PSYCHOLOGICAL STRESS AND NEUTROPHIL FUNCTION

By

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ABSTRACT

Little is known about neutrophil function, an important component of innate immunity, in relation to psychosocial factors. This thesis investigated the effect of acute and chronic psychological stressors on human neutrophil function among young and older adults. The first two studies examined the effects of an acute laboratory psychological stress task on neutrophil function in young and older adults, respectively. Blood samples to determine neutrophil function were taken at resting baseline, during acute stress and during recovery. In the first study (N=40), there was an acute increase in phagocytic ability and a reduction of superoxide production associated with the stress task relative to baseline. In study two (N =17), there was a significant reduction of neutrophil superoxide production associated with the stress task. Study three (N=48) examined the effect of chronic stress, a recent bereavement (<2 months), on neutrophil function in elders. Cortisol and dehydroepiandrosterone-sulphate (DHEAS) levels were determined in serum to assess potential mechanisms. Superoxide production was significantly reduced among the bereaved group when challenged with *E. Coli*; also, the bereaved had a significantly higher cortisol:DHEAS ratio compared to controls. Overall, this thesis shows that human neutrophil function is sensitive to both acute and chronic psychological stress exposures; however, more research is needed to determine the specific underlying mechanisms behind the observed alterations.

LIST OF PAPERS

This thesis comprises the following four original papers:

1. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (2010). Effects of acute psychological stress on human neutrophil function, *Psychosomatic Medicine*, 72, 636-640. IF 3.60
2. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (under review). Reduced neutrophil superoxide production among healthy older adults as a result of acute psychological stress. (in submission).
3. Khanfer, R., Lord, J.M., & Phillips, A.C., (2011). Bereavement is associated with reduced neutrophil function and a raised cortisol:DHEAS ratio in older adults. *Brain, Behavior & Immunity* 25, 1182-1186.
4. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M.(In preparation). The phasic and tonic effects of psychological stress on human neutrophil function.

During the period of postgraduate study at the University of Birmingham, the following were also published/in press:

1. Phillips, A.C., Khanfer, R., & Upton, J. (in press). Stress and neutrophil function. In J. Bosch, A.C. Phillips, & J.M.Lord (Eds). *Immunosenescence: Psychosocial and Behavioural Factors.* New York: Springer.
2. Khanfer, R., (in press). Natural killer cells activity and macrophage sections. In M. Gellman & R. Turner (Eds). *Encyclopedia of Behavioral Medicine*. New York: Springer

In addition, the following published abstracts/conference presentations arose from the material in this thesis:

1. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (2010). Effects of acute psychological stress on human neutrophil function .*Brain, Behavior, and Immunity*, 24,1, S7. Abstract

2. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (2010). Preliminary evidence that bereavement is associated with reduced neutrophil function. *Brain, Behavior, and Immunity*, 24, 1, S68. Abstract.

3. Khanfer, R., Phillips, A.C., Carroll, D., & Lord, J.M. (2010). Preliminary evidence that bereavement is associated with reduced neutrophil function in older adults. *British Society for Research on Ageing, 60th Scientific Meeting, Systems Biology of Ageing* , Newcastle upon Tyne, UK. Invited oral presentation.

4. Phillips, A.C., & Khanfer, R. (2010). Psychological factors and immune function across the life course. *Immunosenescence research conference.* School of Sport and Exercise Sciences, University of Birmingham. Oral presentation

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CONTENTS

ABBREVIATIONS

α- MSH-Alpha Melanocyte Stimulating Hormone

Abs-Antibodies

ACTH -Adenocorticotropic Hormone r

CD-Cluster of Differentiation

C3b-Complement Component 3 Parts

Con A-Concanavalin A

CR-Complements

CRF-Corticotropin Releasing Factor

CRH-Corticotropic Releasing Hormone

CTLs-Cytotoxic T lymphocyte

CTLA-4-Cytotoxic T lymphocyte Antigen 4

DC-Dendritic cell

DHEA-S- Dehydroepiandrosterone -Sulphate

DBP-Diastolic Blood Pressure

DM-Diabetes Mellitus

Fc-Fragment Crytallizable or Constant (Heavy Chain of the IG)

fMLP-Formyl-Methionyl-Leucyll-Phenylalanine

Foxp3-Forkhead-winged helix transcription factor3

FSS-Forward Side Scatter

GITR-Glucocorticoid Induced Tumour necrosis factor Receptor

HR-Heart Rate

HLA-Human Leukocyte Antigen

HPA- Axis-Hypothalamic Pituitary Adrenal Axis

H_2O_2-Hydrogen Peroxide

IFNγ-Interferon gamma

IG-Immunoglobulin (Antibody)

IL-Interleukin

MAP-Mitogen Activated Protein

MHC-Major Histocompatability Complex

MS-Multiple Sclerosis

NK cell-Natural killer cell

NADPH-Nicotinamide Adenine Dinucleotide Phosphate

NBT-Nitro-Blue-Tetrazolium

NO-Nitric Oxide

O_2^--Superoxide

PASAT-Paced Auditory Serial Addition Task

PBMC-Peripheral Blood Mononuclear Cells

PB-Peripheral Blood

PHA-Phytohemagglutinin

PMN-Polymorphonuclear Leukocytes

PNI-Psychoneuroimmunology

ROS-Reactive Oxygen Species

ROI-Reactive Oxygen Intermediates

SAM Axis-Sympatho-Adreno-Medullary Axis

SBP-Systolic Blood Pressure

SL-Systemic Lupus Erythematosus

SSC-Side scatter

TCR-T cell receptor

Th1, 2 -T helper1, 2

TGFβ-Transforming Growth factor Beta

TNFα-Tumour necrosis factor alpha

Tregs-Regulatory T cells

Foreword

Stress

"The word stress does not exist in my life, there is nothing called stress that can affect me and that's always has been the case all my life, I can have little day to day annoying things like delay in the bus or train arrival, but nothing to make me stressed about, even at work I always took the opportunity and turned the anger of the customers I am dealing with to a smile, to show them how good we are in our organization"

One of the older adult participants in my final laboratory study uttered these words at the end of the session. I decided to start my thesis' general introduction with this quotation, rather than with the words of a famous scientist, as this utterance made me think about the way I deal with stress in my own life and what exactly stress means to me personally. The perception of stress and how we believe it will affect us seem to me the first steps in dealing with what we call stress in our own lives.

At the beginning of my nursing training in one of my placements in 1995, I was dealing with a diabetic patient. One day I came to the department and found that his blood sugar was very high, although my colleagues and I had been monitoring his blood sugar, diet and medications in the hospital over several days and his blood sugar was consistently normal. I asked him what happened, and he answered by saying that "my relatives visited me and told me very bad news"; he was clearly very upset. Since that time, and before I knew of the existence of research psychoneuroimmunology (PNI) and psychoneuroendocrinology fields, I realised that the folk wisdom about stress affecting our health and contributing to disease may contain an element of truth. Indeed, studies in the PNI and other fields of both animals and humans have provided strong evidence of associations between psychological stress exposures and health and disease (Ader et al., 2001. ; Tosevski and Milovancevic, 2006), and this is what this thesis is about.

CHAPTER ONE

GENERAL INTRODUCTION

Psychological Stress

   As early as 1936, Selye defined stress as "the nonspecific response of the body to any demand" (Ader et al., 2001). Stress can be defined according to Psychoneuroimmunology (PNI) experts as "environmental events (real or perceived) that perturb one's psychological or physiological homeostasis" or balance (Cannon, 1915). Often we refer to the effects of stress as the "fight-or-flight" reaction, or the stress response (Fink, 2000a). Examples of real life stressors include: loss and bereavement, separation and divorce, marital stress, disease of a spouse, care giving for a demented or disabled person, loneliness or homelessness, academic stress, an earthquake, unemployment or work redundancy, daily work and life hassles, and many others (Ader et al., 2001. ; Fink, 2000b). Stress can be associated with different cognitive, emotional, behavioural, and physical symptoms (Ader et al., 2001. ; Buckingham et al., 1997; Fink, 2000b). Importantly, psychological stress has been shown to be associated with health and disease in humans (Tosevski and Milovancevic, 2006). Indeed, research in the fields of behavioural medicine, PNI, and psychoneuroendocrinology, have produced valuable evidence of how stress, and its intensity and duration, can differentially affect our health, and play a crucial role in many diseases and disorders (Godbout and Glaser, 2006; Tosevski and Milovancevic, 2006; Yang and Glaser, 2002). For instance, studies show that different psychological stressors are associated with poorer vaccination responses (Pedersen et al., 2009). Experts in the PNI field have defined acute stress as stress that results in an increase in stress responsive physiological parameters which last for minutes to hours, whereas chronic stress is defined as stress that persists for several hours a day for a number of days or months (Ader et al., 2001).

   One of the widely accepted stress mechanisms is the activation of the hypothalamic-pituitary-adrenal axis (HPA). Briefly, as a result of psychological stress, cells in the hypothalamus produce the hormone corticotrophin-releasing factor (CRF), which binds to specific receptors on

cells in the brain's pituitary gland, leading to the production of adrenocorticotropic hormone (ACTH). ACTH then travels through the blood stream to its target, the adrenal gland, which when stimulated produces different adrenal hormones (Okuneva et al., 2009). The hormones secreted as a result of the HPA axis activation include CRF, vasopressin, glucocorticoids, mineralocorticoids, ACTH, prolactin, growth hormone, copeptin, dehydroepiandrosterone (DHEA) and its sulphated form (DHEA-S), adrenaline and others (Okuneva et al., 2009; Papadimitriou and Priftis, 2009). However, cortisol is the major stress hormone produced. Figure 1 summarises the steps in HPA axis activation.
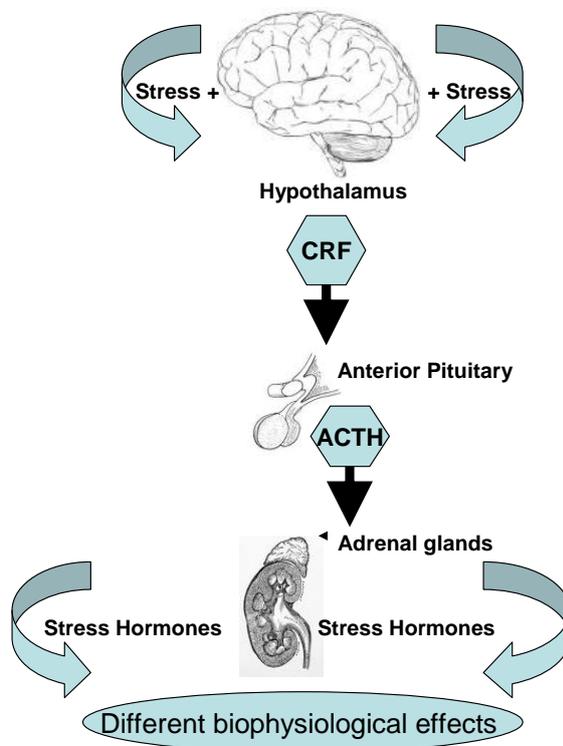
Figure 1: Summary of the HPA axis activation steps.

DHEA-S is steroid hormone, it is the sulphated form of DHEA, a C19 steroid of adrenal origin. In the blood the DHEAS level is much higher than those of free DHEA. Most of the circulating DHEA-S originates from either direct adrenal secretion or by peripheral sulphating of DHEA secreted by the adrenal cortex; DHEA-S does not circulate bound to specific proteins. In response to stimulation by the endogenous hormone adrenocorticotrophin, DHEAS is secreted

from the adrenal cortex alongside DHEA and there is evidence of DHEAs secretion from other parts in the body especially the brain (Barrett-Connor et al., 1986; Hucklebridge et al., 2005; Maninger et al., 2009). Together, these steroids are the most abundant in human circulation, DHEAS circulates at much higher levels than other androgens or related steroids. Levels of DHEAS increase from about the seventh year of life, peak in the third decade, and decrease gradually thereafter(Hucklebridge et al., 2005).

To date, no biological function has been identified for DHEAS independent of its role as a precursor to DHEA, which is widely involved in sex steroid biosynthesis, where it acts a precursor to approximately 30–50% of circulating androgens in men and 100% of circulating estrogens in postmenopausal women. However, the measurements of DHEAS are used in clinical practice. Elevated concentrations of this steroid are found in patients with adrenal hyperplasia, adrenocortical carcinoma, or hirsutism. Low levels of DHEAS are found in patients suffering from adrenal dysfunction and hypopituitarism . There are also some reports of DHEA being a regulator of immune function, triggering immune cells stimulation in vivo, often counteracting the immune-suppressive effects of glucocorticoids. Moreover, it has been proposed that DHEA supplementation may be a novel means by which to minimise immunesenescence. DHEA is also being proposed as a potential therapeutic option for the treatment of autoimmune diseases (Al-Aridi et al., 2011; Barrett-Connor et al., 1986; Hazeldine et al., 2010; Hucklebridge et al., 2005; Maninger et al., 2009).

Brief Description of the Immune System

Immune function can be divided into a specific/acquired and non specific/innate immunity. The components of the two arms of the immune system interact with each other in highly complex ways (Parslow, 2001; Stites and Terr, 1991). The majority of human immune system cells originate in the bone marrow where many of them mature, and then migrate and circulate in the body tissues and blood stream. Immune cells use the specialized system of vessels; the lymphatic system, for migration, maturation, immune cell interaction, and antigen recognition formation (Janeway, 2005). The innate immune system components include; complement, granulocytes or polymorphonuclear leukocytes (PMNs), the group to which neutrophils as well as eosinophils and basophils belong, macrophages/monocytes and natural killer cells (NK cells).

These components are the first to confront pathogens. They operate immediately without specificity or previous antigen exposure and have no memory of pathogens. In contrast, the acquired immune system components on encountering pathogens are highly specific in terms of antigen recognition, require few days to act, improve their affinity to pathogens, and form memory post exposure. It is called adaptive as it happens during the life time as a result of adaptation to infections (Janeway, 2005; Parslow, 2001; Stites et al., 1997). Acquired immunity components include; B lymphocytes which are responsible for secreting antibodies, and T lymphocytes which have two main types (CD4 T helper cells and CD8 cytotoxic T cells). Neutrophils and their function in relation to psychological stress will be the main focus of this thesis.

Neutrophils

   Neutrophils are white blood cells which form a major part of our innate or non-specific immune system. They are circulating phagocytes generating continuously in the bone marrow and have a half life of 6-9 hours. They also form more than 55% of our total circulating white blood cells and more than 85% of circulating phagocytes. Neutrophils also marginate in the tissues in large numbers, and this storage allows them to mobilize rapidly in response to the onset of an infection or inflammation (Janeway, 2005; Nathan, 2006; Parslow, 2001; Stites et al., 1987; Stites and Terr, 1991). By molecular interaction between the cell surfaces of the neutrophil and endothelial cells, neutrophils roll along the blood vessel walls, then adhere and migrate in a process called diapedesis. Neutrophils circulate and migrate between the tissues depending largely on a group of proteins and their ligands called selectins. In addition, neutrophils like other leukocytes rely on different chemo attractants to perform efficient transmigration; these soluble molecules, such as interleukin 8 (IL-8), tumor necrosis factor (TNF), and IL-1, direct the movement of neutrophils in a process called chemotaxis. As neutrophils can be activated by the products of monocytes, macrophages, and T cells, neutrophils also secrete inflammatory cytokines and different chemokines to recruit and activate other immune cells such as monocytes, macrophages, dentritic cells, and lymphocytes (Cooper and Koprowski, 2002; Yokoyama et al., 2005).

   Neutrophil function has been widely studied in Immunology, as its involvement in health and disease processes is significant. Neutrophils are among the first immune cells to reach the

inflammation or infection site in large numbers, and also play a key role in wound healing (Dovi et al., 2004; Nathan, 2006; Nauseef, 2007; Segal, 2005). Research shows that, in addition to the well documented tissue damage that neutrophils can cause in sites of infection and inflammation, neutrophils are also involved in tissue injury in many disorders and also play role in some persistent inflammatory diseases such as asthma (Monteseirin, 2009). There is also increasing evidence that neutrophil accumulation is an early and consistent feature of some antibody-antigen immune complex mediated diseases, such as glomerulonephritis (Keisari and Ofek, 2000; Nathan, 2006; Stites et al., 1997; Tsuboi et al., 2008).

Neutrophil Killing Mechanisms

Neutrophils use different killing mechanisms to eliminate ingested pathogens in complex processes. They have potent antimicrobial means by which they can kill and degrade targets, including different types of intracellular granules which contain bactericidal molecules comprising azurophilic granules also called primary granules; specific or secondary granules; and tertiary granules (Nauseef, 2007; Segal, 2005, 2006). Neutrophils also express different sets of cell surface receptors, including complement and antibody recognition receptors (CR and Fc respectively) and toll like receptors (TLRs). Through these receptors, neutrophils recognize opsonins; proteins which bind to bacteria and enhance phogocytosis in a process called opsonization. Opsonins include the heavy chain of immunoglobulin G (IgG-Fc) and complement components, such as C3b. These important molecules are involved in the initiation of neutrophil phagocytosis and intracellular signaling processes (Akira et al., 2008; Kishore, 2009).

