobilization of $CD8^+$ (Δ% = 48.1 cells/ul; *SD* = 46.1) and γδ T cells

($\Delta\%$ = 49.8 cells/ul; *SD* = 47.3) was also largely driven by an increase in perforin-expressing cells.

Are mobilized pfn$^+$CD4$^+$T cells pfn$^+$CD4$^+$CD8$^+$T cells?

The mobilization of CD8$^+$ T cells during acute stress is well-established and therefore analyses were performed to determine whether the mobilization of cytotoxic (pfn$^+$) CD4$^+$ T cells was explained by CD4$^+$CD8$^+$ T cells, in a subgroup of 18 participants. As can be seen in Table 1, although pfn$^+$CD4$^+$CD8$^+$ T cells increased during the stress task, they represented only a small proportion of total pfn$^+$CD4$^+$T cells; removing CD4$^+$CD8$^+$T cells from the total pfn$^+$CD4$^+$T cell population (yielding CD4$^+$CD8$^-$ T cells only) did not appreciably alter the findings.

Perforin versus CD27 expression in T cells

CD27 expression was determined on pfn$^+$ T cell populations in order to examine the contribution of differentiation status. The expression of CD27 was analysed for CD4$^+$T cells in all participants and for CD8$^+$T cells and γδ T cells in a subgroup of 13 participants.

As displayed in Table 2, for all T cell populations examined (CD4$^+$, CD8$^+$ T and γδ), CD27$^-$ T cells were mobilized, whilst CD27$^+$ T cells were not. As shown in table 2 and Figure 1b, pfn$^+$CD27$^-$ cells were mobilized in all three T cell subsets, whereas pfn$^+$CD27$^+$ subsets did not show statistically significant increases. In CD4$^+$, CD8$^+$ and γδ

T cell subsets $pfn^{-}CD27^{-}$T cells were also mobilized (data not shown), albeit to a lesser extent (Δ% cell/μl = 30.7, 59.8, 36.8; *SD's* = 33.9, 42.8, 21.3, respectively).

Infusion study

Immune parameters

T cell mobilization and perforin expression

As shown in Table 3 and Figure 2a, isoproterenol infusion caused a significant decrease in the total number of $CD4^{+}$T cells (Δ%= –14.3 cells/ul; *SD* = 14.2), while the number of $pfn^{+}CD4^{+}$ T cells increased. These effects were abrogated by the administration of propranolol. As shown in Table 3, and in contrast to the stress study, removal of $CD4^{+}CD8^{+}$ T cells from total $pfn^{+}CD4^{+}$ T cells rendered the increase in $pfn^{+}CD4^{+}$ T cells non-significant, although this was still statistically different from the decrease in total $CD4^{+}CD8^{-}$ T cells (Δ%; $t_{(17)} = -2.25$, $p<.05$.)

In line with findings from the stress study, Table 3 and Figure 2a show that stress-induced mobilization of $CD8^{+}$ (Δ%= 26.7 cells/ul; *SD* = 31.5) and γδ T cells (Δ%= 43.6 cells/ul; *SD* = 29.4) was driven by an increase in perforin-expressing cells. These effects were, again, largely abrogated with propranolol administration (see Table 3 and Figure 2a).

Perforin versus CD27 expression in T cells

As displayed in Table 4, there was a significant decrease in the number of $CD27^{+}CD4^{+}$ T, and no significant change in the number of $CD27^{-}CD4^{+}$T cells. In contrast, there were increases in $CD27^{-}CD8^{+}$T cells and both $CD27^{+}$ and $CD27^{-}\gamma\delta$ T cells during isoproterenol infusion. As shown in Figure 2b, and paralleling stress findings, $pfn^{+}CD27^{-}$T cells were mobilized to a significantly greater extent than $pfn^{+}CD27^{+}$ T cells in all three T cell subsets analysed. In $CD8^{+}$ and $\gamma\delta$ T cell subsets $pfn^{-}CD27^{-}$T cells were also mobilized (data not shown), albeit to a lesser extent (Δ% cells/μl = 60.0 and 70.6; *SD's* = 38.3 and 44.9, respectively). With the exception of $pfn^{+}CD27^{-}$ $\gamma\delta$ T cells, the administration of propranolol almost entirely abrogated these increases (see Table 4 and Figure 2b).

Table 1. Mean (SEM) cell numbers in the psychological stress study during baseline, task and recovery.

Subset (cells/µl)	Baseline	Task	Recovery	$F_{(df)}$
Total lymphocytes	1790.3 (92.4)	2422.6 (147.4)	1977.4 (101.3) ***	24.43 $_{(2,60)}$
Total CD4$^+$T cells	796.3 (53.7)	853.1 (53.5)	837.2 (54.6)	2.21 $_{(2, 56)}$
CD4$^+$pfn$^+$	8.9 (1.7)	14.0 (3.2)	10.6 (2.6)*	4.13 $_{(2,56)}$
CD4$^+$pfn$^-$	787.4 (52.9)	839.1 (52.6)	826.7 (54.1)	1.92 $_{(2,56)}$
Total CD4$^+$CD8$^-$T cells[4]	846.3 (61.9)	899.6 (59.8)	889.2 (68.7)	.70 $_{(2, 34)}$
CD4$^+$CD8$^-$ pfn $^+$	7.6 (1.6)	11.6 (2.4)	9.3 (1.8) *	5.30 $_{(2, 34)}$
CD4$^+$CD8$^-$ pfn $^-$	838.7 (61.1)	888.0 (59.0)	879. 9 (68.2)	0.62 $_{(2, 34)}$
Total CD4$^+$CD8$^+$ T cells	8.5 (1.5)	10.1 (1.7)	8.6 (1.3) *	4.20 $_{(2, 32)}$
CD4$^+$CD8$^+$ pfn $^+$	0.6 (0.3)	1.3 (0.5)	0.8 (0.3) **	5.65 $_{(2, 32)}$
CD4$^+$CD8$^+$ pfn $^-$	7.9 (1.3)	8.9 (1.3)	7.8 (1.2)	2.71 $_{(2, 32)}$
Total CD8$^+$T cells	422.1 (43.9)	620.2 (75.9)	479.6 (44.3) ***	14.01 $_{(2, 38)}$
CD8$^+$pfn$^+$	75.9 (21.6)	170.0 (41.7)	85.5 (20.6) ***	13.38 $_{(2, 38)}$
CD8$^+$ pfn $^-$	346.2 (28.6)	450.3 (43.0)	394.1 (31.6) **	8.01 $_{(2, 38)}$
Total γδ T cells	53.0 (7.9)	76.2 (11.2)	62.0 (8.3) ***	17.29 $_{(2, 36)}$
γδ pfn $^+$	9.8 (2.3)	20.9 (4.4)	13.4 (2.8) ***	15.37 $_{(2, 36)}$
γδ pfn $^-$	43.2 (6.7)	55.3 (8.7)	48.6 (7.0) **	9.07 $_{(2, 36)}$

*** p<.001, **p<.01,*p<.01. (Repeated measures ANOVA comparing baseline, task and recovery)

[4] Analysis of CD4$^+$CD8$^-$ T cells was carried out in a smaller group of 18 participants, which had a higher mean total CD4$^+$ T cells.

Table 2. Mean (SEM) cell number in the psychological stress study during baseline, task and recovery comparing CD27$^+$ and CD27$^-$ subsets.

T cell subset (cells/μl)	Baseline	Task	Recovery	*F (df)*
CD4$^+$CD27$^+$	733.0 (49.8)	767.4 (48.6)	762.3 (52.9)	1.00 $_{(2, 54)}$
CD4$^+$CD27$^-$	64.0 (12.4)	90.1 (20.0)	73.8 (13.0) **	6.38 $_{(2, 56)}$
CD4$^+$ CD27$^+$ pfn$^+$	4.7 (0.8)	5.6 (1.0)	5. 1 (0.7)	0.53 $_{(2, 54)}$
CD4$^+$ CD27$^-$ pfn$^+$	2.4 (0.8)	4.7 (1.5)	2.6 (0.7) **	8.22 $_{(2, 54)}$
CD8$^+$CD27$^+$	308.1 (33.3)	331.3 (29.8)	321.2 (33.1)	0.85 $_{(2, 24)}$
CD8$^+$CD27$^-$	160.0 (57.1)	329.8 (108.3)	193.1 (63.8) **	9.04 $_{(2, 24)}$
CD8$^+$CD27$^+$ pfn$^+$	12.7 (2.1)	16.7 (2.6)	17.5 (5.1)	0.88 $_{(2, 24)}$
CD8$^+$CD27$^-$ pfn$^+$	89.7 (33.5)	197.6 (62.4)	97.4 (32.4) **	9.13 $_{(2, 24)}$
γδ CD27$^+$	25.5 (3.3)	29.9 (3.2)	28.1 (3.5)	2.83 $_{(2, 22)}$
γδ CD27$^-$	14.7 (2.6)	28.6 (5.5)	19.1 (3.3) ***	16.34 $_{(2, 24)}$
γδ CD27$^+$ pfn$^+$	2.3 (0.8)	3.4 (1.2)	3.0 (1.3)	1.85 $_{(2, 22)}$
γδ CD27$^-$ pfn$^+$	7.2(2.0)	16.9 (4.5)	9.7 (2.6) ***	11.17 $_{(2, 24)}$

*** p<.001, **p<.01. (Repeated measures ANOVA comparing baseline, task and recovery)

Table 3. Mean (SEM) cell number during infusion of the β-adrenergic agonist isoproterenol, without (‘placebo’) and with (‘propranolol’) prior administration of the β-adrenergic antagonist propranolol.