Neutrophil Respiratory Burst

Within neutrophils, in a metabolic process termed the respiratory burst, microbes are killed as a result of the production of two highly reactive microbicidal agents called reactive oxygen species (ROS) or reactive oxygen intermediates (ROI). Specifically, upon stimulation of its receptors, the neutrophil becomes activated and starts engulfing the microbes via folding its surface membrane around the pathogen forming a vacuole called a phagosome. In the cytoplasm of the neutrophils, the other granules, mentioned above, called lysosomes, containing various degenerative enzymes, fuse with the phagosome to form a phagolysosome; inside the

phagolysosome, killing and degradation takes place (Nathan, 2006; Nauseef, 2007; Segal, 2005, 2006). Neutrophil granules can also empty their degradative enzymes into the extracellular spaces, attacking bigger extracellular microbes and pathogens. In the respiratory burst, various bactericidal proteins, reactive oxygen species and hydrolytic enzymes are produced including; superoxide (O_2^-), hydrogen peroxide (H_2O_2) , nitric oxide (NO), hypochlorite, myeloperoxidase, lactoferrin, proteases, nucleases, lipases, and many others (Segal, 2005, 2006; Yokoyama et al., 2005). These aid in the complete destruction of engulfed pathogens contained in the phagolysosome.

Clinical Importance of Neutrophils

Neutrophils are key players in fighting invading pathogens and microorganisms which can cause serious infections. The loss of neutrophils in number or of aspects of their function leaves individuals more susceptible to infections. Clinically, the discovery of different rare conditions affecting neutrophils has contributed significantly in our understanding of the importance of neutrophil function in humans. Indeed, conditions such as inherited neutropenias, acquired autoimmune neutropenias, and neutropenias resulting from chemotherapy, leave vulnerable patients at risk of serious life threatening infections. Examples of inherited neutropenias caused by mutations in proteins used by neutrophils during the killing mechanisms include cyclic neutropenia (CN) and severe congenital neutropenia (SCN) (Hager et al., 2010; Nathan, 2006).

A reduction in or lack of neutrophil oxidative burst activity is observed also in some innate immune defects such as chronic granulomatous disease (CGD) which is a heterogeneous group of inherited disorders that usually manifests itself during the first two years of life. This particular disease resulted from abnormalities in the constituent peptides of the NADPH oxidase enzyme system and characterized by repeated life-threatening infections caused by bacterial and fungal organisms. These infections typically consist of pneumonia, lymphadenitis, or abscesses that involve lymph nodes, lungs, and liver(Cowburn et al., 2008; Kumar and Sharma, 2010).

Stress and Immunity

The association of stress and stress hormones with human health and disease is well characterised for many diseases and disorders (de Quervain et al., 2009; Fink, 2000a; Harbuz,

2002; Stojanovich and Marisavljevich, 2008; Yang and Glaser, 2002). Indeed, psychological stress has been shown to be associated with delayed wound healing, poorer cancer prognosis, worsening Multiple Sclerosis (MS) symptoms, and a poorer prognosis following HIV infection (Goforth et al., 2009; Hansen et al., 2009; Mitsonis et al., 2009; Moreno-Smith et al., 2010; Roy et al., 2005; Walburn et al., 2009). Moreover, studies in the PNI field, from human and animals have also shown that stress is associated with many inflammatory and autoimmune diseases (Fink, 2000a; Harbuz, 2002; Stojanovich and Marisavljevich, 2008).

The complex interaction between the HPA axis and the components of the immune system in the body at different levels are well documented. For example, immune cells express different receptors for different stress hormones, and can be activated or altered as a result of activation by the HPA and sympatho-adreno-medullary (SAM) axes; indeed many immune cells express beta-adrenergic receptors (Ader et al., 2001. ; Buckingham et al., 1997; Fink, 2000a). Stress hormones also have important physiological functions that can affect the immune cell growth, proliferation, and differentiation, including having immunosuppressive effects (Bernabe et al., 2010; Bose et al., 2009; Katzung et al., 2009). The interaction of the neuroendocrine and immune systems in the context of autoimmunity and inflammation has also received attention (Capellino and Straub, 2008; Cutolo and Straub, 2006; Harbuz, 2002; Stojanovich and Marisavljevich, 2008). At both the cellular and molecular level, stress can affect gene expression, signalling pathways, and expression of cell surface receptors (Gidron et al., 2010; Kurokawa et al., 2011; Li et al., 2011; Mathews and Janusek, 2011). Many studies have demonstrated these effects, although less is known about the specific underlying mechanisms of these associations.

Acute and Chronic Stress Effects on Immunity

Acute and chronic psychological stress appear to impact differently on the immune system. Studies show that chronic stress can suppress various aspects of immunity, whereas acute stress appears to be immune enhancing (Segerstrom and Miller, 2004). Studies show that acute psychological stress is associated with: lymphocytosis; specific lymphocyte subtypes mobilization; increased NK cell cytotoxicity (Anane et al., 2009; Willemsen et al., 2002); increased salivary and serum immunoglobulin A secretion rate (Ring et al., 2002); stimulation of

aspects of the complement system (Burns et al., 2008); enhanced vaccination responses (Edwards et al., 2006); and faster wound healing (Weinman et al., 2008). The mobilization of lymphocyte subtypes observed with exposure to acute stress includes cytotoxic cells such as NK cells and CD8T cells. Moreover, other immune cell subtypes like T regulatory cells, memory T cells, and immune cells specific to certain pathogens, such as cytomegalovirus, are also sensitive to acute psychological stress (Anane et al., 2009; Anane et al., 2010; Freier et al., 2010).

On the other hand, studies of chronic stress and immunity provide evidence of down-regulation rather than activation (Glei et al., 2007). Chronic stress exposures such as caregiving of individuals with Alzheimer's and children with developmental disabilities, have been found to be associated with: reduced NK cell activity; increased epinephrine and neuropeptide levels (Irwin et al., 1991); poorer antibody response to vaccination (Gallagher et al., 2009a, b) respectively. The stress of caregiving also was shown to be associated with alteration in immune cell function. For instance, spousal dementia caregivers in a longitudinal study, were shown to have less responsive lymphocytes from PBM when stimulated with two mitogens; concanavalin A (Con A) and phytohemagglutinin (PHA) compared to controls (Kiecolt-Glaser et al., 1991), and also associated with alteration in some inflammatory mediators (Bauer, 2008; Gouin et al., 2008). In a recent longitudinal study, there was a significant difference in the pro inflammatory cytokine IL-6 rate of increase, between a group of caregivers (N=119) and their controls (N=106). The stress of caregiving was associated with a four times higher IL-6 increase rate compared to the control group (Kiecolt-Glaser et al., 2003). Other chronic stressors or stressful life events, such as bereavement, have been related to a poorer antibody response to vaccination (Phillips et al., 2006), poorer HIV prognosis (Goforth et al., 2009), lower NK cell activity and higher cortisol levels (Irwin et al., 1987). Chronic stress at work among nurses was shown to be associated with immune dysregulation, including increased expression of the IL-2 receptor on CD4+CD25 T cells (De Gucht et al., 1999). Similarly, two studies have shown that unemployment was related to a significant decrease in proliferation of lymphocytes following stimulation with PHA (Arnetz et al., 1991; Arnetz et al., 1987). Further, the chronic stress of homelessness was associated with lower expression of lymphocyte beta-adrenergic receptors compared to that of control lymphocytes (Dimsdale et al., 1994). Thus, there is a growing

literature testifying that acute and chronic stress may have very different effects on immune function.

Ageing Effects on Immunity

Ageing is associated with a decline in many of the human body's functions, including that of the immune system (Brigham, 1990; Ebbesen, 1984; Frieling, 2011; Grubeck-Loebenstein et al., 2009; Patel et al., 2010; Provinciali et al., 2010; Sue Eisenstadt, 2010; Vasto et al., 2010; Weinberger, 1992). The negative impact of ageing on immunity is called immunosenescence; where the function of various components of both the innate and acquired immune system is attenuated with important consequences for health and disease (Burns, 2004; Ginaldi et al., 1999; Panda et al., 2009; Phillips et al., 2007; Weiskopf et al., 2009; Wessels et al., 2010). It is well documented that ageing is associated with higher morbidity and mortality, specifically from infectious disease (Norman and Toledo, 1992; Simell et al., 2011; Simon et al., 2010), autoimmune and inflammatory diseases (Frieling, 2011; Soubrier et al., 2010), and cancer (Anisimov et al., 2009; Campisi, 2007; Lipschitz et al., 1985; von Figura and Rudolph, 2009). Studies of the effects of ageing on immune function provide evidence of age-related reductions in: CD4 T cell response and proliferation; CD8 T cell proliferation; T cell receptor (TCR) diversity; and T cells signal transduction (Aspinall et al., 2010; Nikolich-Zugich and Rudd, 2010; Zagozdzon et al., 2003). B lymphocytes are also affected by ageing, including reduced: activity of transcription factors; production of low affinity antibodies (Desai et al., 2010); and poorer vaccination responses (Fulop et al., 2009; Maggi, 2010). Macrophage changes with ageing include reduced phagocytosis and reduced cytokine and chemokine secretion (Hajishengallis, 2010).

It has been shown that neutrophils are also affected by ageing; for example, several studies have observed that the ability of neutrophils to fight different pathogens and infections is attenuated in older adults (Christy et al., 2010; Di Lorenzo et al., 1999; Hajishengallis, 2010; Tortorella et al., 2001; Wessels et al., 2010). Interestingly, it would appear that the number of neutrophils in circulation is not affected by age, although there is evidence of decreased phagocytosis and reduced superoxide production (Ginaldi et al., 1999; Ginaldi et al., 2001; Hajishengallis, 2010; Lord et al., 2001; Mishto et al., 2003; Panda et al., 2009; Shaw et al., 2010; Weiskopf et al., 2009).

Ageing and Stress Effects on Immunity

It has been hypothesised that older adults may be more vulnerable to the effects of stress, given co-existing immunosenescence. It has been argued that the combined effect of stress and ageing on the immune system will increase the risk of poor immune function, and consequently, increase disease susceptibility (Phillips et al., 2007). Indeed, several studies have shown a combined negative effect of stress and ageing on the function of the immune system. In an experimental model of influenza, results showed that stressed aged mice had reduced NK cell activity, reduced virus specific T helper cell function, and lower survival rates compared to younger stressed control mice (Padgett et al., 1998). Data from another animal study showed that there was a reduction in the number of activated macrophages in aged rats and higher expression of mRNA for the glucocorticoid receptor in peritoneal macrophages from aged mice, compared to the younger animals, when animals were exposed to the stress of cold (Kizaki et al., 2002). Also it has been proposed that this combined effect of stress and ageing has an impact on inflammatory responses in the older population, potentially via HPA axis dysregulation during both homeostasis and stress situations (Bauer, 2005, 2008; Bauer et al., 2009; De la Fuente, 2008; Heffner, 2011; Kizaki et al., 2002; Padgett et al., 1998). One possible mechanism underlying such effects may be neuroendocrine alterations in ageing; for example, with ageing there is a decline in growth hormone and DHEAS levels and an increase in the cortisol:DHEAS ratio (Buford and Willoughby, 2008; Orentreich et al., 1984; Phillips et al., 2007). This increase in the cortisol:DHEAS ratio, reflects an increase or no change in cortisol, but decrease in DHEAS, has been shown to have impact on various health outcomes (Bauer, 2005; Butcher and Lord, 2004; Orentreich et al., 1984). As explained in more detail below, older patients with higher cortisol:DHEAS ratios were more at risk of developing infections than younger patients (Butcher et al., 2005).

Chronic stress in older individuals has also been associated with decline in a number of immune system functions. For example, bereavement and poor marital quality were related to a poorer influenza vaccination response (Phillips et al., 2006). Caregiving for a spouse with dementia has also been related to poorer vaccination responses compared to the response shown

by age and sex matched non caregivers (Kiecolt-Glaser et al., 1996). Finally, older adults who have suffered the physical stress of hip fracture showed decreased neutrophil bactericidal ability, through a reduction in their neutrophil superoxide production compared to a younger control group with similar fractures, and those with a higher cortisol:DHEAS ratio were also more likely to develop subsequent infections (Butcher et al., 2003; Butcher et al., 2005; Phillips et al., 2006). There is growing evidence to support the theory which suggests that the combined effect of stress and ageing on the immune system contribute and accelerate immunosenescence and the initiation of inflammation and autoimmunity in elders (Bauer, 2005, 2008; Bauer et al., 2009; Butcher and Lord, 2004; Gouin et al., 2008). However, little is known about the combined effect of stress and ageing on neutrophil function. A large part of the current thesis is focused on the effect of psychological stress on neutrophil function in older adults, in an effort to improve our understanding of the mechanisms by which older adults are more susceptible to infection.

Acute versus Chronic Psychological Stress and Neutrophil Function

Although the impact of psychological stress on adaptive immunity is fairly well investigated, there are few studies concerned with innate immune system components. Even less is known about the relationship between psychological stress and neutrophil function. Indeed, only a handful of studies have attempted to explore the effect of stress on neutrophil function. In 1976, in an early trial with a completely different methodology to many contemporary PNI studies, one group examined the effect of stress exposure on PMN phagocytosis. Eight healthy women participated in a 77 hour vigil without rest or sleep. During this vigil they performed on a shooting-range, followed by authentic battle noise from a tape recorder which was amplified, followed by a concentrated 15-min period of completing questionnaires. Participants were required to remain seated on their chairs throughout except when using the toilet or providing blood samples. The PMN during this stress exposure had decreased phagocytosis compared to PMN samples collected on control days before and five days after the vigil; no difference was found in the circulating PMN numbers during and after the vigil (Palmblad et al., 1976). Although this study was not designed exclusively to investigate neutrophil function and included somewhat physical as well as psychological stressors by including lack of sleep, known to be associated with immune dysregulation (Maurovich-Horvat et al., 2008), it is one of the earliest studies to demonstrate the effect of stress on neutrophils.

Studies which have investigated the effect of acute psychological stress on neutrophil function have shown that neutrophil phagocytosis and superoxide production can be altered even with short duration stress, as detailed below (Ellard et al., 2001; Kang and McCarthy, 1994; Segal et al., 2006). For example, Kang et al., have shown in two studies that neutrophil superoxide production increased in healthy and asthmatic students post final exams compared to pre exams period (Kang et al., 1996; Kang et al., 1997). However, these neutrophil changes did not differ between the healthy and asthmatic groups; neither did the exam stress worsen asthmatic students' symptoms. In the above studies, neutrophil superoxide production was assessed by using the superoxide dismutase-inhibitable reduction of ferricytochrome C assay, which is an adequate technique. However, this assay is not optimally sensitive measure of neutrophil function. The superoxide dismutase-inhibitable reduction of ferricytochrome C assay only detects superoxide release that has escaped from the cell into the medium, which may represent only a fraction of the superoxide actually generated. However, it is debatable whether academic stress of this nature lasting for weeks could be considered an acute stressor (lasting minutes to hours), but perhaps is rather more like a chronic stressor (Bosch et al., 2004). In a similar but more recent study, post graduate students subjected to the stress of completing their final thesis examination provided blood samples the day prior to their viva. In comparison to a control no examination group, the stress of the final thesis examination was associated with a significant reduction in neutrophil superoxide production (Ignacchiti et al., 2011). In another study, neutrophil activation was investigated as a result of short mental stress task, which lasted 15 minutes in 25 university students. The technique used depended on neutrophils' oxidative capacity to reduce Nitro-Blue Tetrazolium (NBT). There was a significant increase in neutrophil activation in the stressed group (N= 17) compared to their controls (Ellard et al., 2001). This suggests that acute stress activates neutrophils, whereas more chronic stress such as an examination period is associated with a decline in function. However, the NBT technique is less accurate than current methodology for examining neutrophil function including activation which is by flow cytometry; an advanced and sensitive molecular technique. The NBT assay simply measures, by microscopic inspection of staining, the percentage of non-specifically activated neutrophils; traditionally used to assess neutrophil superoxide release. In contrast assays that utilize flow cytometry technology to measure the effects of stimulation offer a more accurate

means of examining different aspects of neutrophil function such as phagocytosis, superoxide production, and chemotaxis (Kampen et al., 2004; Lehmann et al., 2000; Panasiuk et al., 2005). In addition, in these assays potent biological stimuli are used, such as opsonised bacteria, to trigger the neutrophil response.

An acute increase in the number of neutrophils (neutrophilia) in circulation has also been observed in studies of acute stress (Miller et al., 2005) showing the importance of adjusting assays of neutrophil function for changes in neutrophil number. Finally, a group in Japan investigated the effect of the psychological stress of final exams on spontaneous neutrophil apoptosis in nine medical students by measuring the percentage of apoptotic nuclei in samples taken just prior to examinations and in a control non-stressful period (Sendo et al., 1997). There was significantly higher neutrophil apoptosis in samples prior to examinations compared to the same participants samples taken in non stressful conditions (Sendo et al., 1997). Similar to the paucity of human studies, few studies using animal models have examined the effect of stress on neutrophils. In one, exposure to 1-hour of open field stress, where animals are placed in a vulnerable open space with bright lighting and white noise, rats showed an increase in neutrophil superoxide production (Kang and McCarthy, 1994). In some recent research using a social disruption (SDR) model, where a repeatedly aggressive intruder mouse is allowed to attack and cause disruption among resident mice, it was found that a significant number of neutrophils infiltrated the lungs of the stressed mice and contributed to an inflammatory process; these neutrophils were also found to be highly activated (Curry et al., 2010). Noise stress has been found to be associated with suppressed neutrophil superoxide production in male but not female rats, whereas phagocytosis was suppressed in both (Brown et al., 2008). This suppression of functions was eliminated when rats underwent adrenal medullectomy, leading the researchers to conclude that the HPA axis could be responsible for the neutrophil changes seen during stress. Unfortunately, the above studies overlap in terms of being classified as investigating acute or chronic stressors effects on neutrophils. Repeated stressors could be regarded relatively chronic, especially in animal studies.

It is important for PNI research field to expand its scope to include clinical outcomes as well as immune parameters. Indeed, the findings of the PNI literature can be regarded as invaluable

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to an understanding of how stress can affect health and disease, but this understanding now needs to be applied in a range of clinical conditions which have, as yet, received little attention in the context of psychological stress.

Thesis Summary

While the components of the acquired immune system have been studied in detail in PNI and have attracted the attention of many scientists in the field, the neutrophil, which is an important component of our innate immunity, has received scant attention. Little is known about neutrophil function in relation to different psychosocial factors in health and disease. Comprising three original studies, this thesis investigates the effect of psychological stress (acute and chronic) on human neutrophils.

The next three empirical chapters (two, three and four), describe studies one and two, which examined the effects of an acute laboratory psychological stress exposure on neutrophil function in young and older adults, respectively. Blood samples to determine neutrophil function were taken at the end of resting baseline, during an acute stress task, and during recovery. In the first study, there was an acute increase in neutrophil phagocytic ability but a reduction of superoxide production associated with the stress exposure relative to baseline (chapters two and three). However, the stress sessions were associated with lower neutrophil function on the whole in comparison to neutrophil function during the resting control sessions (chapter two), suggesting tonic longer term effects of stress on neutrophil function as well as short-term phasic effects. In the second study (chapter four), the effects of the acute laboratory psychological stress task on neutrophil function was examined among older adults, to determine whether observed effects change in the presence of immunosenescence. In this study, there was a significant reduction of neutrophil superoxide production associated with the stress task relative to baseline.

In chapter five, the effect of recent bereavement (<2 months) on neutrophil function was examined in participants aged over 65 years old. It was hypothesised that the chronic stress of bereavement would suppress immune function, specifically neutrophil bactericidal activity. The study comprised a between-subjects design with 24 bereaved and 24 age- and sex-matched non-bereaved controls. Cortisol and DHEAS levels were determined in serum to assess potential

mechanisms. Superoxide production was significantly reduced among the bereaved group when challenged with *E. coli*; also the bereaved group had a significantly higher cortisol:DHEAS ratio compared to controls.

   Overall, it seems that human neutrophil function is sensitive to psychological stress. However, more research is needed to determine the specific underlying mechanisms behind the observed alterations. It is also important for PNI to expand its scope to include both immune parameters and clinical outcomes and link the findings of the research field with its clinical aspects.

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CHAPTER TWO

THE PHASIC AND TONIC EFFECTS OF PSYCHOLOGICAL STRESS ON
HUMAN NEUTROPHIL FUNCTION

Abstract

There is mounting evidence that acute stress is associated with increases in a number of immune indices. The present study examined the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis and stimulated superoxide production, in humans. Participants were 40 (20 females) university students who attended counterbalanced stress and control sessions on separate days. In the stress session blood samples to determine neutrophil function by flow cytometry were taken at the end of resting baseline, during an acute stress task and during recovery, and at the same time points in the control session. The stress task was a 10-minute time-pressured mental arithmetic challenge with social evaluation. There was an acute increase in phagocytosis and superoxide production associated with the stress task. In addition, there was evidence of a more tonic reduction in phagocytosis and superoxide production across the stress session. It would appear that neutrophil function is differentially sensitive to psychological stress depending on its chronicity.

Key words: acute psychological stress; neutrophil; phagocytosis, superoxide production

Acute and chronic psychological stress would appear to have a different impact on the immune system. Generally, chronic stress has been shown to have a negative effect on various aspects of immunity, whereas acute stress would seem to be immunoenhancing (Segerstrom and Miller, 2004). For example, acute stress has been shown to elicit lymphocytosis (Anane et al., 2009; Willemsen et al., 2002), increase natural killer cell cytotoxicity (Bosch et al., 2005; Sgoutas-Emch et al., 1994) and secretory immunoglobulin A secretion rate (Ring et al., 2002), stimulate aspects of the complement system (Burns et al., 2008), and boost vaccination response (Edwards et al., 2008). However, little is known about the effects of acute stress on human neutrophils and particularly their function. This is perhaps surprising given that neutrophils are a major component of innate immunity. They form more than 50% of our circulating immune cells and they play a crucial role in fighting invading pathogens, particularly bacteria, and during inflammatory responses (Nathan, 2006). Neutrophils act quickly and without specificity and have the ability to produce cytotoxic and bacteriocidal molecules such as superoxides (Nauseef, 2007).

The effect of stress on neutrophils has been examined in a series of studies on asthma (Kang et al., 1998; Kang et al., 1996; Kang et al., 1997). Academic examinations served as the source of psychological stress and neutrophil function was assessed before, during, and after an examination period, using the superoxide dismutase-inhibitable reduction of ferricytochrome C assay to measure superoxide release. They found some indication that examination stress increased superoxide release. However, it has been argued that a prolonged examination period constitutes chronic rather than acute stress; whereas a single examination elicited an increase in secretory immunoglobulin A, a prolonged period of academic examinations was associated with a decrease (Bosch and Carroll, 2007). More recently, a 15 minute laboratory stress task (Raven`s progressive matrices) was used to examine neutrophil activation using the Nitro-blue Tetrazolium assay which indicates oxidative capacity (Ellard et al., 2001). The acute stress was shown to increase neutrophil activation relative to baseline. However, neither of these assays are optimally sensitive measures of neutrophil function. The superoxide dismutase-inhibitable reduction of ferricytochrome C assay only detects superoxide release that has escaped from the cell into the medium, which may represent only a fraction of the superoxide actually generated (Kang and McCarthy, 1994). The Nitro-blue Tetrazolium assay simply measures, by

microscopic inspection of staining, the percentage of non-specifically activated neutrophils (Ellard et al., 2001). Both of these measures represent a very imperfect proxy of superoxide release. In contrast, assays using flow cytometry technology to measure the effects of stimulation and accordingly offer a more accurate and versatile means of examining different aspects of neutrophil function such as phagocytosis and superoxide production in response to a range of stimuli (Kampen et al., 2004; Lehmann et al., 2000; Panasiuk et al., 2005).

Consequently, the present study examined the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis and stimulated superoxide production, using flow cytometry assays. Self report and cardiovascular measurements were also taken as a manipulation check, since the effects of the stress task on these variables are well characterised (Phillips et al., 2009; Willemsen et al., 2002). It was hypothesised that acute stress would enhance neutrophil function relative to baseline and to a no stress control condition.

Method

Participants

Forty healthy participants (20 men) were recruited from the student population at the University of Birmingham between May 2008 and June 2009. Date of birth, sex, ethnicity, parental occupation, health and smoking status were determined by standard questionnaire. Participants mean (SD) age was 25.9 (4.56) years and mean body mass index was 23.3 (2.53) kg/m^2. The majority (90%) reported their ethnicity as white and that they were non-smokers; and 74% reported that they came from non-manual occupational households. Individuals were excluded if they had an acute infection or suffered from an immune disorder.

Design and Procedure

All participants attended two sessions on separate days: an acute psychological stress session and a control session, which were counterbalanced. They were asked to avoid alcohol and vigorous exercise for 12 hours, and food and caffeine for one hour prior to testing. On each testing day, participants entered the laboratory at 9.30 a.m. Their height and weight were measured. An intravenous cannula was then inserted in the anticubital region of one arm and a blood pressure cuff was attached to the other arm. Each session was of two hours duration.

Participants were told at the outset whether they were attending a control or stress task session. Following instrumentation and laboratory adaptation, there was a baseline resting period of 20 minutes, a 10-minute psychological stress task, and a10-minute resting recovery period. The control condition also lasted 40 minutes, but consisted of quiet sitting throughout. The study was approved by the local Medical Research Ethics Committee, and all participants provided written informed consent.

Acute stress task

Participants undertook an acute psychological mental stress task, the Paced Auditory Serial Addition Test (PASAT), which has been shown to elicit cardiovascular changes in many studies (Burns et al., 2008; Phillips et al., 2009; Willemsen et al., 2002). Participants were presented with a series of single-digit numbers by audio-CD and requested to add each number to the immediately preceding number and say the answer aloud while retaining the second of the pair in memory for addition to the next number presented. The 10-minute task consisted of five consecutive and continuous periods of two minutes, with numbers presented at speeds of 50, 65, 75, 100, and 125 digits at presentation rates of 2.4, 2.0, 1.6, 1.2, and 0.8 seconds, respectively. All participants were given an explanation of the task and an opportunity to practice prior to its commencement. In order to add to the challenging nature of the task, elements of competition and social evaluation were added. A false leader board was in view of the participant, who was instructed to try to beat the scores on the board. A confederate experimenter scored the answers overtly while sitting on a high stool at a distance of 1 metre facing the participant. Participants were seated in front of a large television screen which allowed them to see themselves live throughout the test, and were instructed to look at the screen at all times. They were also informed that they were being video-taped and that videos were to be assessed by "independent body language experts"; no such assessment was made. The confederate experimenter also sounded a loud aversive buzzer once during the first five of every ten trials. Participants began the test with a score of 1000 points; for each incorrect or missed answer 5 points were deducted from their score. Immediately following the task, they rated how stressful, difficult, and engaging the task was on a 7- point scale, where 0 equaled "not at all" and 6 equaled "extremely".

Blood sampling and Immune Assays

Blood samples were taken at three time points: at the end of the resting baseline, in the final minute of the stress task, and at the end of the recovery period. Again, the same sampling times were used in the control condition. At each time point, blood was collected into one EDTA tube and one heparin tube which was placed immediately on ice. The EDTA tubes were used to count lymphocyte and granulocyte numbers using a Coulter® A^C • T diff™ Analyzer (Beckman Coulter, Inc., Miami, Florida, USA). The heparin tubes were used to assess neutrophil function, specifically bacteria-induced phagocytosis and oxidative burst (superoxide production) by flow cytometry.

Phagotest kits (Orpegen Pharma GmbH, Heidelberg, Germany) measured neutrophil phagocytosis with fluorescein isothiocyanate-labeled (FITc) opsonized *Escherichia coli* (*E. coli*) bacteria. Whole blood (100 μL) was incubated with 20 μL of bacteria (2×10^7) at 37°C for 10 minutes, whereas a negative control sample remained on ice. Using a three-laser-Dako Cyan High Performance flow cytometer (Dako, US) with Summit v 4.3 software, neutrophils were gated on forward and side scatter characteristics; the mean fluorescent intensity (MFI) (sample minus control), corresponding to the number of bacteria engulfed by a single cell, was analysed using a blue-green excitation light 488nm argon-ion laser.

The Phagoburst test (Orpegen Pharma GmbH, Heidelberg, Germany) determined the mean fluorescence per neutrophil from the production of reactive oxidants with or without stimulation. In summary, 4 tubes contained 100 μL of heparinized whole blood were incubated for 10 minutes with one of the following stimuli ; 20 μL of *N*-formylmethionyl-leucyl-phenylalanine (fMLP; 5μM), 20 μL of opsonized *E. coli* (2×10^7 bacteria), 20 μL of phorbol-12-myristate-13-acetate (PMA; 8.1 μM) or without a stimulus at 37°C. The formation of reactive oxidants was monitored by the oxidation of the substrate, from non-fluorescent dihydrorhodamine 123 to rhodamine, which produced green fluorescence, and was analysed with flow cytometry as above.

Cardiovascular measurements

Blood pressure and heart rate were assessed using a semi-automatic oscillometric blood pressure monitor (Dinamap 1846, Critikon) every two minutes: in minutes 12, 14, 16, and 18 of the 20 minute baseline period; and minutes 2, 4, 6, and 8 of the stress task period and recovery period. The same schedule of measurements was used in the control condition.

Data analysis

To check the psychological impact of the stress task, ratings of stress, difficulty, and engagement were scrutinised. Blood pressure and heart rate data were averaged separately across baseline, stress task, and recovery periods and analogously for the control condition and analysed using a 2 (session: stress, control) × 3 (time: baseline, task, recovery) repeated measures ANOVA, using the Greenhouse-Geisser correction. The same analytic strategy was applied to lymphocyte and granulocyte numbers and to the neutrophil function data. Partial eta-squared (η^2) was used as a measure of effect size. Slight variations in degrees of freedom reflect occasional missing data.

Results

Psychological and cardiovascular impact of the stress task

The mean (SD) ratings for the PASAT were 4.0 (1.19), 4.1 (1.34), and 4.6 (1.17) for stress, difficulty, and engagement, respectively. There was a significant main effect of session, for systolic blood pressure (SBP), $F(1,39) = 62.12$, $p < .001$, $\eta^2 = .614$, diastolic blood pressure (DBP), $F(1,39) = 38.56$, $p < .001$, $\eta^2 = .497$, and heart rate (HR), $F(1,39) = 53.33$, $p < .001$, $\eta^2 = .578$. There was also a significant main effect of time for SBP, $F(2,78) = 76.69$, $p < .001$, $\eta^2 = .663$, DBP, $F(2,78) = 36.32$, $p < .001$, $\eta^2 = .482$, and HR, $F(2,78) = 66.49$, $p < .001$, $\eta^2 = .591$. Finally, there were significant session × time interaction effects in each case, $F(2,78) = 111.68$, $p < .001$, $\eta^2 = .741$, $F(2,78) = 51.77$, $p < .001$, $\eta^2 = .570$, and $F(2,78) = 56.44$, $p < .001$, $\eta^2 = .591$, for SBP, DBP, and HR, respectively. These effects are shown in Figure 1.
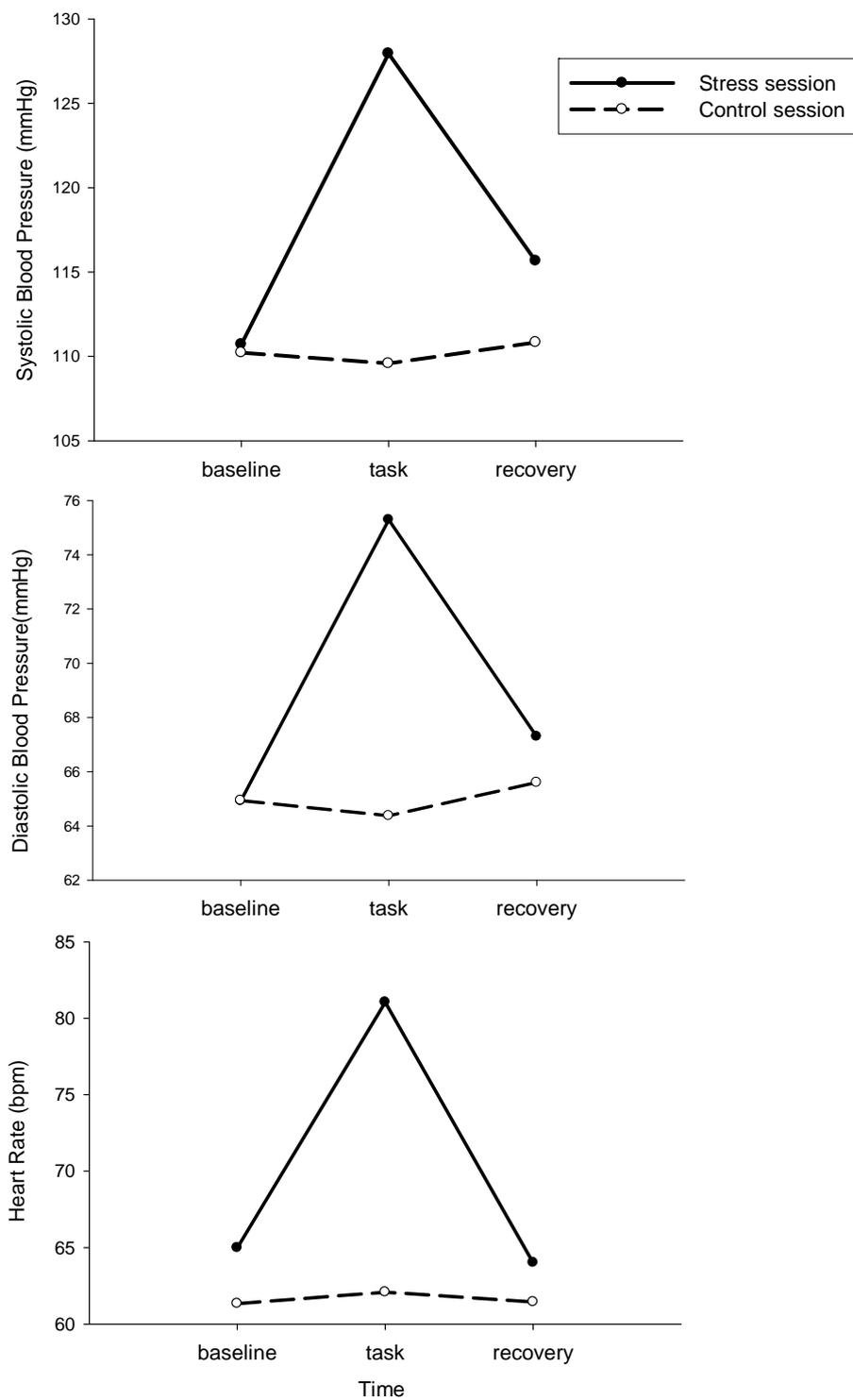
Figure 1: Cardiovascular activity during the stress and control conditions