	Placebo		Propranolol		Placebo	Propranolol
Subset (cells/μl)	Baseline	Infusion	Baseline	Infusion	$F_{(df)}$	$F_{(df)}$
Total lymphocytes	1845.0 (124.5)	2270.3 (149.1) ***	1614.0 (130.4)	1661.4(100.4)	24.09 $_{(1,18)}$	2.06 $_{(1,6)}$
Total CD4$^+$T cells	831.1 (67.3)	708.3 (56.1) ***	712.2 (78.5)	721.9 (71.2)	20.80 $_{(1,18)}$	.24 $_{(1,6)}$
CD4$^+$ pfn$^+$	9.4 (1.3)	11.3 (1.6) *	10.0 (3.2)	10.1 (4.0)	6.10 $_{(1,17)}$	.01 $_{(1,5)}$
CD4$^+$ pfn$^-$	833.1 (69.9)	711.8 (56.9) ***	722.7 (87.8)	728.7 (79.4)	18.9 $_{(1,17)}$	.08 $_{(1,5)}$
Total CD4$^+$CD8$^-$T cells	807.7 (68.1)	685.3 (56.4) ***	700.9 (77.5)	710.4 (70.3)	22.93$_{(1,18)}$	.25 $_{(1,6)}$
CD4$^+$CD8$^-$ pfn$^+$	8.7 (1.3)	9.6 (1.4)	9.9 (3.4)	9.3 (3.8)	3.18 $_{(1,17)}$	.14 $_{(1,5)}$
CD4$^+$CD8$^-$ pfn $^-$	817.8 (69.0)	695.2 (55.8)***	711.3 (86.6)	717.8 (78.5)	20.82$_{(1,17)}$	.09 $_{(1,5)}$
Total CD4$^+$CD8$^+$ T cells	14.0 (2.3)	16.2 (2.6)	11.5 (2.5)	10.8 (2.8)	1.40 $_{(1,18)}$	0.26 $_{(1,6)}$
CD4$^+$CD8$^+$ pfn$^+$	0.8 (0.14)	2.0 (0.5)**	0.7 (0.2)	0.8 (0.2)	9.50 $_{(1,18)}$	0.75 $_{(1,6)}$
CD4$^+$CD8$^+$ pfn$^-$	13.2 (2.2)	14.2 (2.4)	10.9 (2.4)	10.0 (2.6)	0.38 $_{(1,18)}$	0.40 $_{(1,6)}$
Total CD8$^+$T cells	440.5 (42.1)	547.2 (60.2)**	385.7 (54.8)	395.9 (50.3)	10.27 $_{(1,18)}$	1.06 $_{(1,6)}$
CD8$^+$ pfn$^+$	48.0 (13.2)	127.7 (36.3)**	25.4 (7.3)	33.0 (8.0)*	11.69 $_{(1,18)}$	6.70 $_{(1,6)}$
CD8$^+$ pfn$^-$	392.5 (37.8)	419.6 (39.3)	360.3 (53.2)	362.9 (48.7)	2.32 $_{(1,18)}$	.08 $_{(1,6)}$
Total γδ T cells	43.4 (6.1)	58.6 (7.3) ***	48.7 (17.7)	50.5 (18.5)	51.80 $_{(1,18)}$	2.62 $_{(1,6)}$
γδ pfn$^+$	5.7 (1.2)	11.8 (1.9) ***	6.4 (2.2)	8.1 (2.8) *	45.63 $_{(1,18)}$	7.21 $_{((1,6)}$
γδ pfn$^-$	37.7 (5.2)	46.8 (6.0) ***	42.3 (15.7)	42.5 (15.9)	33.4 $_{(1,18)}$	.03 $_{(1,6)}$

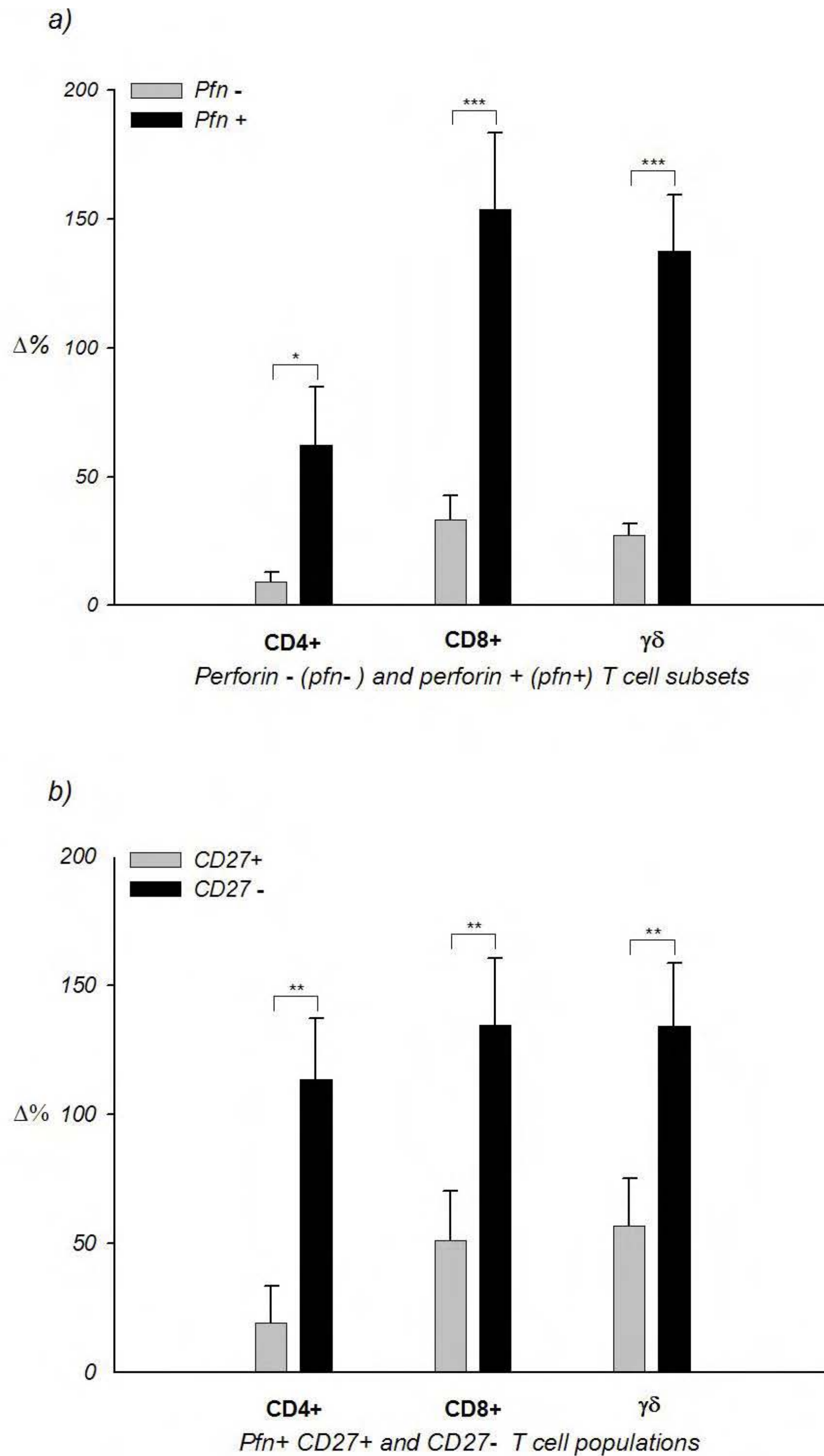
*** *p*<.001, ** *p*<.01,* *p*<.05 (Repeated measures ANOVA comparing Baseline with isoproterenol infusion, in the placebo and propranolol conditions.)

Table 4. Mean (SEM) cell number during infusion of the β-adrenergic agonist isoproterenol, without (‘placebo’) and with (‘propranolol’) prior administration of the β-adrenergic antagonist propranolol comparing $CD27^{+}$ and $CD27^{-}$ subsets.

	Placebo		Propranolol		Placebo	Propranolol
T cell subset (cells/µl)	Baseline	Infusion	Baseline	Infusion	$F_{(df)}$	$F_{(df)}$
$CD27^{+}CD4^{+}$	756.4 (67.0)	626.8 (54.4) ***	658.4 (74.8)	666.5 (68.7)	30.72 $_{(1,18)}$	.19 $_{(1,6)}$
$CD27^{-}$ $CD4^{+}$	75.2 (11.4)	82.1 (14.3)	54.1 (13.5)	55.8 (13.5)	1.64 $_{(1,18)}$	.61 $_{(1,6)}$
$CD27^{+}CD4^{+}$ pfn^{+}	8.5 (1.0)	7.8 (1.0)	11.6 (3.2)	11.4 (3.9)	1.47 $_{(1,18)}$	.01 $_{(1,6)}$
$CD27^{-}CD4^{+}$ pfn^{+}	1.5 (0.3)	3.6 (1.0)*	1.4 (0.4)	1.6 (0.5)	8.36 $_{(1,18)}$	1.61 $_{(1,6)}$
$CD27^{+}CD8^{+}$	348.3 (36.3)	359.5 (33.6)	316.1 (52.7)	320.9 (49.7)	1.24 $_{(1,17)}$	.28 $_{(1,6)}$
$CD27^{-}CD8^{+}$	84.0 (18.2)	186.5 (44.0) **	69.6 (20.8)	75.0 (22.9)	15.42 $_{(1,18)}$	3.46 $_{(1,6)}$
$CD27^{+}$ $CD8^{+}$ pfn^{+}	9.8 (1.5)	20.4 (3.2) ***	8. 9 (1.5)	10.4 (1.6)	26.80 $_{(1,18)}$	2.20 $_{(1,6)}$
$CD27^{-}CD8^{+}$ pfn^{+}	38.3 (12.8)	107.4 (34.8) **	16.5 (6.8)	22.6 (8.5)	9.74 $_{(1,18)}$	4.56 $_{(1,6)}$
$CD27^{+}\gamma\delta$	35.3 (5.1)	43.9 (6.1) ***	39.7 (16.2)	40.7 (16.5)	26.61 $_{(1,18)}$	1.36 $_{(1,6)}$
$CD27^{-}$ $\gamma\delta$	8.1 (1.8)	14.8 (2.4) ***	9.0 (3.2)	9.9 (3.5)	48.79 $_{(1,18)}$	2.29 $_{(1,6)}$
$CD27^{+}\gamma\delta$ pfn^{+}	3.0 (0.8)	4.8 (1.3) **	3.6 (1.7)	4.3 (2.0)	11.04 $_{(1,18)}$	4.42 $_{(1,6)}$
$CD27^{-}\gamma\delta$ pfn^{+}	2.7 (0.6)	6.7 (1.2) ***	2.7 (1.0)	3.7 (1.2) *	28.14 $_{(1,18)}$	8.27 $_{(1,6)}$

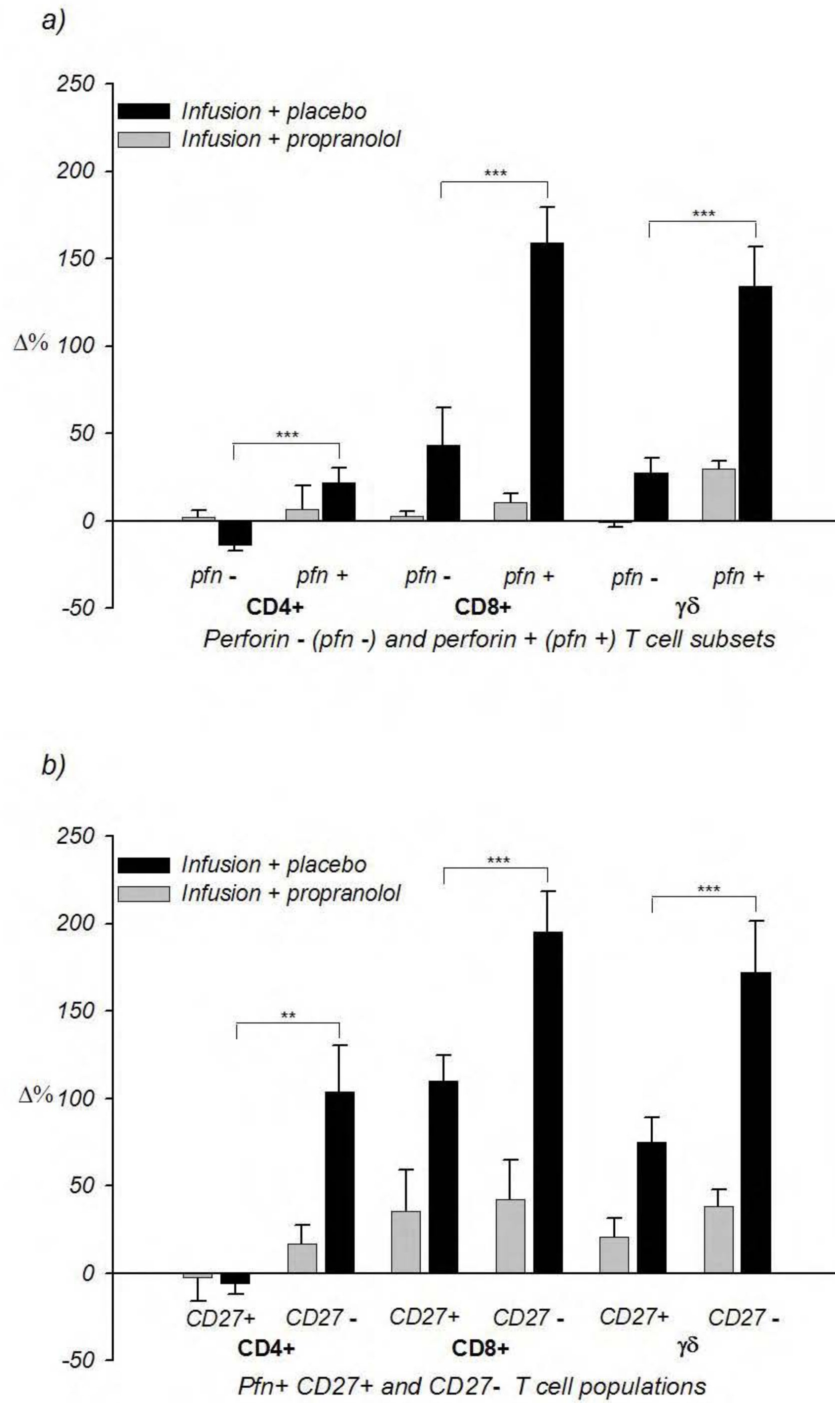
***p<.001, **p<.01, *p<.05 (Repeated measures ANOVA comparing Baseline with isoproterenol infusion in the placebo and propranolol conditions.)

Figure 1. Mean (SEM) change in cell number (Δ%) for (a) perforin (pfn)$^{+}$ and pfn^{-} T cell subsets, and (b) pfn^{+} CD27^{+} and CD27^{-}T cell populations during psychological stress.



***p<.001, **p<.01, *p<.05 (paired t-test analysis).

Figure 2. Mean (SEM) change in cell number (Δ%) for (a) perforin (pfn)$^+$ and pfn$^-$ T cell populations, and (b) pfn$^+$CD27$^+$ and CD27$^-$T cell populations during isoproterenol infusion.



***p<.001, **p<.01 (paired t-test analysis).

Discussion

The present study examined the hypothesis that specifically cytotoxic lymphocytes are mobilized during psychological stress and investigated the impact of differentiation status on T cell mobilization. Consistent with the hypothesis that cytotoxic cells are preferentially mobilized, only $CD4^{+}$T cells that expressed perforin were mobilized in response to psychological stress and β-infusion. We could exclude that these observations were explained by increases in $CD8^{+}$ T cells that co-express CD4, demonstrating that bona fide cytotoxic $CD4^{+}$ T cells are mobilization sensitive cells. The study also revealed that stress-induced increases in $CD8^{+}$ and γδ T cells were likewise largely driven by pfn^{+} cells, further confirming that stress enriches peripheral blood with cytotoxic lymphocytes. The effects observed in the isoproterenol infusion condition were almost entirely abrogated by the administration of a β-blocker (propranolol), strongly suggesting β-adrenergic mechanisms of mobilization.

The results in the above paragraph demonstrate that cytotoxic potential is indeed an important feature of mobilized cells and support the initial hypothesis that cytotoxicity is the key predictor of cells that become mobilized. Subsequent analyses shed new light on this hypothesis, however, revealing that a differentiated ($CD27^{-}$) phenotype, rather than cytotoxic potential (pfn^{+}), more consistently identified mobilized cells. Indeed, stress induced mobilization was most pronounced when pfn^{+} T cells were also $CD27^{-}$. Furthermore, $CD27^{-}$ T cells were mobilized even when they were pfn^{-}. There could be several explanations for the apparent superiority of CD27 in predicting mobilization. For

example, the less robust mobilization of $pfn^{+}CD27^{+}$ may reflect that these cells, despite being pfn^{+}, have not yet fully acquired a cytotoxic ability, as they are still missing other essential effector molecules such as granzymes (Heusel et al., 1994; Takata and Takiguchi, 2006). It could therefore be that loss of CD27 better predicts a cell's actual cytotoxic capability than perforin expression. In support of this, studies have shown that cytotoxicity increases alongside differentiation, thus less differentiated cells ($CD27^{+}$), have a ten times lower killing capacity than highly differentiated $CD27^{-}$ T cells despite expressing perforin and granzymes (at lower levels) (Romero et al., 2007). Finally, it became apparent that although a lytic ability is an important feature of mobilized cells, it does not exclusively identify cells recruited during stress as $pfn^{-}CD27^{-}$ T cells still become mobilized (data not shown), even though it is known that perforin expression is critical for the killing activity of cytotoxic T cells (Kagi et al., 1994; Takata and Takiguchi, 2006). The mobilization of pfn^{-} $CD27^{-}$ T cells may be explained by the fact that $CD27^{-}$ T cells also possess other effector functions in addition to perforin-mediated cytotoxicity, such as inhibition of viral infection by secretion of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). These abilities can be present in T cells with lower cytolytic abilities (Angelini. et al., 2004; Guidotti and Chisari, 1996; Hamann et al., 1997; Romero et al., 2007; Shresta et al., 1998). In addition, $CD27^{-}$ T cells are primed to migrate into the tissues, where antigen encounter typically occurs, whilst $CD27^{+}$ T cells tend to be lymph node homing (Appay et al., 2002; De Rosa et al., 2004; Hamann et al., 1997). Thus, it is likely that tissue migration is also an important characteristic of mobilized cells.

The mobilization of lymphocytes during stress is thought to be an adaptive mechanism aimed at enhancing immune protection in times of threat, where antigen exposure is more likely (Dhabhar, 2002). Indeed, stress may facilitate the migration of mobilized cells into sites of tissue damage or infection (Viswanathan and Dhabhar, 2005). We have shown here that stress mobilizes a range of pfn$^+$ T cell types, i.e. CD4$^+$, CD8$^+$ and $\gamma\delta^+$. This could increase the repertoire of antigens that could be recognized, hence maximizing immune responsiveness. For example, cytotoxic CD4$^+$T cells recognize MHC II, rather than MHC I, giving them the ability to recognize peptides that cannot be picked up by CD8$^+$T cells (Brown, 2010; van de Berg et al., 2008).

The finding that mobilized pfn$^+$ T cells are predominantly of a CD27$^-$ phenotype fits with an adaptive mechanism of mobilization. Cells lacking CD27 have a less stringent requirement for activation and, as such, these cells can rapidly initiate immune responses such as target killing. A short response time may be beneficial under conditions of fight or flight. We show here that pfn$^+$ CD27$^-$T cells are mobilized most robustly during acute stress. A cytotoxic T cell's primary mechanism of defense against viral infection and cancer is exerted predominantly through cytotoxic granules, including perforin (Grossman et al., 2004). The pfn$^+$CD27$^-$T cells likely play a role in the early prevention of pathogenic secondary infection by immediate lysis of target cells (Barber et al., 2003; Champagne et al., 2001; Hamann et al., 1997).