Lymphocyte and granulocyte numbers

The summary lymphocyte and granulocyte number data are presented in Figure 2. For lymphocytes there was a significant main effect of session, $F(1,39) = 14.52$, $p < .001$, $\eta^2 = .271$, and time, $F(2,78) = 12.24$, $p < .001$, $\eta^2 = .239$. There was also a significant session × time interaction, $F(2,78) = 14.39$, $p < .001$, $\eta^2 = .269$. For granulocytes, there was no main effect of session. However, there was a significant main effect of time, $F(2,78) = 9.88$, $p < .001$, $\eta^2 = .202$, and a session × time interaction, $F(2,78) = 8.94$, $p = .001$, $\eta^2 = .186$. In both cases, it is clear that the acute stress was associated with an increase in circulating leukocytes.
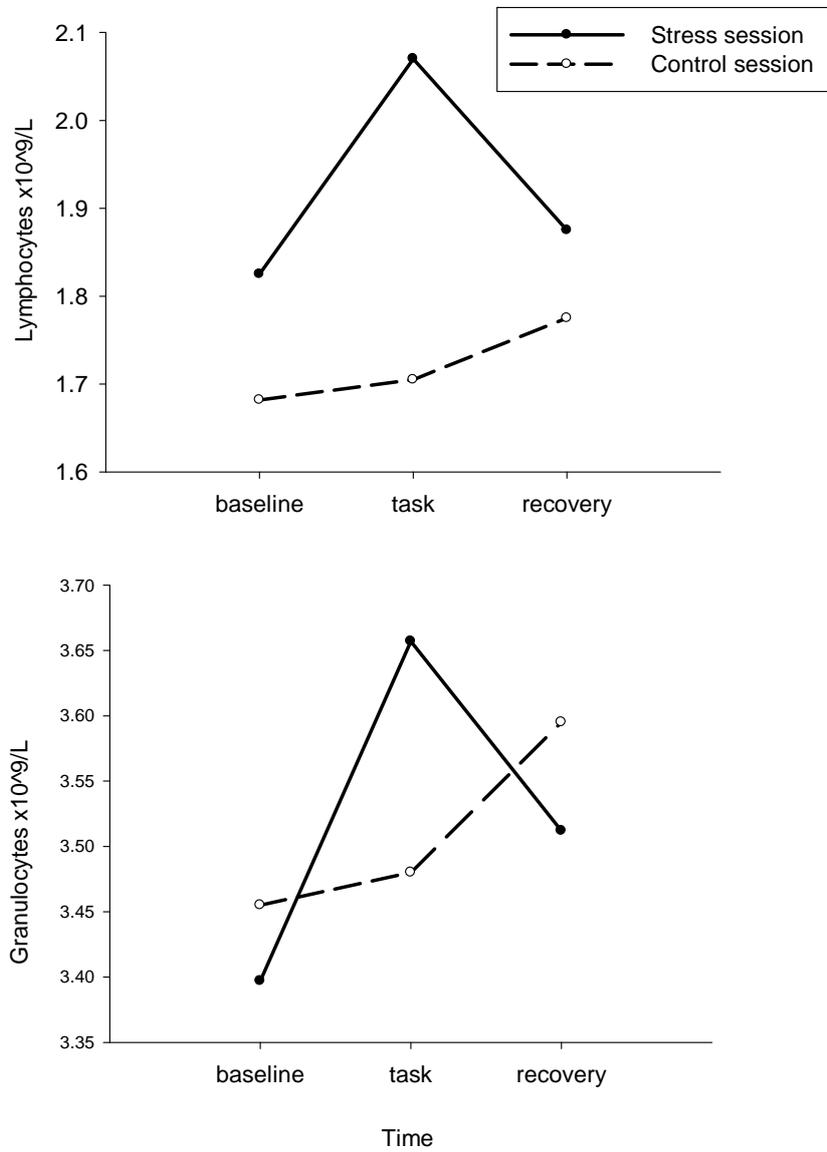
Figure 2: Lymphocyte and granulocyte numbers during the stress and control conditions

Neutrophil function

For neutrophil phagocytosis, there were no main effects of session or time, nor did the session × time interaction effect meet the conventional criteria for statistical significance, $F_{(2,70)} = 2.48$, $p = .09$, $\eta^2 = .066$. However, subsequent orthogonal contrasts revealed a significant session × time interaction for the quadratic component, $F_{(1,35)} = 4.94$, $p = .03$, $\eta^2 = .124$; such that the quadratic component of the trend was more pronounced and convex in the stress condition compared to the control condition, as shown in Figure 3. Thus, the stress task would appear to have enhanced phagocytosis. On the other hand, the overall stress condition would seem to be associated with a decrease in phagocytosis, as reflected in a significant baseline difference between conditions, $F_{(1,38)} = 4.02$, $p = .05$, $\eta^2 = .096$.
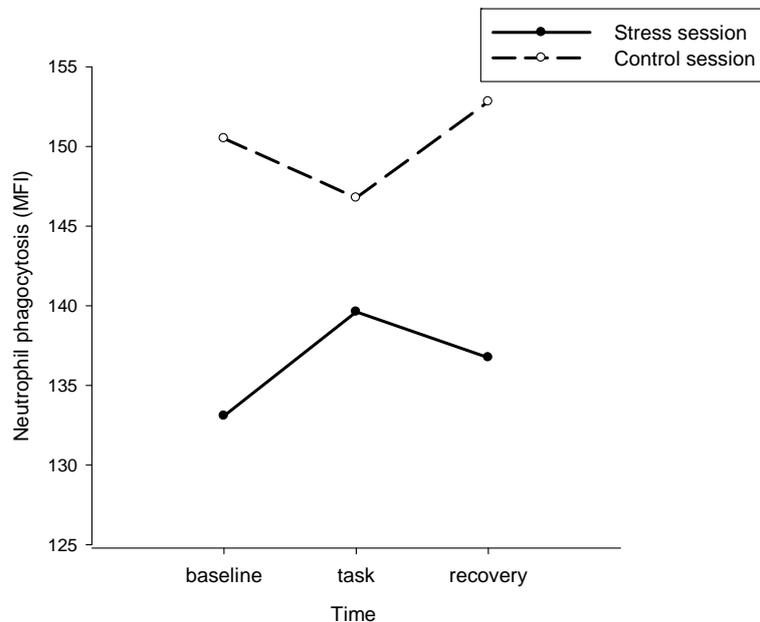
Figure 3: Neutrophil phagocytosis during the stress and control conditions

Much the same pattern of effects emerged for neutrophil superoxide production, when stimulated by fMLP. The session × time interaction effect was significant, $F_{(2,72)} = 4.54$, $p = .02$, $\eta^2 = .112$, and the orthogonal contrasts revealed a significant session × time interaction for the quadratic component, $F_{(1,36)} = 6.83$, $p = .01$, $\eta^2 = .160$. The quadratic component of the trend was convex in the stress condition and was concave in the control condition. This is illustrated Figure 4, which shows that the acute stress increased superoxide production. In this

case, the overall stress condition was not associated with a tonic reduction in superoxide production.

There was no evidence that the acute stress task perturbed neutrophil superoxide production upon stimulation with either *E.coli* or PMA. However, for superoxide production with PMA, there was a trend towards an overall session effect, $F(1,32) = 3.23$, $p = .08$, $\eta^2 = .092$. As shown in Figure 4, the stress condition tended to be associated with a tonic reduction in superoxide production. For *E.coli*, there was a significant time effect, $F(2, 64) = 3.19$, $p = .05$, $\eta^2 = .091$; in both conditions superoxide production declined over time (see Figure 4).
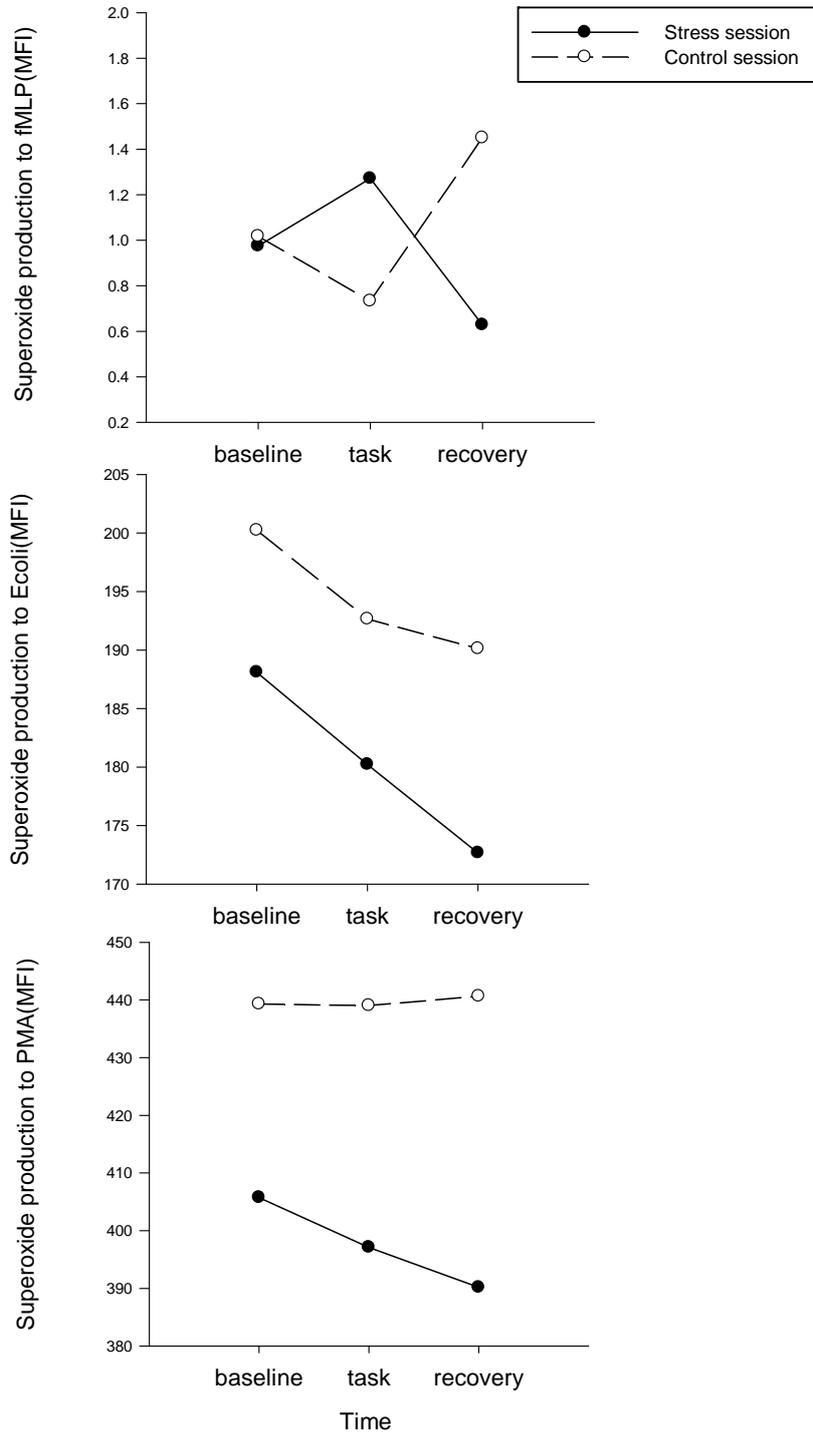
Figure 4: Neutrophil superoxide production upon stimulation with fMLP, *E.coli,* and PMA
respectively.

Association between cardiovascular and neutrophil activity during acute stress

There were no significant correlations between cardiovascular activity during stress and neutrophil function. Neutrophil phagocytosis was positively but not significantly related to superoxide production upon stimulation with fMLP, r (37) = .26, *p* =.11, during the acute stress task.

Discussion

The self report and cardiovascular data confirmed that the acute stress task provoked emotional and physiological responses. Similarly, as expected, there was an acute stress-induced increase in lymphocyte and granulocyte numbers. The effects of stress on neutrophil function, however, were more complex. For neutrophil phagocytosis, there was an acute increase in phagocytosis associated with the stress task. However, we also observed a more tonic reduction in phagocytosis across the stress session, particularly evident in the significant baseline difference. The acute stress task also elicited an increase in neutrophil superoxide production to the bacterial peptide fMLP. Finally, there was some evidence of a tonic reduction in neutrophil superoxide production to PMA during the stress session.

The present study is the first we know of to examine the effects of psychological stress in humans using assays which measure neutrophil function via flow cytometry techniques. Accordingly, the existing literature offers little help in establishing a precedent for our findings. However, the effects of the acute stress task on neutrophil phagocytosis and superoxide production observed here are consistent with the increase in neutrophil activation to a short mental stressor reported previously (Ellard et al., 2001). Similarly, our phasic results can be considered broadly in line with the increase in neutrophil superoxide release with fMLP stimulation during examination stress (Kang et al., 1996; Kang et al., 1997). However, it should be conceded that academic examination stress may have more in common in terms of chronicity with our stress session as a whole. Thus, given the apparent elevation of superoxide release reported during academic stress, we might have expected to find an overall increase in neutrophil function throughout the stress session, whereas we actually observed a relative suppression of

neutrophil phagocytosis of *E.coli* and superoxide production to PMA. It is possible that this tonic suppression of neutrophil function resulted from the instructions given to participants at the beginning of each session making it explicit whether they were attending a control or a stress session. Therefore, in retrospect it is likely that in addition to the acute stress exposure, we also imposed a more tonic stress load. Although cardiovascular activity and circulating lymphocyte numbers did not seem to be affected, neutrophils, and particularly aspects of their function, appeared to be specifically sensitive to this mild tonic stress. Although there are no published studies of unambiguously chronic psychological stress and neutrophil function, the tonic suppression of superoxide generation has been observed in response to the chronic physical stress of hip fracture in older adults (Butcher et al., 2005).

The present study might be considered to have a number of limitations. First, the sample size was fairly modest. However, it was of the same order of magnitude as previous studies of stress and human neutrophil function (Ellard et al., 2001; Kang et al., 1996; Kang and Fox, 2000) and larger than studies examining the effects of acute psychological stress on other immune outcomes (Bosch et al., 2005; Burns et al., 2008). Further, study numbers were reasonable given the temporal demands of the current protocol, with cannulation, two testing sessions, and same-day flow cytometry assays. Second, the within subject design could be considered another limitation. However, such a design increases the power to detect effects and it is possible that without such a design we would not have detected the tonic effects. The inclusion of a control session would seem necessary to discount the possibility that changes in neutrophil function simply reflected temporal variation or the impact of cannulation (Burns et al., 2008). Finally, despite the advantages of the flow cytometry assays, they are sensitive to diurnal and day-to-day variation (Hirt et al., 1994; Kampen et al., 2004). However, such effects were minimised by counterbalancing the sessions, testing at the same time of day, and the use of internal controls for each assay.

In conclusion, the present study revealed both phasic and tonic effects of psychological stress on neutrophil function. The acute stress task elicited a short-term elevation phagocytosis and superoxide production. In addition, there was evidence that the more tonic stress load of the stress session as a whole was associated with suppressed phagocytosis and superoxide

production. Clearly, the present results must be regarded as preliminary and replication is necessary. Given that neutrophil function has been shown to decline with age (Lord et al., 2001), future studies might consider the effects of age on the tonic and phasic effects of acute psychological stress.

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CHAPTER THREE

ALTERED HUMAN NEUTROPHIL FUNCTION IN RESPONSE TO ACUTE
PSYCHOLOGICAL STRESS

Abstract

There is mounting evidence that acute stress is associated with short-term increases in a number of immune indices. The present study examined the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis of *E.coli* and stimulated superoxide production, in human neutrophils. Participants were 40 (20 female, 20 male) university students. Blood samples to determine neutrophil function by flow cytometry were taken at the end of resting baseline, during an acute stress task and during recovery. The stress task was a 10-minute time-pressured mental arithmetic challenge with social evaluation. There was an acute increase in phagocytic ability and a reduction of superoxide production associated with the stress task relative to baseline. It would appear that neutrophil function is sensitive to acute psychological stress. However, different aspects of neutrophil bactericidal function appear to be differentially affected by acute stress.

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Acute and chronic psychological stress appear to have a differential impact on the immune system. Generally, chronic stress has been shown to have a negative effect on various aspects of immunity, whereas acute stress would seem to be immune enhancing (Segerstrom and Miller, 2004). For example, acute stress has been shown to elicit lymphocytosis (Anane et al., 2009; Willemsen et al., 2002), increase natural killer cell cytotoxicity (Bosch et al., 2005; Sgoutas-Emch et al., 1994) and secretory immunoglobulin A secretion rate (Ring et al., 2002), stimulate aspects of the complement system (Burns et al., 2008), and boost vaccination responses (Edwards et al., 2008). However, little is known about the effects of acute stress on human neutrophils and particularly their bactericidal function. This is perhaps surprising given that neutrophils are a major component of innate immunity and are the dominant leukocyte in the circulation. These cells play a crucial role in killing invading pathogens, particularly rapidly dividing bacteria, and are key cellular components of the early phase of inflammatory responses (Nathan, 2006). Neutrophils act quickly and without specificity and have the ability to produce a range of cytotoxic and bactericidal molecules such as reactive oxygen species (superoxide production) and proteolytic enzymes (Nauseef, 2007). Superoxide production is a means by which neutrophils eliminate pathogens, and thus is key in combating infections, by for example pneumococcal bacteria (Segal et al., 2006). However, excessive neutrophil activation and superoxide production can cause local tissue inflammation and damage, an example of oxidative stress (Cossette et al., 2008; Mayadas et al., 2009). Further, tissue damage via neutrophil activation is implicated in the aetiology and aggravation of inflammatory disease (Mayadas et al., 2009; Nathan, 2006). Finally, neutrophils also play a role in acquired immunity acting as antigen presenting cells (Cowburn et al., 2008).