The mobilization of pfn$^+$ T cells may also have detrimental consequences, however, as perforin expression is thought to play a role in a number of autoimmune and inflammatory diseases, such as contact dermatitis (Yawalkar et al., 2001), asthma (Arnold

et al., 2000) and Rheumatoid Arthritis (van de Berg et al., 2008). If acute stress does indeed enhance migration of leukocytes into sites of inflammation, it is possible that recruitment of pfn^{+} T cells into sites of aberrant inflammation could exacerbate symptoms (Chen and Miller, 2007; Dhabhar et al., 2010; Kimata, 2003; Straub et al., 2005; Viswanathan and Dhabhar, 2005). Future research into the clinical consequences of stress induced effector cell mobilization is warranted.

A marked difference between the effects in the stress and infusion studies is that the latter shows a decrease in total $CD4^{+}$ T cells, which replicates earlier findings (Goebel et al., 2000; Mills et al., 2000; Mills et al., 1997). We note that despite the decrease in $CD4^{+}$ T cells, $pfn^{+}CD4^{+}CD8^{-}$ T cells showed a slight increase. The mechanism for the decrease in $CD4^{+}$ T cells during infusion remains elusive at this point, however it is clear that this effect, and the slight increase in $pfn^{+}CD4^{+}CD8^{-}$ T cells, was most probably β-adrenergically mediated.

Indeed, the present study demonstrated that all T cell subset mobilization was likely to have been mediated by β-adrenergic mechanisms. Recent work by Dimitrov and colleagues suggest this mobilization is caused by epinephrine induced detachment of cytotoxic cells from endothelium (Dimitrov et al., 2010). Further elucidation of the molecular pathways involved in this phenomenon is an important research target. In addition, a number of studies have shown that acute stress leads to an increase in in-vitro NK cell cytotoxicity (Benschop et al., 1996; Dopp et al., 2000; Schedlowski et al., 1993), although this may be due to a stress induced change in the proportion of cells with cytolytic ability. Future studies should utilize functional assays, e.g. cytotoxicity assays,

and cell sorting methods to purify T cell subsets and determine whether the increase in T cells with cytolytic potential actually translates into increased cytotoxic function. As the present infusion study implicates β-adrenergic mechanisms, studies may also further explore the effects of beta-adrenergic receptor stimulation on cytotoxicity in functional assays.

In summary, this study shed new light on the hypothesis that exclusively cytotoxic T cells are mobilized; although cytotoxic potential (pfn^{+}) was important, differentiation status ($CD27^{-}$) more consistently identified mobilization sensitive cells. Used in combination, a $pfn^{+}CD27^{-}$ T cell phenotype always identified cells that showed the largest mobilization. The current report provides new insight that may help understand the exact functions of stress-induced T cell mobilization.

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CHAPTER FIVE

MOBILIZATION OF $CD16^{+}$ MONOCYTES IN RESPONSE TO PSYCHOLOGICAL STRESS AND ISOPROTERENOL INFUSION

Abstract

Monocytes are a heterogeneous population of innate immune cells that can be divided into several functionally distinct subsets. Although monocytes have been shown to be mobilized in response to acute psychological stress, little is known about the relative sensitivity of these subsets. The current study investigated the effects of acute psychological stress (speech task) and pharmacological β-adrenergic stimulation (isoproterenol infusion) on individual monocyte subsets including; $CD14^{++}CD16^{-}$; $CD14^{++}CD16^{+}$; and $CD14^{+}CD16^{+}$ monocytes. In the speech task, total monocytes were further analysed on the expression of adhesion molecules CD11a, CD11b, CD49d, CD62L and CD162. Flow cytometric analysis was used to examine monocyte subsets. All monocyte subsets increased during the speech task and, as expected, there were significant differences in the sensitivity of monocyte subsets to stress; $CD14^{+}CD16^{+}$ monocytes showed the greatest increase, followed by $CD14^{++}CD16^{+}$ cells, and finally $CD14^{++}CD16^{-}$ monocytes. Adhesion molecule analysis revealed an increase in cells expressing CD49d and a decrease in those expressing CD62L. In addition, $CD14^{+}CD16^{+}$ monocytes were selectively mobilized by isoproterenol infusion, which was abrogated by administration of

a β-adrenergic receptor antagonist (propranolol), suggesting β- adrenergic mechanisms of mobilization. The current findings indicate that acute psychological stress and isoproterenol infusion primarily mobilize the pro-inflammatory $CD14^{+}CD16^{+}$ monocytes. These findings are consistent with the theory that stress selectively recruits cells with a high tissue migrating and inflammatory potential, which may provide rapid protection in contexts of increased risk of infection and tissue damage.

Introduction

Mammals possess the remarkable capacity to rapidly change the composition and numbers of leukocytes in the blood and tissues in response to acute psychological stress (Benschop et al., 1996; Segerstrom and Miller, 2004). Human studies have characterized this phenomenon *in extenso* for peripheral blood lymphocytes, showing that acute stress robustly increases the numbers of cytotoxic lymphocytes (i.e. NK cells, CD8 T cells, γδ T cells) via beta (β)-adrenergic mechanisms (Anane et al., 2009; Anane et al. 2010; Atanackovic et al., 2006; Benschop et al., 1996; Bosch et al., 2005; Campbell et al., 2009; Dimitrov et al., 2010; Mills et al., 2003; Riddell et al., 2009; Schedlowski et al., 1996; Segerstrom and Miller, 2004). These mobilized cytotoxic cells also display a strong tissue migrating potential, as evidenced by their adhesion molecule expression profile (e.g. $CD62L^{lo}$, $CD11a^{hi}$) (Anane et al., 2010; Bosch et al., 2003; Bosch et al., 2005; Goebel and Mills, 2000; Mills et al., 2003; Mills et al., 2000a). This observation is consistent with the idea that stress selectively mobilizes lymphocytes capable of rapid migration into peripheral tissues, presumably to enhance immune preparedness (Dhabhar, 2002).

Monocyte numbers also increase during acute stress, although this response is not as well characterized as acute-stress induced lymphocytosis (Bosch et al., 2003; Goebel and Mills, 2000; Mills et al., 2003). Monocytes belong to the innate arm of the immune system that recognize microorganisms and dying cells through expression of various pattern recognition receptors (Auffray et al., 2009; Serbina et al., 2008), and are the precursors of tissue macrophages. It is well recognized that circulating monocytes are a

heterogeneous population of cells that can be divided into distinct subsets, which differ in their functional and migratory ability (Passlick et al., 1989). The $CD14^{++}CD16^{-}$ subset, also known as 'classical monocytes', comprise the majority (80-90%) of monocytes. They express high phagocytic and myeloperoxidase activity, but a lower ability to produce inflammatory cytokines such as IL1-β and TNF-alpha(α) (Auffray et al., 2009). In contrast, $CD16^{+}$ monocytes readily produce these inflammatory cytokines, and are therefore sometimes dubbed 'pro-inflammatory'. Indeed, these cells are one of the major producers of TNF-α in human blood (Belge et al., 2002). Another characteristic of the $CD16^{+}$ subset is the expression of adhesion molecules that promote tissue migration, such as CD11a and VLA-4 (CD49d/CD29) (Ancuta et al., 2003; Steppich et al., 2000; Ziegler-Heitbrock, 1996). More recent work demonstrated that these $CD16^{+}$ monocytes could be further divided into a $CD14^{+}CD16^{+}$ population and a smaller $CD14^{++}CD16^{+}$ subset (Ancuta et al., 2003; Crowe and Ziegler-Heitbrock, 2010; Skrzeczyńska-Moncznik et al., 2008). The latter cells were recently shown to produce the highest amounts of IL-10 of all monocyte subsets, suggesting a possible anti-inflammatory role for this subset, whilst in response to LPS the $CD14^{+}CD16^{+}$ subset produces the greatest levels of TNF-α, suggesting a pro-inflammatory role for this population (Skrzeczyńska-Moncznik et al., 2008).

Studies have found that pro-inflammatory monocytes become mobilized during exercise (Gabriel et al., 1994; Hong and Mills, 2008; Simpson et al., 2009; Steppich et al., 2000) and infusion of adrenaline (Dimitrov et al., 2010), whilst classical monocytes remain largely unchanged. It is likely that this mobilization is mediated by adrenergic

mechanisms (Dimitrov et al., 2010; Steppich et al., 2000). How these monocyte subsets respond to acute psychological stress, however, is yet to be examined. In addition, it has also been shown that adrenergic stimulation and stress are associated with changes in adhesion molecule expression largely consistent with a mobilization of inflammatory monocytes (Dimitrov et al., 2010; Greeson et al., 2009; Nielsen and Lyberg, 2004), however CD11a expression analysis on total monocytes does not appear to follow this pattern (Goebel and Mills, 2000; Mills et al., 2003; Mills et al., 2002; Mills et al., 2000a; Mills et al., 2000b; Redwine et al., 2003; Steppich et al., 2000).

The aims of the current study were, therefore, to investigate the mobilization of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{+}$ monocytes in response to acute psychological stress and infusion of the β-adrenergic receptor agonist isoproterenol. In conjunction the expression of adhesion molecules CD11a, CD11b, CD49d, CD62L and CD162 was explored on total monocytes during psychological stress.

Methods

Participants

The stress study involved 61 university undergraduates from the Ohio State University (mean age = 19.9 (*SD* = 2.01) years, 29 women), 21 of which (Mean age = 20.5 (*SD* = 1.4) years, 10 women) were randomly allocated to a no-stress control condition where they read magazines. In the isoproterenol infusion study, 19 participants (mean age

= 36.3 (*SD* = 9.4) years, 7 women) were recruited from the local community and students and staff from the University of California San Diego. Of these, six participants (mean age = 38.0 (*SD* = 4.6) years, 1 woman) repeated the experiment after treatment with the β-adrenergic blocking agent propranolol (see procedures below for details). All participants reported to be in good health and were non-medicated with the exception of the contraceptive pill. For both studies, 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. Volunteers received monetary compensation for their participation. Participants provided informed consent and study protocols were approved by the Ohio State University, and University of California San Diego Institutional review boards.

Stress study

Procedure

On arrival at 12pm 1) Informed consent was obtained, 2) a 19-gauge indwelling catheter was placed in the antecubital vein of the non-dominant arm, 3) participants were served a standardised lunch (350kcal; 9g protein, 50g carbohydrate, 16g fat) and water ad libitum, and 4) electrodes for electrocardiography were attached. Following this, participants filled out questionnaires and then engaged in leisurely reading. At 1.30 pm a baseline blood sample was obtained and the stress task was initiated.

The stress task involved participants delivering two consecutive speeches, each with two minutes preparation and four minutes of delivery (Bosch et al., 2003). Speech tasks were videotaped and performed in front of an audience to enhance social stress (Bosch et al., 2009). In order to standardise instruction and timing task instructions were presented on a computer screen. The task has been well validated and induces robust physiological activation and negative mood increase (Bosch et al., 2009; Bosch et al., 2007; Mills et al., 2009; Redwine et al., 2004). A second ('task') blood sample was obtained during the final two minutes of the speech task. In the control condition, participants were asked to remain quietly reading following the baseline blood draw.

Questionnaires

Affective responses were assessed with Profile of Mood States (POMS) (McNair et al., 1992), which was completed at baseline and immediately post-task. Instructions for the post-task questionnaire were adapted to reflect how the participants felt during the task. Health and lifestyle information was also obtained by self-report questionnaire.

Catecholamines

Blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes, kept on ice, and spun down within 20 minutes after collection and plasma was stored at −80°C. Plasma catecholamine concentrations were determined by HPLC (ESA, Inc., Chelsford,

MA) using standards and chemicals purchased from ChromSystems (Thermo-Alko, Beverly, MA). Samples from the same participant were analyzed in a single run. Intraassay variation for norepinephrine was 3% (interassay variation 6%). Intraassay variation for epinephrine was 6% (interassay variation 13%). Sensitivity for norepinephrine and epinephrine was 15 pg/mL and 6 pg/mL, respectively.

Cardiovascular assessment

To assess autonomic nervous system activation, cardiovascular activity was recorded throughout the experiment. Indices of sympathetic and parasympathetic drive were obtained by analysis of electrocardiogram (ECG) and thoracic impedance (ICG) signals (Berntson et al., 1997; Sherwood et al., 1990). The ICG and ECG signals were recorded from six Ag/AgCl spot-electrodes (Conmed corporation, UTICA, NY, USA) using a VU-AMD device (Vrije Universiteit, Amsterdam, Holland). Reliability and validity of the VU-AMD device have been reported elsewhere (de Geus et al., 1995; De Geus and van Doornen, 1996; Willemsen et al., 1996). The ICG complexes were ensemble averaged with reference to the ECG R-wave across 1-minute periods. From these 1-minute ensembles, averages were computed for heart rate (HR), and the pre-ejection period (PEP). These minute-by-minute means were averaged over the last 6-minutes of baseline, each 6-minute speech, and the last 6-minutes of recovery.

Interbeat intervals (IBI) were checked and edited for artifacts by a detection algorithm developed by Bernston and colleagues (Bernston et al., 1990). Respiratory sinus

arrhythmia (RSA) was derived by the method of Porges (Porges and Bohrer, 1990), using the MXedit program (Delta Biometrics, Bethesda MD). This program converts the heart period series into a time series, applies a moving polynomial filter (polynomial=3, COEFFICIENTS=21) to remove slow non-stationarities in the data, applies a band-pass filter (.12–.40 Hz) to the residual series, and then derives the natural log of the band variance. This corresponds to the statistical variance of the time sampled heart period data within the respiratory frequency band.

RSA was used to index changes in vagal tone, whereas changes in PEP were used to index changes in cardiac sympathetic drive (Sherwood et al., 1990).

Infusion study

Procedure

Isoproterenol infusion was performed according to a standardized protocol described elsewhere (Goebel et al., 2000; Mills et al., 2002; Mills et al., 2000a; Mills et al., 1997). In brief, upon arrival at the laboratory height and weight measurement were taken and body surface area (BSA) was used to calculate the isoproterenol infusion rate. Following placement of ECG electrodes and two i.v. 22-gauge catheters, into an antecubital vein of each arm, participants lay supine for 15 min. Following this, the baseline blood sample was collected through one i.v. catheter, followed by isoproterenol infusion through the other for 15 minutes at a rate of 1µg/min/1.73 m^2 BSA. A blood sample was again acquired in the final minutes of isoproterenol infusion. The infusion is

generally well-tolerated and all participants successfully completed the protocol. The half-life of isoproterenol in the body is approximately 2 minutes (Goebel et al., 2000). For safety purposes, ECG was monitored throughout the infusion.

β-blockade

A group of six participants performed the isoproterenol infusion procedure twice; 1) following five consecutive days of the oral administration of 80mg of the β-adrenergic receptor antagonist propranolol (propranolol condition) and, 2) following 5 days of the administration of a placebo (placebo condition). Drug administrations were single-blinded and order was counter balanced across participants.

Flow cytometry

Blood was collected in EDTA vacutainer tubes (BD, BD Biosciences), kept at room temperature and prepared within 2 hours of collection. Monocyte subsets were identified by immunofluorescent antibody staining of whole blood using multi-colour flow cytometry (FACS-Calibur, FACS-Canto II, BD Biosciences). The antibodies used were CD11a PE, CD11b PE, , CD16 PE, CD49d PE, CD62L PE, CD162 PE (Parmingen, Oxford, UK) and CD14 FITC (Beckman Coulter, UK). Matched isotype controls were used to set negative staining criteria, IgG1 PE, IgG2b FITC. Briefly, 50μl whole blood samples were incubated with the appropriate amounts of antibody (as determined by titration) for 30 minutes in the dark at room temperature (RT), and erythrocytes were

subsequently lysed using FACS Lysing Solution (Becton Dickinson). Following lysis, samples were centrifuged (217 x g, 6 minutes, at RT) and fixed in 1% paraformaldehyde/PBS without Ca^{2+} and Mg^{2+} (w/v). All fixed cell preparations were stored in the dark at 4°C and read within 24 hours. Data was analysed using FlowJo software v7.5 (Tree Star, Inc., Ashland, OR). Monocyte numbers were determined using a Coulter GEN-S haematology analyzer (Beckman Coulter, Miami, FL).

Gating procedure

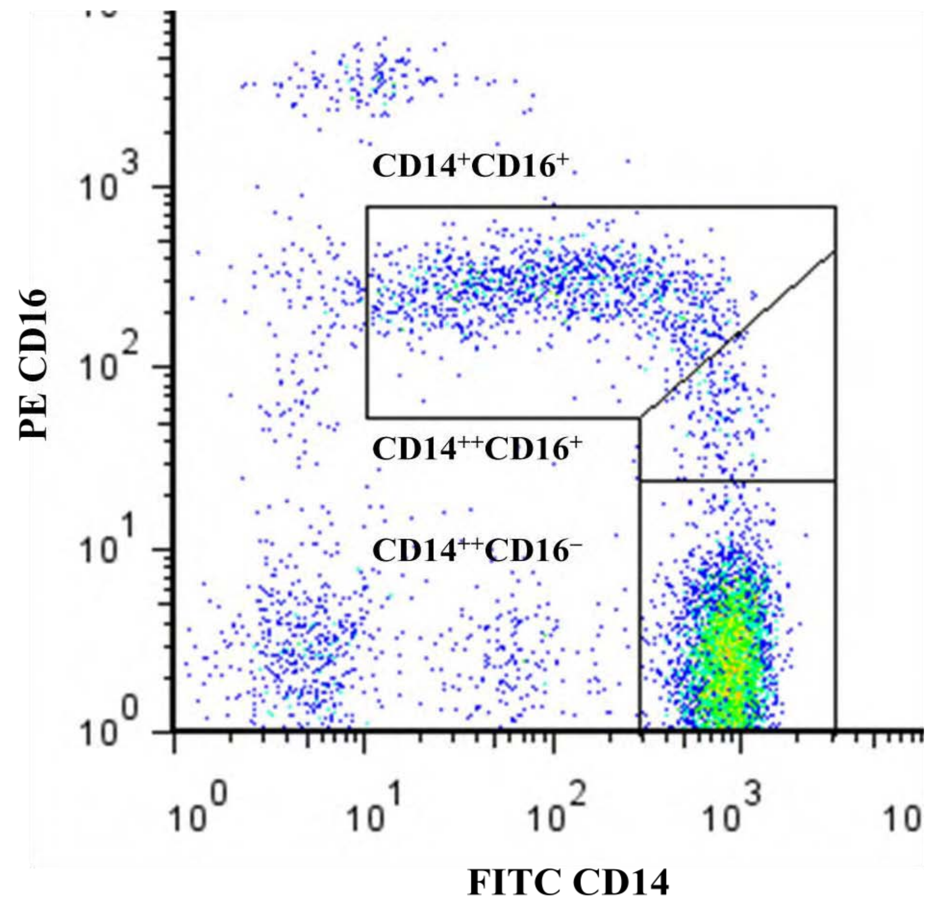
Total monocytes were identified using forward versus side scatter and, as a portion of $CD16^{+}$ monocytes are found within the lymphocyte gate, a small portion of lymphocytes were also included in the monocyte gate, as recommended (www.monocytes.de). Isotype controls were used to set negative staining criteria and monocyte subsets were identified using the markers CD14 and CD16. Three phenotypes can be identified (see Figure 1); $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$ (www.monocytes.de) (Crowe and Ziegler-Heitbrock, 2010; Skrzeczyńska-Moncznik et al., 2008). The Median Fluorescence Intensity (MFI) was computed for CD11a, CD11b, CD49d, CD62L and CD162 on total monocytes.