The effect of stress on neutrophils has been examined in a series of studies of asthma (Kang et al., 1998; Kang et al., 1996; Kang et al., 1997). Academic examinations served as the source of psychological stress and neutrophil function was assessed before, during, and after an examination period, using the superoxide dismutase-inhibitable reduction of ferricytochrome C assay to measure superoxide release. These authors found some indication that examination stress increased superoxide release. However, it has been argued that a prolonged examination period constitutes chronic rather than acute stress; whereas a single examination elicited an

increase in secretory immunoglobulin A, a prolonged period of academic examinations was associated with a decrease (Bosch and Carroll, 2007). More recently, a 15 minute laboratory stress task (Raven`s progressive matrices) was used to examine neutrophil activation using the Nitro-blue Tetrazolium assay which indicates oxidative capacity (Ellard et al., 2001). The acute stress was shown to increase neutrophil activation relative to baseline. However, neither of these assays are optimally sensitive measures of neutrophil function. The superoxide dismutase-inhibitable reduction of ferricytochrome C assay only detects superoxide release that has escaped from the cell into the medium, which may represent only a fraction of the superoxide actually generated (Kang and McCarthy, 1994). The Nitro-blue Tetrazolium assay simply measures, by microscopic inspection of staining, the percentage of non-specifically activated neutrophils (Ellard et al., 2001). Both of these measures represent a very imperfect proxy of superoxide release. In contrast, assays that exploit flow cytometry technology to measure the effects of stimulation and accordingly offer a more accurate and versatile means of examining different aspects of neutrophil function such as phagocytosis and superoxide production in response to a range of stimuli (Kampen et al., 2004; Lehmann et al., 2000; Panasiuk et al., 2005). In addition, physiologically relevant agents such as opsonised bacteria, can be used to elicit the neutrophil response.

Consequently, the present study examined the effects of an acute laboratory psychological stress task on neutrophil function, specifically phagocytosis and stimulated superoxide production, using flow cytometry based assays. Self report and cardiovascular measurements were also taken as a manipulation check, since the effects of the stress task on these variables are well characterised (Phillips et al., 2009; Willemsen et al., 2002). It was hypothesised that acute stress would enhance neutrophil function relative to baseline.

Method

Participants

Forty healthy participants (20 female, 20 male) were recruited from the student population at the University of Birmingham between May 2008 and June 2009. Date of birth, sex, ethnicity,

parental occupation, health and smoking status were determined by standard questionnaire. Participants mean (SD) age was 25.9 (4.56) years and mean body mass index was 23.3 (2.53) kg/m^2. The majority (90%) reported their ethnicity as white and that they were non-smokers and 74% reported that they came from non-manual occupational households. Individuals were excluded if they had an acute infection or suffered from an immune disorder.

Design and Procedure

All participants attended a two-hour laboratory acute psychological stress session. They were asked to avoid alcohol and vigorous exercise for 12 hours, and food and caffeine for one hour prior to testing. On the testing day, participants entered the laboratory at 9.30 a.m. Their height and weight were measured. An intravenous cannula was then inserted in the anticubital region of one arm and a blood pressure cuff was attached to the other arm. Following instrumentation and laboratory adaptation, there was a baseline resting period of 20 minutes, a 10-minute psychological stress task, and a10-minute resting recovery period. The study was approved by the local Medical Research Ethics Committee, and all participants provided written informed consent.

Acute stress task

Participants undertook an acute psychological mental stress task, the Paced Auditory Serial Addition Test (PASAT), which has been shown to elicit cardiovascular changes in many studies (Burns et al., 2008; Phillips et al., 2009; Willemsen et al., 2002). Participants were presented with a series of single-digit numbers by audio-CD and requested to add each number to the immediately preceding number and say the answer aloud while retaining the second of the pair in memory for addition to the next number presented. The 10-minute task consisted of five consecutive and continuous periods of two minutes, with numbers presented at speeds of 50, 65, 75, 100, and 125 digits at presentation rates of 2.4, 2.0, 1.6, 1.2**,** and 0.8 seconds, respectively. All participants were given an explanation of the task and an opportunity to practice prior to its commencement. In order to add to the challenging nature of the task, elements of competition and social evaluation were added. A false leader board was in view of the participant, who was

instructed to try to beat the scores on the board. A confederate experimenter scored the answers overtly while sitting on a high stool at a distance of 1 metre facing the participant. Participants were seated in front of a large television screen which allowed them to see themselves live throughout the test, and were instructed to look at the screen at all times. They were also informed that they were being video-taped and that videos were to be assessed by "independent body language experts"; no such assessment was made. The confederate experimenter also sounded a loud aversive buzzer once during the first five of every ten trials. Participants began the test with a score of 1000 points; for each incorrect or missed answer 5 points were deducted from their score. Immediately following the task, they rated how stressful, difficult, and engaging the task was on a 7- point scale, where 0 equalled "not at all" and 6 equalled "extremely".

Blood sampling and Immune Assays

Blood samples were taken at three time points: at the end of the resting baseline, in the final minute of the stress task, and at the end of the recovery period. At each time point, blood was collected into one EDTA tube and one heparin tube which was placed immediately on ice, in addition to a plain tube for serum cortisol level measurements. The EDTA tubes were used to count lymphocyte and granulocyte numbers using a Coulter$^{®}$ A^{C} • T diff™ Analyzer (Beckman Coulter, Inc., Miami, Florida, USA). The heparin tubes were used to assess neutrophil function, specifically bacteria-induced phagocytosis and oxidative burst (superoxide production) by flow cytometry. Serum was collected and frozen from the plain tube sample at -20°C until assay for cortisol levels, performed by ELISA using commercially available kits (IBL International, Hamburg, Germany)

Phagotest kits (Orpegen Pharma GmbH, Heidelberg, Germany) measured neutrophil phagocytosis of fluorescein isothiocyanate-labeled (FITC) opsonized *Escherichia coli* (*E. coli*) bacteria. Whole blood (100 μL) was incubated with 20 μL of bacteria (2×10^7) at 37°C for 10 minutes, whereas a negative control sample remained on ice. Using a three-laser-Dako Cyan High Performance flow cytometer (Dako, US) with Summit v 4.3 software, neutrophils were gated on forward and side scatter characteristics; the mean fluorescent intensity (MFI)

corresponding to the number of bacteria engulfed by a single cell, was measured using a blue-green excitation light 488nm argon-ion laser.

The Phagoburst test (Orpegen Pharma GmbH, Heidelberg, Germany) determined the mean fluorescence per neutrophil from the production of reactive oxidants with or without stimulation. In summary, 4 tubes contained 100 µL of heparinized whole blood were incubated for 10 minutes with one of the following stimuli; 20 µL of N-formylmethionyl-leucyl-phenylalanine (fMLP; final concentration 0.83 µM), 20 µL of opsonized $E.\ coli$ (2×10^7 bacteria), 20 µL of phorbol-12-myristate-13-acetate (PMA; final concentration 1.35 µM) or without a stimulus at 37°C. The formation of reactive oxidants was monitored by the oxidation of the substrate, from non-fluorescent dihydrorhodamine 123 to rhodamine, which produced green fluorescence, and was analysed with cytometry as described above.

Cardiovascular measurements

Blood pressure and heart rate were assessed using a semi-automatic oscillometric blood pressure monitor (Dinamap 1846, Critikon) every two minutes: in minutes 12, 14, 16, and 18 of the 20 minute baseline period; and minutes 2, 4, 6, and 8 of the stress task period and recovery period.

Statistical analysis

To check the psychological impact of the stress task, ratings of stress, difficulty, and engagement were scrutinised. Blood pressure and heart rate data were averaged separately across baseline, stress task, and recovery periods and analysed using a repeated measures ANOVA (baseline, task, recovery), with the Greenhouse-Geisser correction. The same analytic strategy was applied to lymphocyte and granulocyte numbers and to the neutrophil function data. Partial eta-squared (η^2) was used as a measure of effect size. Slight variations in degrees of freedom reflect occasional missing data.

Results

Psychological and cardiovascular impact of the stress task

The mean (SD) ratings for the PASAT were 4.0 (1.19), 4.1 (1.34), and 4.6 (1.17) for stress, difficulty, and engagement, respectively. There was a significant main effect of time, for systolic blood pressure (SBP), $F(2,78) = 119.53$, $p < .001$, $\eta^2 = .754$, diastolic blood pressure (DBP), $F(2,78) = 52.05$, $p < .001$, $\eta^2 = .572$, and heart rate (HR), $F(2,78) = 67.83$, $p < .001$, $\eta^2 = .635$. These effects are shown in Figure 1.

Lymphocyte and granulocyte numbers

The summary lymphocyte and granulocyte number data are also presented in Figure 1. For lymphocytes there was a significant main effect of time, $F(2,78) = 14.21$, $p < .001$, $\eta^2 = .267$. For granulocytes, there was also a significant main effect of time, $F(2,78) = 10.22$, $p < .001$, $\eta^2 = .208$. In both cases, it is clear that the acute stress was associated with a transient increase in circulating leukocytes which decreased following cessation of the task.
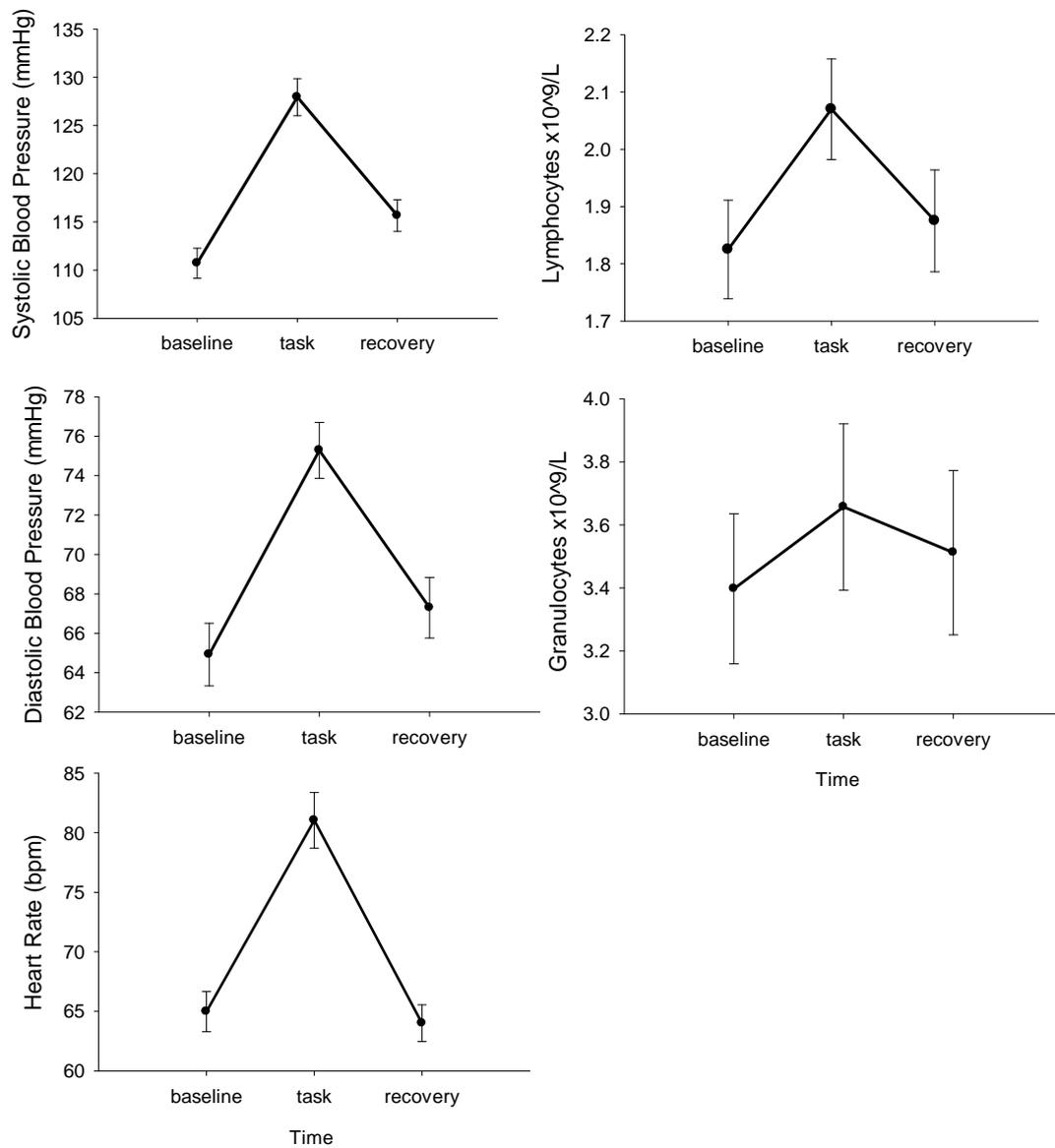
Figure 1: Cardiovascular activity and lymphocyte and granulocyte numbers during baseline, stress, and recovery.

Neutrophil function

For neutrophil phagocytosis measurements, all samples showed that 100% of the neutrophils were able to phagocytose the *E.coli* and differences seen were in the number of bacteria ingested (indicated by differences in MFI). There was a significant main effect of time on neutrophil phagocytic capacity, $F(2,78) = 3.20$, $p = .047$, $\eta^2 = .076$, as shown in Figure 2. Thus, the stress task appeared to have enhanced phagocytosis.

For superoxide production, when granulocytes were stimulated by *E.coli*, the opposite pattern of effects emerged. There was a significant reduction in superoxide production across the session, $F(2,72) = 4.03$, $p = .026$, $\eta^2 = .101$. This is illustrated Figure 2. There was evidence of a similar decline in superoxide production to fMLP, $F(2,72) = 1.18$, $p = .31$, $\eta^2 = .032$, and PMA, $F(2,68) = 0.76$, $p = .44$, $\eta^2 = .022$, but these decreases were not statistically significant (see Figure 2). There was no effect of gender on neutrophil responses to the stress task.
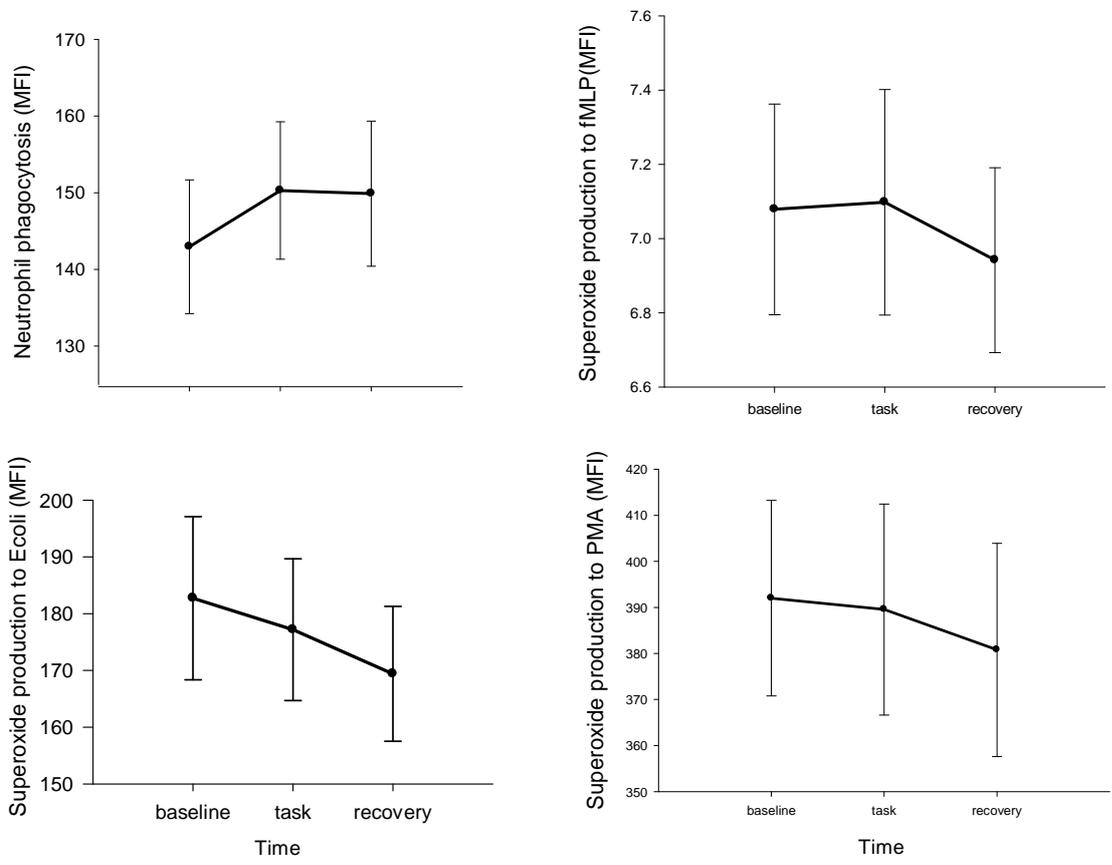
Figure 2: Neutrophil phagocytosis of *E.coli* and superoxide production upon stimulation with fMLP, *E.coli* or PMA during baseline, stress, and recovery.