Data analysis

The effects of the manipulations on immune, endocrine, cardiovascular and psychological measures were assessed using repeated-measures analysis of variance (ANOVA). Paired t-tests were used for further post-hoc analyses. Associations between cardiovascular, endocrine and immune cell responses were explored using Pearson's

correlation coefficients. Where means are presented, standard deviation is given in brackets. Data was analyzed using SPSS 16 (SPSS, Chicago, IL). Occasional data was missing, and degrees of freedom were adjusted accordingly.

Figure 1. Gating strategy used to identify monocyte populations.



Results

Stress study

<u>Affective and physiological responses</u>

Analysis of the POMS tension anxiety subscale confirmed that the speech tasks were perceived as stressful, whilst during the control condition there was no significant change. As shown in Table 1, and confirming sympathetic activity, the speech task, but not the control, led to increases in heart rate (HR), decreases in pre-ejection period (PEP) and increases in epinephrine and norepinephrine. Further, the speech task, but not the control, led to a significant decrease in Respiratory Sinus Arrhythmia (RSA), indicating vagal withdrawal.

<u>Immune parameters</u>

Monocyte subsets

As shown in Table 2, total monocyte numbers increased in response to the stress task, but not the control condition. All three monocyte populations; $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{+}$, were mobilized during the stress task. However, as shown in Figure 2a, $CD14^{+}CD16^{+}$ monocytes showed the greatest increase (Δ%), followed by $CD14^{++}CD16^{+}$ cells, whereas $CD14^{++}CD16^{-}$ monocytes showed the smallest relative increase.

In order to confirm that the observed increased in monocyte subsets was not driven by contaminating NK cells, the analysis was repeated selecting only HLA-DR positive

monocytes (Ziegler-Heitbrock, 2000). These analyses yielded virtually identical results (data not shown).

Within the stress group, there were significant correlations between change in cardiac sympathetic drive (PEP) and the percentage change in total monocytes ($r = -.28$, $p<.05$), $CD14^{++}CD16^{-}$ monocytes ($r = -.28$, $p<.05$), and $CD14^{++}CD16^{+}$ monocytes ($r = -.41$, $p<.01$). No other significant correlations were observed. This provides some evidence of sympathetic involvement in stress-induced monocyte mobilization.

Monocyte adhesion molecule expression

As can be seen in Table 3, CD49d MFI increased in response to the stress task, while CD62L MFI decreased. No effects were observed for CD11b, CD11a or CD162. There were no significant changes in the control condition.

Infusion study

Immune parameters

As can be seen in Table 4 and Figure 2b, total monocytes did not change significantly in response to isoproterenol infusion. However, there was a robust increase in $CD14^{+}CD16^{+}$ monocytes and a trend towards mobilization of $CD14^{++}CD16^{+}$ monocytes, whereas no change was seen in $CD14^{++}CD16^{-}$ monocytes. Administration of a β-adrenergic receptor blocker (propranolol) entirely abrogated any isoproterenol induced increases.

Table 1. Mean (SEM) changes in affective, endocrine and cardiovascular parameters during baseline and task in the speech and control conditions.

Parameter	Condition	Baseline	Task	*F (df)*
POMS	Speech	3.1 (0.4)	8.9 (0.8)	$55.66_{(1, 37)}$ ***
	Control	3.4 (0.9)	2.0 (0.5)	$2.12_{(1,17)}$
HR	Speech	74.3 (1.8)	89.1 (2.5)	$110.0_{(1, 37)}$ ***
(beats/min)	Control	73.8 (2.1)	74.8 (2.2)	$2.17_{(1,19)}$
PEP	Speech	104.2 (3.6)	93.9 (3.1)	$28.09_{(1,37)}$ ***
(ms)	Control	111.4 (5.0)	110.8 (4.8)	$0.63_{(1,19)}$
RSA	Speech	6.9 (0.2)	6.4 (0.2)	$13.06_{(1, 35)}$ **
(ms)	Control	6.8 (0.2)	6.7 (0.2)	$1.03_{(1, 13)}$
Epinephrine	Speech	20.9 (1.8)	46.7 (4.0)	$60.31_{(1,39)}$ ***
(pg/ml)	Control	21.3 (2.7)	23.3 (3.3)	$2.10_{(1,20)}$
Norepinephrine	Speech	306.1 (15.7)	448.9 (22.3)	$70.86_{(1,39)}$ ***
(pg/ml)	Control	325.3 (33.2)	335.0 (20.9)	$0.15_{(1,20)}$

**p<.01, ***p<.001 (repeated measures ANOVA)

Table 2. Mean (SEM) cell number (cells/μl) and results of repeated measures ANOVA for total monocytes and subsets during baseline and task in the speech and control conditions.

Monocytes (cells/μl)	Condition	Baseline	Task	*F (df)*
Total	Speech	442.5 (22.3)	540.0 (29.9)	$40.11_{(1, 39)}$ ***
	Control	400.0 (30.0)	423.8 (27.5)	$3.05_{(1,20)}$
$CD14^{++}CD16^{-}$	Speech	369.6 (19.3)	431.9 (24.2)	$31.54_{(1, 39)}$ ***
	Control	349.9 (30.0)	368.9 (28.0)	$2.46_{(1, 18)}$
$CD14^{++}CD16^{+}$	Speech	13.7 (1.2)	17.7 (1.4)	$29.08_{(1, 39)}$ ***
	Control	17.8 (2.2.)	18.5 (2.1)	$0.47_{(1, 18)}$
$CD14^{+}CD16^{+}$	Speech	59.2 (6.0)	90.4 (9.7)	$27.27_{(1, 39)}$ ***
	Control	48.0 (5.5.)	49.5 (5.7)	$0.52_{(1, 18)}$

***p<.001 (repeated measures ANOVA)

Table 3. Mean (SEM) Median Fluorescence Intensity (MFI) and results of repeated measures ANOVA for adhesion molecules expressed on total monocytes during baseline and task in the speech and control conditions.

Adhesion molecule	Condition	Baseline	Task	*F (df)*
CD49d	Speech	111.4 (5.3)	115.2 (4.9)	$11.33_{(1, 39)}$ **
	Control	114.2 (7.3)	114.7 (7.4)	$0.04_{(1, 20)}$
CD11a	Speech	992.3 (24.6)	991.6 (22.2)	$0.01_{(1, 37}$
	Control	1026.7 (43.6)	1021.9 (41.3)	$0.09_{(1, 19)}$
CD11b	Speech	609.7 (39.5)	585.1 (31.9)	$0.67_{(1, 39)}$
	Control	692.9 (72.8)	687.7 (61.5)	$0.03_{(1, 20)}$
CD162	Speech	269.5 (13.8)	279.6 (22.1)	$0.30_{(1, 39)}$
	Control	294.4 (47.6)	288.1 (48.0)	$2.70_{(1, 20)}$
CD62L	Speech	284.3 (26.2)	260.5 (22.0)	$5.40_{(1, 39)}$ *
	Control	253.3 (31.8)	257.5 (34.2)	$0.14_{(1, 19)}$

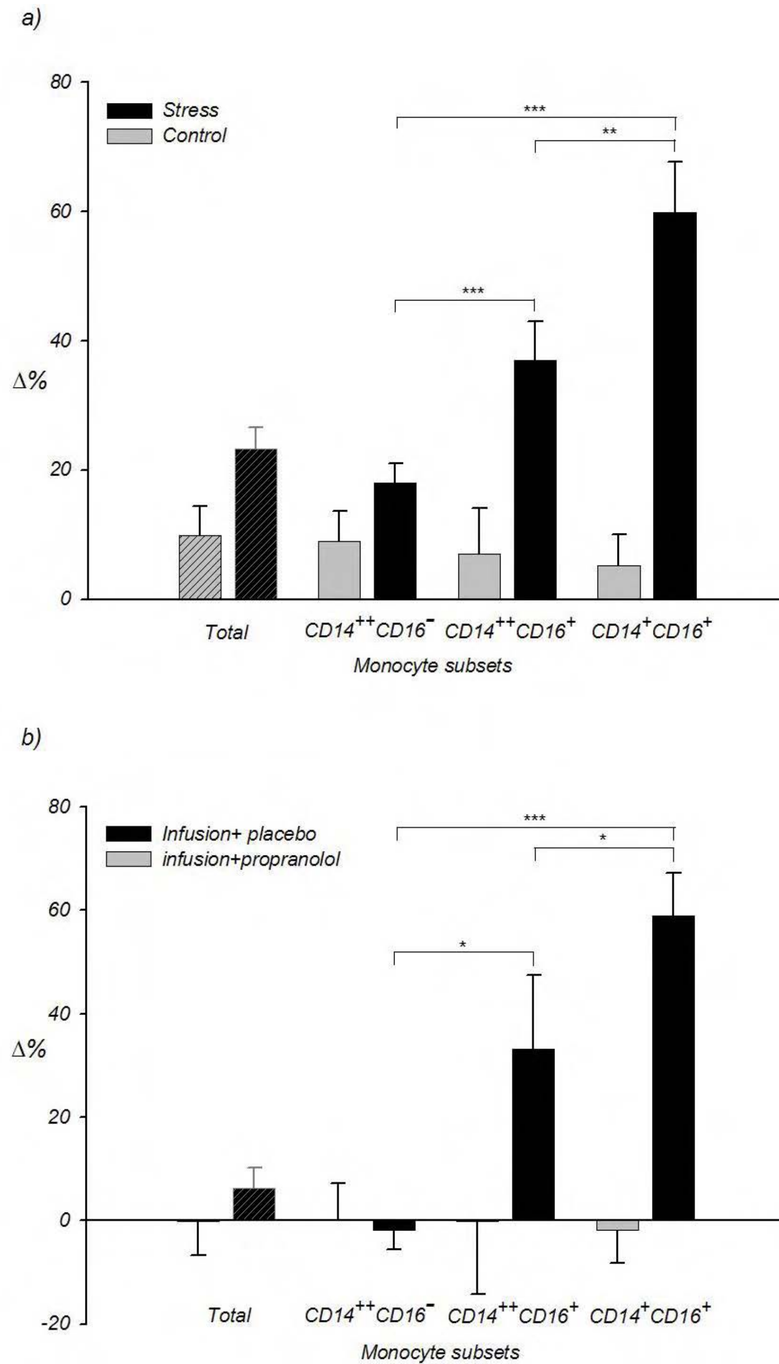
* p<.05, **p<.01 (repeated measures ANOVA)

Table 4. Mean (SEM) cell number (cells/μl) for total monocytes and subsets during baseline and isoproterenol infusion in the placebo and propranolol conditions.

Monocytes (cells/μl)	Condition	Baseline	Isoproterenol infusion	*F (df)*
Total	Placebo	479.6 (30.8)	502.4 (34.3)	$1.22_{(1, 17)}$
	Propranolol	458.3 (55.3)	447.5 (40.4)	$0.15_{(1, 5)}$
$CD14^{++}CD16^{-}$	Placebo	410.7 (28.7)	397.5 (28.8)	$0.64_{(1, 17)}$
	Propranolol	400.3 (53.1)	390.1 (38.0)	$0.15_{(1, 5)}$
$CD14^{++}CD16^{+}$	Placebo	8.7 (1.0)	11.9 (2.2)	$3.92_{(1, 17)}$≠
	Propranolol	7.0 (1.4)	7.2 (1.9)	$0.07_{(1, 5)}$
$CD14^{+}CD16^{+}$	Placebo	60.2 (6.0)	92.9 (9.0)	$47.28_{(1, 17)}$ ***
	Propranolol	51.1 (6.9)	50.2 (7.7)	$0.07_{(1, 5)}$

*** p<.001, ≠ = p < .07 (Repeated measures ANOVA)

Figure 2. Mean (SEM) change in cell number (Δ%) for total monocytes and monocyte subsets in a) the stress and control conditions and b) the placebo and propranolol isoproterenol infusion conditions.



* indicates p<.05, ** p<.01, ***p<.001 (Paired t-test analysis).

Discussion

The present study examined the mobilization of three monocyte subsets, which differ in inflammatory and tissue migrating potential, during acute psychological stress and β-adrenergic agonist (isoproterenol) infusion. The results showed that whilst all monocyte subsets increased during stress, the two CD16 expressing 'pro-inflammatory' monocyte populations, i.e. $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{+}$, showed a substantially larger increase than the 'classical' $CD14^{++}CD16^{-}$ monocytes. These stress effects were almost exactly replicated by infusion of isoproterenol. Further, this effect was abrogated by administration of the β-blocking agent propranolol, confirming β-adrenergic involvement in monocyte mobilization (Dimitrov et al., 2010; Steppich et al., 2000).

The pattern of responses, whereby $CD14^{+}CD16^{+}$ monocytes showed the largest relative increase, and $CD14^{++}CD16^{-}$ the smallest, closely mirrors their potential to produce the pro-inflammatory cytokine TNF-α. It has been demonstrated that $CD14^{+}CD16^{+}$ monocytes produce 90% of the TNF-α secreted by peripheral blood monocytes (Belge et al., 2002). These inflammatory cells also express a strong tissue migrating profile (Ancuta et al., 2003; Steppich et al., 2000; Ziegler-Heitbrock, 1996). Acute stress and isoproterenol infusion, therefore selectively recruit monocytes with a pro-inflammatory and tissue migrating signature, which extends prior observations of responses to exercise and infusion (Dimitrov et al., 2010; Gabriel et al., 1994; Hong and Mills, 2008; Simpson et al., 2009; Steppich et al., 2000). In mice, $CD16^{+}$ monocytes exhibit a 'patrolling behavior' around blood vessels, and are the first to locate to sites of infection, secreting TNF within

1 hour of infection (Auffray et al., 2007). The mobilization of $CD16^+$ monocytes could therefore comprise part of an adaptive mechanism designed to maximize immune defense (Anane et al., 2009; Dhabhar, 2002), by facilitating migration into sites of tissue damage and infection. Indeed, animal studies have shown that stress-induced leukocyte redistribution is associated with an enhanced immune defense (Dhabhar and McEwen, 1996; Dhabhar and Viswanathan, 2005; Viswanathan and Dhabhar, 2005). The recruitment of effector-type cells to the peripheral circulation during stress appears to be conserved across other leukocyte subtypes. For example, within cytotoxic lymphocytes, subsets with a strong effector and tissue migrating potential, such as effector-memory CD8 T cells and $CD56^{dim}$ NK cells, are selectively mobilized during stress (Anane et al., 2009; Anane et al., 2010; Atanackovic et al., 2006; Bosch et al., 2003; Bosch et al., 2005; Riddell et al., 2009). Thus, acute stress primes both the innate and adaptive arms of the immune system for enhanced immunosurveillance.

The selective mobilization of pro-inflammatory monocytes could, however, be detrimental in inflammatory diseases, such as atherosclerosis. Monocytes play an integral role in the pathogenesis of this disease (Gautier et al., 2009) and $CD16^+$ monocytes are found in increased numbers in the circulation of patients with atherosclerosis (Ziegler-Heitbrock, 2007). It has been shown that $CD16^+$ monocytes adhere avidly to activated endothelial cells (Dimitrov et al., 2010) and it is possible that stress induced $CD16^+$ monocyte mobilization could enhance the migration of these cells into the sub-endothelia, exacerbating this disease. In the present study the administration of a β-blocker abrogated this mobilization, and it is known that β-blockers have anti-atherosclerotic properties

(Donetti et al., 1998; Feuerstein and Ruffolo, 1996; Kopecky, 2006). Elevated sympathetic activation is common to most cardiovascular diseases and is a feature shared by risk factors for cardiovascular disease, such as hypertension, diabetes, and autoimmune disease (Fisher et al., 2009). Preventing the recruitment and surveillance of inflammatory monocytes under conditions of elevated sympathetic drive may present an additional mechanism explaining the anti-atherosclerotic effects of β-blockers. The mobilization of $CD16^+$ monocytes during stress may also impact other inflammatory diseases, such as rheumatoid arthritis (Straub et al., 2005; Ziegler-Heitbrock, 2007). Thus, elucidation of the clinical consequences of monocyte mobilization appears warranted.

The findings presented here may provide an explanation for prior observations that stress acutely alters functional parameters, such as cytokine production and adhesion molecule expression. For example, the changes in monocyte adhesion molecule expression seen here and by others (Greeson et al., 2009) likely reflect the selective increase in $CD16^+$ monocytes, which are $CD49d^{high}$ and $CD62L^{low}$ (Ancuta et al., 2003; Steppich et al., 2000; Ziegler-Heitbrock, 1996), and thereby affect the 'averaged' adhesion molecule expression of the entire monocyte population. In a similar vein, prior studies have reported that stress, as well as exercise, rapidly alter in vitro stimulated inflammatory cytokine production (Ackerman et al., 1998; Goebel et al., 2000; Maes et al., 1998), typically in response to LPS. This may be explained by a change in the cellular composition of the blood (i.e., more pro-inflammatory $CD16^+$ monocytes in the blood), rather than a change in functionality of cells. Although convincing, more direct evidence is required to confirm this hypothesis.

A possible limitation of the present study is the lack of functional assays that could determine whether the mobilization of the inflammatory $CD16^+$ subset indeed predisposes to elevated inflammatory activity. For example, it should be directly tested whether stress induced mobilization of $CD16^+$ monocytes subsets leads to increased production of proinflamatory cytokines, such as TNF-α and IL-6 (Goebel et al., 2000). Future studies should further examine which mechanisms govern the release of $CD16^+$monocytes into peripheral blood, and identify the specific cellular and molecular pathways involved. For example, it is known that Natural Killer (NK) cell mobilization is mediated by the action of catecholamines on β-adrenergic receptors expressed by NK cells, which stimulates their detachment from endothelium (Benschop et al., 1993; Schedlowski et al., 1996). It is possible that similar mechanisms are in operation in $CD16^+$ monocytes, although initial investigation into monocyte detachment from endothelium by Dimitrov and colleagues could not confirm this mechanism (Dimitrov et al., 2010).

In summary, the present report demonstrates that $CD16^+$ monocytes are selectively mobilized in response to acute psychological stress and infusion of the β-adrenergic receptor agonist isoproterenol. These findings are consistent with the idea that autonomic activation selectively recruits cells with a high tissue migrating and inflammatory potential, possibly to provide rapid protection in contexts of increased risk of infection and tissue damage.

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CHAPTER 6
GENERAL DISCUSSION

Summary of main findings

The data presented in chapter two showed that γδ T cells mobilize during acute stress; this response was larger than that of $CD8^+$ T cells and less than NK cells. γδ T cell mobilization appears to be mediated by β-adrenergic mechanisms as it correlated with indices of sympathetic cardiac activation, exercise intensity, and β-agonist (isoproterenol) dose. Detailed phenotypic analysis of these cells, carried out in chapter three showed that mobilized γδ T cells were of a tissue migrating ($CD11a^{hi}$) and $CD45RA^+$ effector memory (EMRA; $CD45RA^+CD27^-$) phenotype, and expressed the NK marker $CD94^+$. EMRA γδ T cells are known to express the highest levels of perforin (pfn) and granzymes (Angelini. et al., 2004; Caccamo. et al., 2005), thus the robust mobilization of these cells provides support for the hypothesis that cytotoxicity is the key determinant of which lymphocytes become mobilized during stress.

The results of the study presented in chapter four were threefold. Firstly, we showed that $CD4^+$ T cells were only mobilized when they expressed perforin, and, secondly, that this response was not confounded by an increase in $CD8^+$ T cells that expressed CD4. Thirdly, it was shown that perforin expression identified $CD8^+$ and γδ T cells that become mobilized, which is consistent with the idea that cytotoxicity is a determinant of mobilization sensitive lymphocytes. However, further analysis using the

differentiation marker (CD27), shed new light on this hypothesis. It was found that mobilized pfn^{+} T cells were largely represented by $CD27^{-}$ T cells, thus $pfn^{+}CD27^{-}$ T cells were consistently found to give the most robust mobilization, whilst $pfn^{+}CD27^{+}$ T cells were not. In addition, $pfn^{-}CD27^{-}$ T cells were also mobilized, indicating that although cytotoxicity is an important feature of mobilized cells, it is not essential. A differentiated phenotype ($CD27^{-}$) more consistently identified T cells that become mobilized during stress. Isoproterenol infusion produced an almost identical pattern of mobilization to stress, suggesting β-adrenergic mechanisms of mobilization.