Association between cardiovascular, cortisol and neutrophil activity during acute stress

There was no significant main effect of time on serum cortisol level across the session, $F(2,76) = 1.11$, $p = .32$, $\eta^2 = .028$, as shown in Figure 3. The mean (SD) of the raw cortisol data in nmol/l, at the three time points were: 138.2(73.7), 139.7(64.2) and 146.0(59.3) at baseline, task and recovery, respectively. Figure 3 shows the pattern of cortisol across the session.
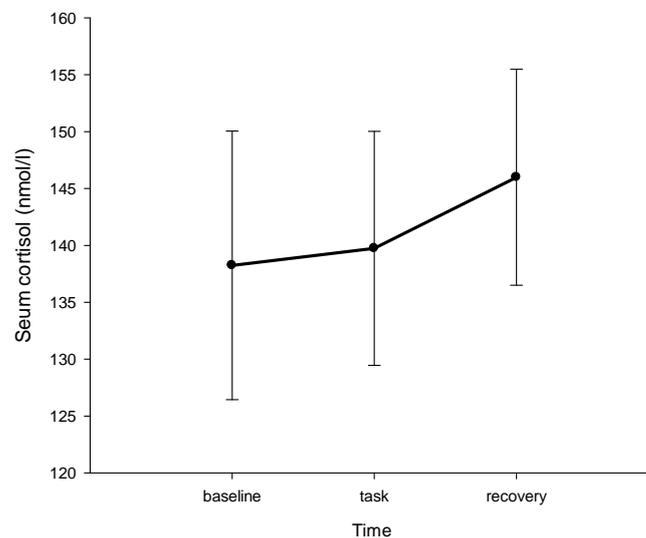
Figure 3: Serum cortisol levels at baseline, stress, and recovery in nmol/l (error bars are standard error of the mean).

Cortisol reactivity (calculated as recovery minus baseline value) was not significantly correlated with cardiovascular reactivity, or with neutrophil phagocytosis, superoxide production, or granulocyte reactivity.

Discussion

The self report and cardiovascular data confirmed that the acute stress task provoked emotional and physiological responses. Similarly, as expected, there was an acute stress-induced increase in lymphocyte and granulocyte numbers. The effects of stress on neutrophil function, however, were more complex. For neutrophil phagocytosis, there was an acute increase in phagocytosis associated with the stress task. However, the acute stress task was associated with reduction in neutrophil superoxide production when stimulated with *E.coli*. The present study is the first we know of to examine the effects of psychological stress in humans using assays which measure neutrophil function via flow cytometry techniques and in response to physiologically relevant stimuli. Accordingly, the existing literature offers little help in establishing a precedent for our findings. However, the effects of the acute stress task on neutrophil phagocytosis observed here are consistent with the increase in neutrophil activation to a short mental stressor reported previously (Ellard et al., 2001). Superoxide production in the present study decreased in contrast to the increase with examination stress shown in previous reports (Kang et al., 1996; Kang et al., 1997). However, it should be noted that academic examination stress over several weeks cannot be considered to be acute psychological stress. Further, studies evaluating neutrophil function and regulation have shown that phagocytosis and intracellular bacterial killing are independent activities of neutrophil-mediated antibacterial defense, thus can show different patterns of effect (Pechkovsky et al., 1996; Vollebregt et al., 1998).

Neutrophil function was differentially sensitive to acute psychological stress in the present study. However, cardiovascular activity did not appear to be related to neutrophil function. This is surprising, given that neutrophils have been shown to have alpha and beta adrenergic receptors (Abraham et al., 1999; Gosain et al., 2009), and changes in adrenalin and noradrenalin have been shown to relate to circulating neutrophil numbers (Abraham et al., 1999). We hypothesised that the effects observed in the present study might be mediated by other stress pathways such as the HPA axis. Indeed, many psychoneuroendocrinology studies have shown that the HPA axis can be activated within 10-20 minutes of different laboratory stress tasks (Dickerson and Kemeny, 2004; Kudielka and Kirschbaum, 2005). A meta-analysis in 2004 investigated 208 laboratory-based stress studies, have showed that majority of stress tasks elicited HPA axis activation and

cortisol responses(Dickerson and Kemeny, 2004). Others have shown *in vitro* that cortisol and other steroids like dexamethasone can decrease superoxide production (Hoffmann-Jagielska et al., 2003; Ignacchiti et al., 2011). Consequently, the declining superoxide production to *E.coli* observed here might relate to the effects of the acute stress task on cortisol. However, cortisol reactivity was not significantly correlated with neutrophil phagocytosis or superoxide production in this study, when cortisol reactivity was calculated as the recovery concentration minus baseline due to the known delay in the cortisol response to acute stress. Thus, it is difficult to elucidate the exact mechanisms behind the different phagocytosis and superoxide production results observed here, given that the regulation of neutrophil function is under the control of many different receptors for a variety of cytokines (Pechkovsky et al., 1996), which are also differentially affected by stress (Steptoe et al., 2001; Yamakawa et al., 2009). Further, the question where the neutrophils are coming from should be considerd given the significant granulocytosis observed as a result of acute stress. It is known that neutrophil homeostasis is maintained by a fine balance between granulopoiesis, bone marrow storage and release, intravascular margination, clearance and destruction (von Vietinghoff and Ley, 2008). So the granulocytosis as a result of acute stress here is perhaps due to the demargination of neutrophils already in the vasculature . Margination is a process refer to is the prolonged transit of neutrophils through specific organs, which results in discrete intravascular marginated pools these can be found within the spleen, liver, bone marrow and the lung. It is therefore considered that a proportion of the granulocytes that exit the circulation could be mobilized back into this freely circulating pool. Also other steroids like prednisolone increases the size of both the circulating and marginated pools, while exercise and adrenaline cause a shift of cells from the marginated to the circulating pool. Whether this is also involved in the acute stress effect on neutrophil number, remains unknown, and whether the changes observed in the present study in neutrophil function is affected by the arrival of demarginated neutrophils which might have different functional capacity is also a possibility(Mantovani et al., 2011; Nakagawa et al., 1998; von Vietinghoff and Ley, 2008).

The present study might be considered to have some limitations. First, the sample size was fairly modest. However, it was of the same order of magnitude as previous studies of stress and

human neutrophil function (Ellard et al., 2001; Kang et al., 1996; Kang and Fox, 2000) and larger than studies examining the effects of acute psychological stress on other immune outcomes (Bosch et al., 2005; Burns et al., 2008). Further, study numbers were reasonable given the temporal demands of the current protocol, with cannulation, time of testing session, and same-day flow cytometry assays. Second, despite the advantages of the flow cytometry assays, they are sensitive to diurnal and day-to-day variation (Hirt et al., 1994; Kampen et al., 2004). However, such effects were minimised by, testing at the same time of day, and the use of internal controls for each assay.

   In conclusion, the present study revealed the effects of psychological stress on neutrophil function. The acute stress task elicited a short-term elevation in phagocytosis and a reduction of superoxide production. Clearly, the present results must be regarded as preliminary and replication is necessary. Given that neutrophil function has been shown to decline with age (Lord et al., 2001), future studies might consider the effects of age on the effects of acute psychological stress. In addition, the underlying mechanisms of acute stress effects on neutrophil function warrant further investigation.

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CHAPTER FOUR

REDUCED NEUTROPHIL SUPEROXIDE PRODUCTION AMONG HEALTHY
OLDER ADULTS AS A RESULT OF ACUTE PSYCHOLOGICAL STRESS

Abstract

Ageing is known to be associated with higher morbidity and mortality, and with declining immune function parameters (immunesenescence) including neutrophils, key players in innate immunity. The present study examined both the effects of an acute psychological stress task on neutrophil function in older adults and whether there is a combined effect of ageing and acute stress on neutrophil function.

Participants were 17 (11 female) older adults (mean age 75.7±7.06). Blood samples to determine neutrophil function by flow cytometry were taken at the end of resting baseline, during an acute stress task and during recovery. The two functional assays measured were phagocytosis of *E.coli* and stimulated superoxide production. The stress task was an 8-minute time-pressured mental arithmetic challenge with social evaluation. Results were compared to those from a previous study in younger adults tested using the same protocol.

For the older sample, there was a significant reduction in neutrophil superoxide production, $p =.017$, $\eta^2_p= .240$, associated with the stress task relative to baseline, but no effect on phagocytosis. In comparison to the younger adults, older participants had lower superoxide production and showed a more pronounced decline in superoxide production in response to acute stress. They also had higher overall cortisol and cortisol:DHEAS ratio.

The results of this study could contribute to explaining the increased risk of infection in older adults, particularly those subject to frequent stress exposures. Future research is needed to explore the underlying mechanisms of acute stress effects on human neutrophil function in both young and older adults in greater detail.

Key words: acute psychological stress**;** neutrophil; phagocytosis; superoxide production

Neutrophils are short-lived white blood cells that are produced in large numbers in the bone marrow and circulate in the blood, forming a major part of our non-specific innate immunity, particularly combating infections by rapidly dividing bacteria, yeast and fungi. They are equipped with a variety of antimicrobial mechanisms in order to eradicate pathogens via different killing mechanisms, including cytoplasmic granules containing bactericidal agents and the production of reactive oxygen and nitrogen species; neutrophils can also cause collateral tissue damage when these tissues are exposed to these cytotoxic components (Cowburn et al., 2008; Nauseef, 2007; Segal, 2005, 2006).

With ageing, in a process called immunesenescence, different components of the immune system are reduced in function (Shaw et al., 2010). The ability of neutrophils to ingest and kill different pathogens is significantly attenuated in older adults (Christy et al., 2010; Di Lorenzo et al., 1999; Hajishengallis; Lord et al., 2001; Tortorella et al., 2001; Wessels et al., 2010), which has implications for the rates of morbidity and mortality from infectious disease in this population (Adib-Conquy et al., 2008; Asberg et al., 2008; Atzpodien and Reitz, 2008; Butcher et al., 2005; Glynn et al., 1999; Korkmaz et al., 2010). Several studies have shown that neutrophil phagocytosis of microbes (Ginaldi et al., 1999; Ginaldi et al., 2001; Hajishengallis, 2010; Schroder and Rink, 2003; Shaw et al., 2010; Weiskopf et al., 2009),and subsequent production of reactive oxygen species (Ginaldi et al., 1999; Ginaldi et al., 2001; Schroder and Rink, 2003; Shaw et al., 2010) is reduced with ageing and our own studies revealed that the decline in phagocytosis is associated with reduced expression of the phagocytic receptor CD16 (Butcher et al., 2001). However, some studies show that normal phagocytic capacity is retained in older age (Pawelec et al., 1998; Wessels et al., 2010).

Studies in the psychoneuroimmunology field and others have shown that neutrophils are sensitive to different physical and psychological stressors. For instance, the psychological stress of an examination period among students was shown to be associated with altered neutrophil functions in two studies (Kang et al., 1997; Kang and McCarthy, 1994). In a more recent study, psychological stress among postgraduate students who were completing their final thesis examination was associated with significant reduction in neutrophil superoxide production compared to matched controls (Ignacchiti et al., 2011). Other studies which have investigated

the effect of acute psychological stress on neutrophil function have shown that neutrophil phagocytosis and superoxide production can be altered even with short duration stress (Ellard et al., 2001; Khanfer et al., 2010; Saigo et al., 2008) and our group has shown recently that in young healthy students, neutrophil phagocytosis increased whereas superoxide production decreased as a result of a brief laboratory acute mental stress task (Khanfer et al., 2010).

One of the mechanisms by which neutrophil function might be altered in response to stress may be via the action of the hypothalamic pituitary adrenal (HPA) axis. Both cortisol and DHEAS are hormonal outputs of the HPA axis. It is known that cortisol has mainly immunosuppressive actions while DHEAS is immune enhancing (Hazeldine et al., 2010). Further, previous research indicates that with ageing there is imbalance between the two hormone levels, due to the reduction in DHEAS levels from age 30 years onwards (adrenopause), and relatively stable cortisol levels (Orentreich et al., 1984; Phillips et al., 2007). This increased cortisol:DHEAS ratio is thought to contribute to immunesenescence (Buford and Willoughby, 2008). Crucially, we have shown recently that neutrophil superoxide generation can be enhanced directly by DHEAS *in vitro* (Radford et al., 2010), and DHEAS can overcome the suppressive effects of cortisol on neutrophil function (Butcher et al., 2005; Radford et al., 2010). Alterations in levels of cortisol and DHEAS may therefore underlie any changes seen in neutrophil function with acute stress, particularly in older participants.

Although there are many studies on adaptive immune system changes with ageing, and the impact of psychological stress on adaptive immunity, there are relatively few examining the effect of psychological stress on the innate immune system with age. The effect of chronic stress, including a physical stress such as hip fracture, was associated with reduced neutrophil superoxide generation in older adults (Butcher et al., 2003; Butcher et al., 2005). Our group has also recently demonstrated that the stress of bereavement was also associated with reduced neutrophil superoxide production and an increased cortisol:DHEAS ratio among bereaved older adults compared to their healthy sex and age-matched controls (Khanfer et al., 2011). As far as we are aware, the effect of acute psychological stress on neutrophil function in older adults has not been previously investigated. The present novel study examined the effects of acute stress on neutrophil function among older adults, and investigated levels of cortisol, DHEAS, and their

ratio during acute stress as a potential mechanism. It also sought to examine whether there is a combined effect of ageing and psychological stress on neutrophil function among older adults by comparing the present study in an older subject sample with our previous young subject sample as a control group. To our knowledge this is the first study investigating these associations.

Method

Participants

Seventeen participants (11 females and 6 males) were recruited from the community in Birmingham, UK, between May 2010 and February 2011. Date of birth, sex, ethnicity, current/previous occupation, health, and smoking status were determined by standardised questionnaire. Participants' mean (SD) age was 75.7 (7.1) years and body mass index (BMI) was 24.9 (1.9) kg/m^2. All participants (100%) reported their ethnicity as white and 88% reported that they were non-smokers; 88% reported that they came from non-manual occupational households. Individuals were excluded if they had an acute infection or suffered from an immune disorder. These older participants were compared to a younger control group (N = 40) with a mean (SD) age of 25.9 (4.6) years tested using an identical protocol; the younger sample details are published elsewhere (Khanfer et al., 2010).

Design and procedure

All participants attended one laboratory session; an acute psychological stress session. They were asked to avoid alcohol and vigorous exercise for 12 hours, and food and caffeine for one hour prior to testing. Each session was of one hour and half duration. On the testing day, participants entered the laboratory at 9.30 a.m. Their height and weight were measured using standard equipment. An intravenous cannula was then inserted in the anticubital region of one arm and a blood pressure cuff was attached to the other arm. Following instrumentation and laboratory adaptation, there was a formal baseline resting period of 20 minutes, an 8-minute psychological stress task, and a 10-minute resting recovery period. The study was approved by the local NHS Research Ethics Committee, and all participants provided written informed consent. Assays for neutrophil function (phagocytosis and superoxide production) were performed on the same day. Serum was frozen for later assay of the levels of the stress

hormones cortisol and DHEAS. During the baseline and recovery periods, all participants completed a questionnaire pack.

Acute stress task

During the session all participants undertook an acute psychological mental stress task, the Paced Auditory Serial Addition Test (PASAT), which has been shown by our group and others to elicit cardiovascular changes (Bosch et al., 2005; Hirvikoski et al., 2011; Khanfer et al., 2010; Phillips et al., 2009; Willemsen et al., 2002). Participants were presented with a series of single-digit numbers by audio-CD and requested to add each number to the immediately preceding number and say the answer aloud while retaining the second of the pair in memory for addition to the next number presented. The 8-minute task consisted of four consecutive and continuous periods of two minutes, with numbers presented at speeds of 50, 65, 75, and 100 digits at presentation rates of 2.4, 2.0, 1.6, and 1.2 seconds, respectively. All participants were given an explanation of the task and an opportunity to practice prior to its commencement. In order to add to the challenging nature of the task, elements of competition and social evaluation were added. A false leader board was in view of the participant, who was instructed to try to beat the scores on the board. A confederate experimenter scored the answers overtly while sitting on a high stool at a distance of 1 metre facing the participant. Participants were seated in front of a large television screen which allowed them to see themselves live throughout the test, and were instructed to look at the screen at all times. They were also informed that they were being video-taped and that videos were to be assessed by "independent body language experts"; no such assessment was made. The confederate experimenter also sounded a loud aversive buzzer once during the first five of every ten trials. Participants'` total scores were calculated by multiplying the number of correct answers by five. Immediately following the task, they rated how well did they perceived they performed on the task and how stressful, difficult, exciting, confusing, and engaging the task was on a 7-point scale, where 0 equalled "not at all" and 6 equalled "extremely".

Blood sampling and immune assays

Blood samples were taken at three time points: at the end of the resting baseline, in the final minute of the stress task, and at the end of the recovery period. At each time point, blood was

collected into one EDTA tube, one plain tube, and one heparin tube which was placed immediately on ice. The EDTA tubes were used to count lymphocyte and granulocyte numbers using a Coulter® A^C • T diff™ Analyzer (Beckman Coulter, Inc., Miami, Florida, USA). Serum was collected and frozen from the plain tube sample at -20°C until assays for cortisol and DHEAS levels, performed by ELISA using commercially available kits (IBL International, Hamburg, Germany). The heparin tubes were used to assess neutrophil function, specifically *E.coli*-induced phagocytosis and oxidative burst (superoxide production) by flow cytometry.

Neutrophil phagocytic ability and efficiency were assayed in whole blood using a commercial kit (Phagotest, Glycotope Biotechnology GmbH, Heidelberg, Germany) which measures uptake of fluorescein isothiocyanate-(FITC) labeled opsonized *E. coli*. Flow cytometry (Dako Cyan, Carpinteria, California) was used to gate on neutrophils and determine the mean fluorescence intensity (MFI) corresponding to the amount of bacteria engulfed per cell and the number of cells with phagocytic function. Production of reactive oxidant species following stimulation with opsonised *E.coli* was measured using a commercial lucigenin-based kit and according to the manufacturer's instructions (Phagoburst, Glycotope Biotechnology GmbH). The formation of reactive oxidants was monitored by the oxidation of the substrate dihydrorhodamine 123 to rhodamine, detected by flow cytometry.

Cardiovascular measurements

Blood pressure and heart rate were assessed using a semi-automatic oscillometric blood pressure monitor (Dinamap 1846, Critikon) every two minutes: in minutes 12, 14, 16, and 18 of the 20 minute baseline period; and minutes 1, 3, and 5 of the stress task period and in minutes 1, 3, 5 and 7 of the 10-minute recovery period.

Questionnaires
Socio-demographics and general health behaviours

Standard socio-demographic questions including date of birth, sex, ethnicity, presence of chronic disease, medication usage, and occupational classification were asked as part of a questionnaire pack presented during the testing session. Health behaviours over the year preceding entry to the study were assessed using questions adapted from the Whitehall II study (Marmot et al., 1991). Participants were asked, on average how much they smoked (0, 1-5, 6-10,

11-20, 21+ cigarettes per day); how much alcohol they drank (0, 1-5, 6-10, 11-20, 21-40, 40+ units per week); how long they slept (0-3, 4-5, 6-7, 8-9, 10-11, 12+ hours per night); and how often they take vitamin/mineral supplements (never, once a month, once a week, a few per week, every day, more than one per day). A simple categorical scoring system was used in all cases. Participants also reported how much time they spent in activities of light, moderate and vigorous exercise intensity (0, 1-2, 2-5, 6-8, 9-10, 11+ hours per week). The category scores (0,1,2,3,4, or 5), derived from the above were multiplied by a weighting of 1,2, and 3 for light, moderate, and vigorous intensity activity respectively, and the products summed to yield a composite exercise score. Participants also reported how often (never, less than once a week, once or twice a week, most days, once a day, two or three times a day, four or more times a day) they ate each of a list of foods. From this dietary information, two main measures were derived: scores for fresh fruit and cooked vegetables were summed to give a measure of fruit and vegetable consumption; and scores for chips/fried food, crisps/similar, sweets/chocolate, biscuits/cakes/puddings, full fat dairy products and processed meat were summed to provide an index of fat intake.

Data analysis

To check the psychological impact of the stress task, ratings of stress, difficulty, and engagement were scrutinised. Blood pressure and heart rate data were averaged separately across baseline, stress task, and recovery and analysed using repeated measures ANOVA (baseline, task, recovery), with the Greenhouse-Geisser correction. The same analytic strategy was applied to lymphocyte and granulocyte numbers, cortisol, DHEAS, cortisol:DHEAS ratio, and the neutrophil function data. The neutrophil function data were logged due to the skew in the distributions but raw values are presented in the text. Repeated measures ANOVA was used to assess the differences in neutrophil function and cortisol, DHEAS, and cortisol:DHEAS ratio at baseline, during stress, and in recovery between our previous young and the present older adult groups. Partial eta-squared (η^2) was used as a measure of effect size. Slight variations in degrees of freedom reflect occasional missing data.

<center>**Results**</center>

Psychological and cardiovascular impact of the stress task

The mean (SD) ratings for the PASAT were 3.6 (1.75), 3.7 (1.84), and 3.7 (1.61) for stress, difficulty, and engagement, respectively. There was a significant main effect of time, for SBP, $F(2,32) = 20.38$, $p < .001$, $\eta^2 = .560$, DBP, $F(2,78) = 11.93$, $p = .002$, $\eta^2 = .427$, and HR, $F(2,78) = 23.45$, $p < .001$, $\eta^2 = .594$. Clearly the stress task provoked significant cardiovascular changes. These effects are shown in Figure 1.

Lymphocyte and granulocyte numbers

The summary lymphocyte and granulocyte numbers data are also presented in Figure 1. For lymphocytes there was a significant main effect of time, $F(2,32) = 12.66$, $p < .001$, $\eta^2 = .442$. There was also a significant main effect of time for granulocytes, $F(2,32) = 10.60$, $p < .001$, $\eta^2 = .399$. In both cases, it is clear that the acute stress was associated with a transient significant increase in circulating leukocytes which decreased following cessation of the task.
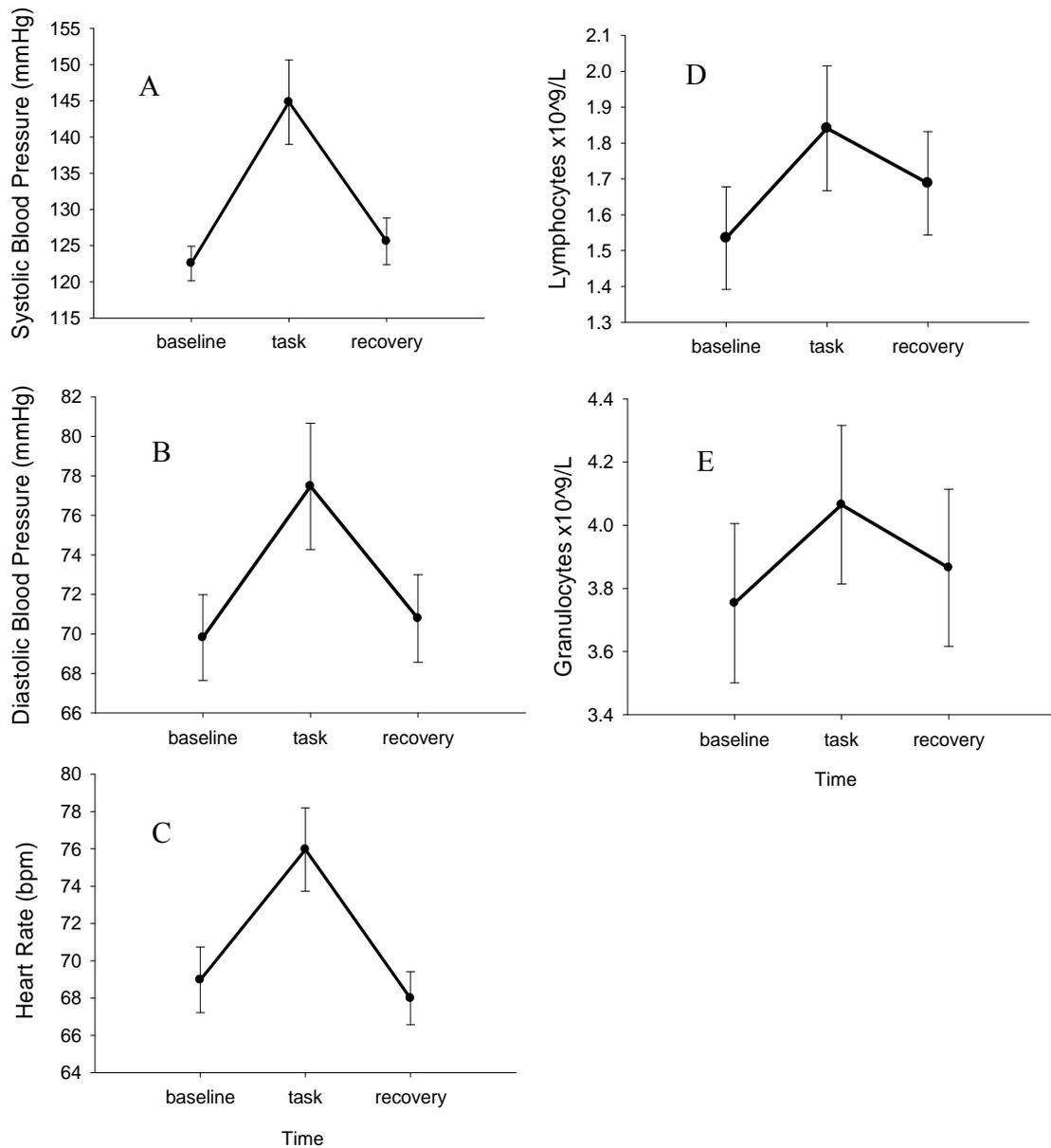
Figure 1: Cardiovascular activity (A- systolic BP, B- diastolic BP, C- heart rate), and lymphocyte (D) and granulocyte (E) numbers during baseline, stress, and recovery (error bars are standard error of the mean).

Neutrophil function

There was a significant reduction in neutrophil superoxide production during acute stress, when granulocytes were stimulated by *E.coli*, $F(2,32) = 5.05$, $p = .017$, $\eta^2 = .240$. The raw mean (SD) fluorescent intensities for superoxide production against *E.coli* at baseline, task and recovery were; 110.5 (55.57), 102.6 (56.69), and 109.7 (57.53), respectively. There was no main effect of time on neutrophil phagocytic function, $F(2, 32) = 0.97$, $p = .39$, $\eta^2 = .057$. Thus, the stress task did not affect phagocytosis (see Figure 2).
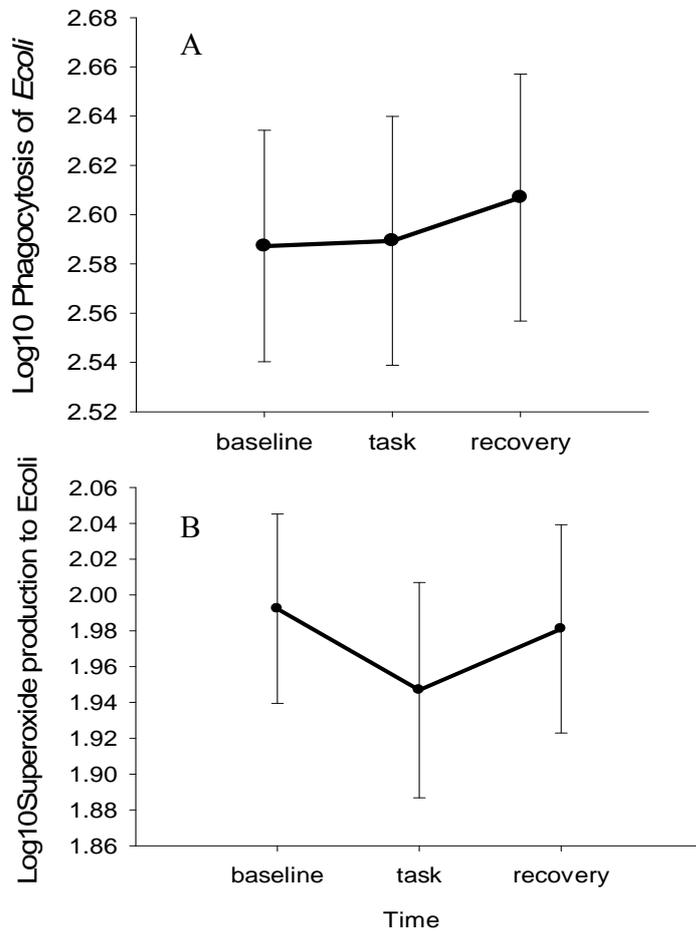
Figure 2: A-Neutrophil phagocytosis of *E.coli* (Log_{10} of phagocytosis MFI) and B-superoxide production (Log_{10} of MFI) upon stimulation with *E.coli* during baseline, stress, and recovery (error bars are standard error of the mean).

Neutrophil function reactivity to acute stress among the older group was not associated with gender, body mass index, smoking status, or any other health behaviours. Further, baseline neutrophil function was not significantly correlated with the extent of neutrophil function

reactivity. Finally, there were no significant correlations between cardiovascular activity during stress and neutrophil function at baseline or in response to stress.

Cortisol and DHEAS

Cortisol and DHEAS values were normally distributed, so were not subject to logarithmic transformation. Analysis revealed a trend towards increased cortisol concentrations during the task, but this did not reach significance, $F(2,30) = 2.90$, $p = .07$, $\eta^2 = .162$, as shown in Figure 3. There was no significant change in DHEAS levels (Figure 3) or in the cortisol:DHEAS ratio (data not shown).
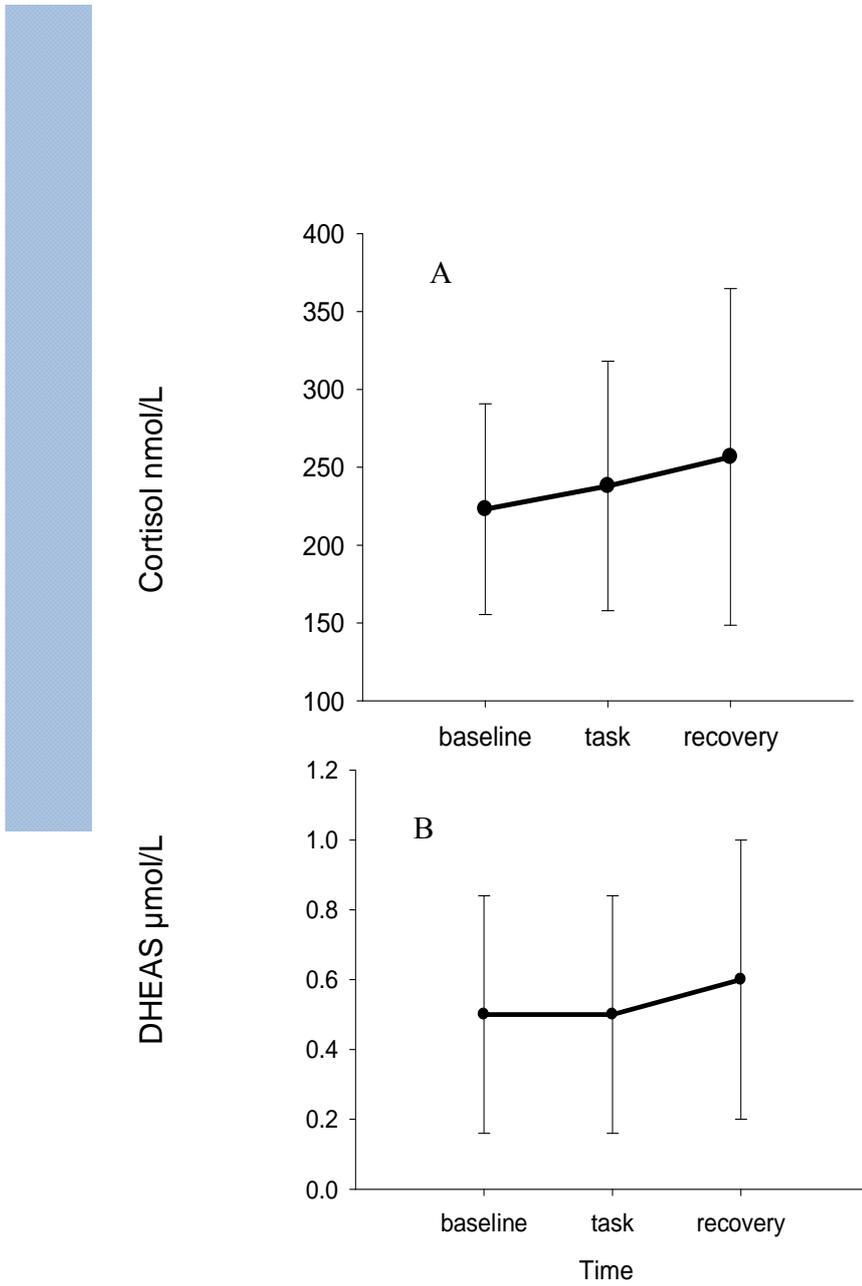
Figure 3: Cortisol (A) and DHEAS (B) at baseline, stress, and recovery (error bars are standard error of the mean).

Cortisol reactivity was calculated as the recovery concentration minus baseline due to the known delay in the cortisol response to acute stress. All other reactivity variables were calculated as task minus baseline values. Cortisol reactivity was not significantly correlated with cardiovascular reactivity, nor with neutrophil phagocytosis, superoxide production, or granulocyte reactivity. However, cortisol reactivity was positively correlated with the increase in lymphocyte numbers during stress $r(14) = .56$, $p = .02$.

Comparison of neutrophil superoxide generation in response to acute stress in older and younger adults

As neutrophil superoxide generation was the neutrophil function that was affected by acute stress we wanted to determine if this was an ageing specific effect. We were able to compare neutrophil superoxide response between young and older adults across the stress session, using data from our previous study (Khanfer et al., 2010). Older participants showed significantly lower superoxide production overall in response to *E.coli* compared to the younger adults, $F(1,52) = 14.96$, $p < .001$, $\eta^2 = .223$. Older adults also showed a significant effect of time for the quadratic component; $F(1,16) = 16.07$, $p = .001$, $\eta^2 = .501$; such that the quadratic component of the trend was pronounced and convex during the session. When comparing the two age groups, there was a tendency for the older adults to show a more pronounced decrease during the acute stress task than younger adults, which was evidenced by a significant time x age group quadratic component, $F(1,52) = 4.24$, $p = .04$, $\eta^2 = .075$. The raw means (SD) for the young and older adults are shown in Table 1.