Chapter five was the first study to examine the response of functional monocyte subsets, identified using CD14 and CD16 expression, during psychological stress. This chapter demonstrated that the tissue migratory and 'proinflammatory' $CD14^{+}CD16^{+}$ monocytes were robustly mobilized during psychological stress and isoproterenol infusion. The mobilization of these cells reveals that stress selects for monocytes of a pro-inflammatory nature and is consistent with the observations in lymphocytes described in prior chapters.

Key features of acute stress induced mobilization: is there a pattern?

The phenotype and likely β-adrenergic mechanism of mobilized γδ T cells is in line with previous findings in $CD8^{+}$ T cells and NK cells which show that cytotoxic subsets with a tissue migrating (e.g., $CD11a^{high}$) and high effector potential are mobilized during

various types of adrenergic stimulation (Atanackovic et al., 2006; Bachen et al., 1995; Benschop et al., 1996a; Benschop et al., 1996b; Bosch et al., 2005; Landmann. et al., 1984; Maisel et al., 1990; Mills et al., 1995; Murray et al., 1992; Riddell et al., In preparation ; Schedlowski et al., 1996). Previously this was interpreted as representing an increase in cytotoxic populations that have the ability to rapidly kill target cells. However, findings in chapter four of this thesis suggest that the pattern might be more complex. We showed that advanced differentiation ($CD27^{-}$), which includes EMRA T cells, actually better predicted which T cells became mobilized than a cytotoxic marker (pfn^{+}). This raises important questions about the function of $CD27^{-}$ T cells in stress leukocytosis. $CD27^{-}$ T cells have a lower threshold for activation, express a range of effector functions, and are tissue migrating. Apart from perforin mediated cytotoxicity, these cells can also kill targets by surface molecules like Fas-L, and cytokine secretion, such as TNF-α and IFN-γ (Angelini. et al., 2004; Guidotti and Chisari, 1996; Hamann et al., 1997; Shresta et al., 1998). Thus, stress appears to select for cells that can initiate immediate responses, as opposed to cells which have a higher threshold for activation, and usually require proliferation and further differentiation to acquire effector function (Appay et al., 2008; Hamann et al., 1997).

As shown in chapter five, this model appears to generalize to monocytes also. $CD14^{+}CD16^{+}$ monocytes have been shown to exhibit patrolling behavior in blood vessels potentially seeking out stressed or damaged tissue (Auffray et al., 2007), avidly secrete TNF-α (Belge et al., 2002), express up-regulated tissue migration receptors (Ancuta et al., 2003; Steppich et al., 2000; Ziegler-Heitbrock, 1996) and are involved in scavenging and

responses to viral nucleic acids via Toll like receptors (Saha and Geissmann, 2011). Hence it appears that effector capabilities and a tissue migrating nature are features common to mobilized lymphocytes and monocytes.

Clinical implications of stress leukocytosis

Stress leukocytosis is part of the fight or flight response and is thought to be an adaptive mechanism, aimed at maximizing host survival during times of threat (Benschop et al., 1996b; Dhabhar, 2002). Enhancement of the immune system would be adaptive as, in nature, wounding and infection often accompany fight-or-flight initiating situations. In the words of Dhabhar, it is unlikely that evolution would select for a system that allows an animal to escape the jaws of a lion, only to succumb to the jaws of bacteria (Dhabhar, 2002). In animals, stress induces a short-lived lymphocytosis followed by a lymphopenia, accompanied by increased cellularity in various tissues (Engler et al., 2004; Krüger et al., 2008; Stefanski et al., 2003). Although acute stress studies in humans do not show a lymphopenia, possibly due to the comparatively low intensity stressors used, intensive exercise induces a similar lymphocytosis, followed by lymphopenia (Pedersen and Hoffman-Goetz, 2000; Shephard, 2003; Simpson et al., 2007). This suggests that the ultimate purpose of the evolved stress response is to recruit cells into the tissues. In support of this, animal studies have shown that leukocytosis may be associated with an enhanced immune defense, such as an increased delayed-type hypersensitivity response (Dhabhar and McEwen, 1996), an increased migration of leukocytes into wounded tissue

(Viswanathan and Dhabhar, 2005), and an enhanced response to immunization (Dhabhar and Viswanathan, 2005). Further, a recent study demonstrated that stress leukocytosis predicted surgical recovery in humans (Rosenberger et al., 2009). The authors showed that patients exhibiting a greater magnitude of lymphocyte and monocyte redistribution during surgery stress showed a better recovery.

Mobilized leukocytes can thus be thought of as 'patrolling' cells. For example, γδ T cells have recently been suggested as key players in the 'lymphoid stress surveillance response' (Hayday, 2009). As a result of their immediate effector function, recognition of both stress and microbial antigens, and their ability to present antigen to adaptive immune cells, these cells are ideally placed to rapidly respond to damaged cells and to signal, or activate, other immune cells accordingly. The mobilization of these cells during psychological stress and exercise may contribute to this stress surveillance. Assuming that stress facilitates the targeted migration of cells into the tissues, the mobilization of leukocytes characterized in this thesis could: provide immediate effector functions at the infection site, such as perforin-mediated cytotoxicity, INF-γ and TNF-α secretion ($CD27^{-}CD4^{+}$, $CD27^{-}CD8^{+}$ and $CD27^{-}$γδ T cells and $CD14^{+}CD16^{+}$ monocytes); initiate adaptive immune responses via the presentation of antigen (γδ T cells); secrete chemokines, such as RANTES , recruiting other immune cells ($CD8^{+}$ and γδ T cells); and even aid wound healing (γδ T cells and $CD14^{+}CD16^{+}$ monocytes) (Angelini. et al., 2004; Barber et al., 2003; Belge et al., 2002; Brandes et al., 2005; Hamann et al., 1997; Jameson et al., 2002; Nahrendorf et al., 2007; Romero et al., 2007; Saha and Geissmann, 2011; Tikhonov et al., 2006). In aberrant inflammatory contexts, such as atherosclerosis, or

rheumatoid arthritis, however, the mobilization of these cells may have a detrimental impact. For example, it is possible that the stress induced mobilization of proinflammatory monocytes, which are found in increased numbers in the blood of atherosclerosis patients (Ziegler-Heitbrock, 2007), may facilitate the migration of these cells into the sub-endothelia, exacerbating this disease. The clinical impact of stress leukocytosis, therefore, is likely to depend on the context in which this response takes place: it could represent an early intervention in new immune responses, and yet may amplify ongoing immune responses.

Future directions

The present thesis provided a detailed characterization of leukocytes mobilized during acute psychological stress. An important next step is the elucidation of whether, or how, functional parameters are altered in tandem with this stress induced mobilization. For example, cytotoxicity assays could be employed to determine whether the stress-induced enhanced mobilization of cells expressing perforin translates into enhanced target cell killing. In parallel with this, while we have shown that these cells are sensitive to β-adrenergic stimulation, understanding how catecholamine stimulation may impact cytotoxicity is also an important step. A further question to arise from this thesis is why differentiation status is so important in mobilized T cells. As discussed in chapter four, there may be a number of reasons, and dissecting and separating out these reasons will be a future research challenge. The enhanced stress sensitivity of differentiated T cells is

likely explained by up-regulated β-adrenergic receptor expression (Dimitrov et al., 2009; Holmes et al., 2005). The function of the β-adrenergic receptor on these cells however remains elusive. Understanding which pathways and processes are impacted by activation of these receptors will perhaps help to answer this question. For example, microarray experiments could be employed to examine which genes are up or down regulated upon adrenergic stimulation of differentiated cells.

Another issue which is yet to be fully clarified is where stress leukocytes become mobilized from. In animals this has been better characterized than in humans (Kanemi et al., 2005; Krüger et al., 2008), as human studies have focused mainly on demonstrating that the spleen is not the major source of mobilized cells (Benschop et al., 1996a; Schedlowski et al., 1996). Other potential sources of mobilized cells include the marginal pool and bone marrow, where effector memory T cells are known to localize (Benschop et al., 1996b; Di Rosa and Pabst, 2005). Bone marrow samples could be treated with catecholamines to assess whether this initiates the release of these cells, as has been shown with cells bound to endothelium in vitro (Benschop et al., 1993; Dimitrov et al., 2010).

Finally, perhaps the most important question pertains to the clinical impact of leukocytosis. For example, we have shown here that β- blockade abrogates monocyte recruitment. These cells are integral in the pathogenesis of atherosclerosis and it is known that β-blockers have anti-atherosclerotic properties (Donetti et al., 1998; Feuerstein and Ruffolo, 1996; Gautier et al., 2009; Kopecky, 2006). Investigating the links between leukocyte recruitment during stress, the use of β-blockers and the pathogenesis of disease is warranted. Perhaps a step towards understanding this impact would be to utilize

migration assays to test whether catecholamine treatment alters the migration behavior of monocytes towards chemokines known to be released by inflamed endothelium.

In conclusion,

This thesis examined the hypothesis that specifically cytotoxic lymphocytes are mobilized during stress. The findings shed new light on this theory, revealing that although cytotoxic potential is an important feature of mobilized cells, the characteristics governing stress induced leukocytosis are more complex. Indeed, chapter four demonstrated that differentiation is perhaps an even more important characteristic of mobilized T cells. We have demonstrated that stress preferentially recruits effector cells from a range of T cell and monocyte populations. It is possible that the stress induced mobilization of a range of leukocytes in times of threat, represents an adaptive mechanism that maximizes the diversity of the immune response, and hence survival.

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