Further, the older adults had significantly higher cortisol overall, $F(2,106) = 5.65$, $p = .005$, $\eta^2 = .096$, and lower DHEAS, although this was not a statistically significant difference. There was also a significant difference in the cortisol:DHEAS ratio between the old and young samples, such that the older adults had a much higher ratio overall, $F(2,106) = 5.60$, $p = .019$, $\eta^2 = .096$, and also a significant time x age group interaction, showing an increase in the ratio across the stress session among the older adults in comparison to the younger group whose ratio did not change over time, $F(2,106) = 5.07$, $p = .03$, $\eta^2 = .087$. The raw means (SD) are shown in Table 1.

Table 1: Comparison of neutrophil function and hormone levels between young and older adults at baseline, during stress and recovery.

	Mean (SD)					
	Young			Older adults		
	Baseline	Task	Recovery	Baseline	Task	Recovery
Superoxide production (MFI)	182.7 (87.4)	177.2 (75.9)	169.4 (72.2)	110.5(55.6)	102.6(56.7)	109.7(57.5)
Cortisol (nmol/L)	138.2(73.7)	139.7(64.2)	146.0(59.2)	223.1(67.6)	238.0(80.1)	256.6(108.1)
DHEAS (µmol/L)	2.3 (1.0)	2.4(1.0)	2.3(1.0)	0.5(0.3)	0.5(0.3)	0.6(0.4)
Cortisol:DHEAS	0.07(0.6)	0.07(0.06)	0.08(0.6)	0.7(0.7)	0.7(0.7)	0.9(1.0)

Discussion

In the current study, the data show that the psychological stress task caused acute physiological changes among older adults, manifested by changes in the cardiovascular parameters during the session. Also, as expected, the stress task provoked significant granulocytosis and lymphocytosis in comparison to baseline. To our knowledge, this study is the first to investigate the effect of acute psychological stress on human neutrophil function in older adults. The acute stress task was associated with significant reduction in neutrophil superoxide production when stimulated with *E.coli*. However, for neutrophil phagocytosis, there was no significant change associated with the stress task.

The present results are consistent with our previous findings and others (Ellard et al., 2001; Ignacchiti et al., 2011; Khanfer et al., 2011; Khanfer et al., 2010), which examined the effect of psychological stress on neutrophil function. We have shown previously that acute stress in younger adults elicited similar changes in neutrophil function, namely reduced superoxide production (Khanfer et al., 2010). Bereaved older adults also showed a decrease in their superoxide production compared to their sex and age matched non-bereaved controls (Khanfer et

al., 2011). Similarly, older adults who experienced the physical stress of hip fracture had attenuated neutrophil superoxide generation, which was associated with increased susceptibility to infection following surgery (Butcher et al., 2005). The neutrophil superoxide response thus seems susceptible to both acute and chronic stressors in young and older adults. Direct comparison with our younger adults exposed to the same protocol revealed that older adults had lower superoxide production overall and a more pronounced decrease during acute stress, as well as higher phagocytosis overall. The finding that superoxide production was lower in the older adults is consistent with several previous studies (Ginaldi et al., 1999; Ginaldi et al., 2001; Schroder and Rink, 2003; Shaw et al., 2010). What is novel is our finding that superoxide production decreases with stress exposure in the older adults. The increase observed in the lymphocyte and granulocyte numbers as a result of acute stress is also consistent with previous studies (Anane et al., 2009; Anane et al., 2010; Bosch et al., 2005; Khanfer et al., 2010). What is notable is that the increase elicited in granulocyte and lymphocyte numbers observed in the present study among older adults in response to stress is somewhat larger than seen in our previous study of younger adults, although the cardiovascular changes are not greater (Khanfer et al., 2010). Given that limited research has focused on acute stress effects on immunity in older adults, replication of these results is necessary.

   The underlying mechanism driving the changes in neutrophil function remains to be fully determined. There has been speculation about the role of the neuroendocrine system, specifically the HPA axis and the sympathetic-adrenal-medullary axis (SAM axis). In *vitro* studies have shown a reduction in superoxide production when neutrophils were incubated with cortisol (Butcher et al., 2005; Ignacchiti et al., 2011). The study by Ignachiti et al., also reported that neutrophil superoxide production was reduced and serum cortisol levels were elevated as a result of academic stress among postgraduate students (Ignacchiti et al., 2011). In the present study there was a trend for an increase in cortisol in response to the stress task. However, this did not reach significance and was not associated with the stress-related decrease in neutrophil superoxide production suggesting that these effects may be in part independent of one another. We have previously reported an association between a raised cortisol:DHEAS ratio and reduced neutrophil superoxide generation in older adults after hip fracture and also showed that DHEAS was able to counteract the inhibition of superoxide generation by cortisol *in vitro* (Butcher et al.,

2005). We have also shown a raised cortisol:DHEAS ratio among older adults showing the stress of bereavement (Khanfer et al., 2011). Differences between these earlier studies and the present findings may reflect the difference in effects of the duration of raised cortisol, namely acute versus chronic elevation, or that neutrophil superoxide production decreases with acute stress reflect a different neuroendocrine mechanism. Our findings that the older adults had overall higher cortisol levels and cortisol:DHEAS ratio than the younger group confirm the usual expected hormone changes associated with ageing (Phillips et al., 2007).

Although neutrophils are known to have adrenergic receptors (Harvath, 1991; LaBranche et al., 2010; Weglarz et al., 2003), in the present study and previously with our younger adults, cardiovascular reactivity was not related to the change neutrophil function and thus we suggest that the effects seen are unlikely to be due to modulation of the SAM axis. The present study suffers from a few limitations. First, the sample size was relatively small compared to previous studies. However, it should be noted that recruitment of an older group to perform a 1.5 hour session in the laboratory proved difficult. Second, although the flow cytometry assays are considered to be state-of-the-art and sensitive, they also are subject to diurnal and day-to-day variation. However, such effects were minimised by testing at the same time of day and the use of internal controls for each assay. The study design could also have been strengthened by inclusion of a no stress control session, although counterbalancing of this within subjects proved impractical.

In conclusion, in older adults, acute stress elicited a short term reduction of neutrophil superoxide production and increase in circulating granulocyte and lymphocyte numbers, but with no significant effect on neutrophil phagocytosis. These changes were independent of cortisol and DHEAS. Compared with younger adults exposed to the same acute stress task, older participants had lower superoxide production overall and a more pronounced decrease during acute stress. The results of this study could contribute to explaining the increased risk of infection in older adults, particularly those subject to frequent stress exposures. The present results must be regarded as preliminary and replication is necessary. Further research is needed to explore the underlying mechanisms of acute stress effects on human neutrophil function in both young and older adults in greater detail.

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CHAPTER FIVE

NEUTROPHIL FUNCTION AND THE CORTISOL:DHEAS RATIO IN
BEREAVED OLDER ADULTS

Abstract

Bereavement is a common life event for older adults and is associated with increased risk of morbidity and mortality, though the underlying reasons for this link are poorly understood. Although physical and emotional stressors and ageing are known to suppress immunity, few studies have explored the impact of bereavement upon immunity in the older population. We therefore hypothesised that the emotional stress of bereavement would suppress immune function, specifically neutrophil bactericidal activity, in older adults. A between-subjects design was used to examine the effect of recent bereavement (< two months) on neutrophil function in elders. Participants were 24 bereaved and 24 age- and sex-matched non-bereaved controls all aged 65+ years. Neutrophil phagocytosis of *Escherichia coli* (*E. coli*) and stimulated superoxide production were assessed. Cortisol and dehydroepiandrosterone-sulphate (DHEAS) levels were determined in serum to assess potential mechanisms. Depressive and anxiety symptoms were measured by questionnaire. Neutrophil superoxide production was significantly reduced among the bereaved when challenged with *E. coli* ($p =0.05$), or phorbol 12-myristate 13-acetate ($p =0.009$). Further, the bereaved group had a significantly higher cortisol:DHEAS ratio compared to controls ($p =0.03$). There was no difference in neutrophil phagocytosis between the two groups. The psychological questionnaire results showed that the bereaved had significantly greater depressive and anxiety symptoms than the non-bereaved. The emotional stress of bereavement is associated with suppressed neutrophil superoxide production and with a raised cortisol:DHEAS ratio. The stress of bereavement exaggerates the age-related decline in HPA axis and combines with immune ageing to further suppress immune function, which may help to the explain increased risk of infection in bereaved older adults.

***Key words*:** Bereavement**;** neutrophil; phagocytosis; superoxide production

Bereavement is considered to be one of the most stressful life events, becoming more frequent as we age, yet the impact upon health is under researched. This is due in large part to the difficulty of accessing this vulnerable group. What is clear is that bereavement, particularly in older adults, is associated with higher risk of morbidity and mortality (Biondi and Picardi, 1996; Clayton, 1990; Manor and Eisenbach, 2003; Stroebe et al., 2007). To try to understand the basis of this association a small number of studies have explored the impact of bereavement upon immunity (Calabrese et al., 1987; Gerra et al., 2003; Goodkin et al., 1996). Bereaved women showed reduced Natural Killer cell activity and increased plasma cortisol levels compared to non-bereaved controls (Irwin et al., 1987). Bereaved parents showed significantly decreased numbers of T-regulatory cells, and significantly increased T-helper cells compared to their matched controls and this effect persisted over eight months (Spratt and Denney, 1991). More recently, it was found that HIV patients who experienced maladaptive grief following bereavement showed more rapid losses of CD4 T-cells over time, even when statistically adjusting for age, health status, and use of antiretroviral medications (Goforth et al., 2009).

However, there are very few studies that have examined the impact of bereavement upon immunity in older adults despite the fact that the chances of experiencing a significant bereavement increase with age. This is an important issue as it is now well established that ageing itself results in a decline in immune status, termed immunosenescence (Shaw et al., 2010), with significant consequences for susceptibility to infection. These age-related changes include decreased neutrophil function, with older adults showing a reduction in neutrophil phagocytic ability and superoxide production (Butcher et al., 2001; Hajishengallis; Lord et al., 2001; Wessels et al.). As stress also suppresses immune function (Segerstrom and Miller, 2004), we propose that stress and ageing combined will have an additive and deleterious effect upon immunity, with significant health consequences for the bereaved older adult.

In support of this notion, we have previously shown that older adults who have suffered bereavement in the past 12 months had a poorer antibody response to the annual influenza vaccination in comparison to non-bereaved older adults (Phillips et al., 2006). We have also shown that a significant physical stress, hip fracture, can worsen neutrophil bactericidal ability in older adults, which was associated with increased susceptibility to infection following surgery (Butcher et al., 2003). One suggested mechanism for this effect was the increased

cortisol:DHEAS ratio observed in older adults as a result of adrenopause (Orentreich et al., 1984), which is then exaggerated in older patients by the stress of hip fracture (Butcher et al., 2005). Both cortisol and DHEAS are outputs of the hypothalamic pituitary adrenal (HPA) axis. It is known that cortisol has mainly immunosuppressive actions while DHEAS is immune enhancing. Further, previous research indicates that with ageing there is imbalance between the two hormones levels, due to reducing DHEAS levels from age 30 years onwards, and relatively stable cortisol levels (Phillips, et al., 2007). This reported increased cortisol:DHEAS ratio is thought to be a contributing factor to the process of immunosenescence (Buford and Willoughby, 2008). Importantly, we showed very recently that neutrophil function was enhanced by DHEAS *in vitro* (Radford et al., 2010), and that this steroid can overcome the suppressive effects of cortisol on neutrophil function (Butcher et al., 2003; Radford et al., 2010).

As yet, it is unknown whether a psychological stressor such as bereavement can influence neutrophil function. Such an association might underlie the link between bereavement and morbidity and mortality, particularly from pneumonia, in older adults. Further, the biological mechanisms by which bereavement modulates immune function are unknown. Consequently, the present study examined the association between bereavement and neutrophil function in healthy older adults by comparing those who had recently suffered bereavement to an age- and sex-matched non-bereaved control group. Cortisol and DHEAS levels were also measured to examine whether any group differences in neutrophil function were associated with either hormone or differences in the cortisol:DHEAS ratio.

Method

Participants

Forty-eight healthy older adults (32 females) were recruited from St Mary`s Hospice and from the community population in Birmingham, UK, between 2008 and 2010. Their mean (SD) age and body mass index (BMI) was 72.7 (5.30) years and 25.7 (3.56) kg/m^2. All but one Asian participant reported their ethnicity as white. The majority were non-smokers (93.7%), and 77% were classified as from a manual occupational household. Individuals were excluded if they had an acute infection or suffered from an immune disorder. Twenty-four of the participants had suffered a significant bereavement in the past two months.

Study design and procedure

The study was a between-subjects design. Blood samples were taken in the morning (09.00 - 10.00) within two months of bereavement of a close family member or friend. On the same day an age- and sex-matched non-bereaved control participant was recruited to provide a blood sample. Assays for neutrophil function (phagocytosis and superoxide production) were performed on the same day. Serum was frozen for later assay of the levels of the stress hormones cortisol and DHEAS. Following blood sampling, all participants completed a questionnaire pack. The study was approved by the South Birmingham NHS Local Research Ethics Committee and all participants provided their written informed consent.

Blood sampling and immune assays

Blood was collected into a heparin containing tube for analysis of neutrophil function on the same day and in another anti-coagulant free tube for later hormone analysis. Serum from this tube was frozen at -20°C until assays for cortisol and DHEAS were performed by ELISA (IBL International, Hamburg, Germany). Neutrophil phagocytic ability and efficiency were assayed in whole blood using a commercial kit (Phagotest, Orpegen Pharma GmbH, Heidelberg, Germany) which measures uptake of fluorescein isothiocyanate-(FITC) labeled opsonized *E.coli*. Flow cytometry (Dako Cyan, Carpinteria, California) was used to gate on neutrophils and determine the mean fluorescence intensity (MFI) corresponding to the amount of bacteria engulfed per cell and the number of cells with phagocytic function. These values were used to define the phagocytic index (% phagocytic cells x MFI). Production of reactive oxidant species following stimulation with either opsonized *E.coli* or 12-phorbol, 13-myristate (PMA) was measured using a commercial lucigenin-based kit and according to the manufacturer's instructions (Phagoburst, Orpegen Pharma). The formation of reactive oxidants was monitored by the oxidation of the substrate dihydrorhodamine 123 to rhodamine, detected by flow cytometry.

Questionnaires

Standard socio-demographic and health behavior questions including date of birth, sex, ethnicity, and occupational classification were asked as part of a questionnaire pack presented during the testing session. Health behaviours over the year preceding entry to the study were

assessed using questions adapted from the Whitehall II study (Marmot et al., 1991), regarding smoking, alcohol consumption, exercise, sleep quantity, and diet.

Psychological morbidity was measured using the Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith, 1983). The scale contains 14 four-point items, from 0 (not present) to 3 (considerable), with seven assessing largely the anhedonic rather the somatic aspects of depression (e.g., "I have lost interest in my appearance") and seven assessing anxiety (e.g., "I feel tense or wound up"). The HADS has good concurrent validity and performs well as a psychiatric screening device (Herrmann, 1997; Snaith, 2003). For the present sample, Cronbach's alpha was 0.90 for depressive symptoms and 0.88 for anxiety symptoms.

Statistical Analysis

The differences between the bereaved and non-bereaved groups, in the demographic, health behaviours, and psychological variables were analysed using t-test or chi-square. Neutrophil function and hormone levels were skewed, so were subject to log transformation. Univariate analysis of variance (ANOVA) was used to assess the differences in neutrophil function and hormone levels between the bereaved and control groups. The cortisol:DHEAS ratio was calculated and compared between the groups using ANOVA. Effect sizes are reported in terms of η^2. Correlations were then used to ascertain whether the cortisol:DHEAS ratio or any other significant demographic or psychological variables were potential mechanisms underlying the association between bereavement and neutrophil function.

Results

Descriptive statistics

Table 1 shows the descriptive statistics of demographic information and questionnaire scores for the two groups. The bereaved group had significantly greater depressive symptoms, $t(45) = -2.662$, $p = 0.01$, and anxiety symptoms, $t(44) = -3.406$, $p = 0.001$, than the non-bereaved controls. None of the participants were taking medication that has been described as modifying immune function.

Table 1: Descriptive statistics for participant demographics and questionnaire scores

Variable	Total participants	Bereaved	Non-bereaved	
		Mean (SD)		p
Age	72.7 (53.00)	72.0 (53.80)	73.3 (5.25)	.39
HADS Depression	3.9 (4.31)	5.5 (5.26)	2.4 (2.37)	.01
HADS Anxiety	6.2 (4.47)	8.2 (4.80)	4.2 (3.02)	.001
BMI	25.7 (3.56)	25.8 (3.74)	25.7 (3.46)	.87
Alcohol units intake per week	1.2 (0.94)	1.5 (0.99)	1.0 (0.83)	.06
Exercise score	6.9 (6.98)	5.6 (4.68)	8.4 (8.57)	.17
		N (%)		
Current Smoker	3 (6.3)	2 (8.3)	1 (4.2)	.55
Takes vitamins	23 (43.7)	11 (45.8)	10 (41.7)	.67
Sleep (<6 hours per night)	9 (18.8)	3 (12.5)	6 (25.0)	.27
Occupation - Manual	37 (77.1)	18 (75.0)	19 (79.2)	.94
Ethnicity - White	47 (97.9)	24 (100.0)	23 (95.8)	.31

Neutrophil Function

Bereaved participants showed significantly lower superoxide production in response to *E.coli*, $F(1,45) = 4.07$, $p = 0.05$, $\eta^2 = 0.083$, and PMA, $F(1,46) = 7.46$, $p = 0.009$, $\eta^2 = 0.139$, as illustrated in Figure 1. The raw means (SD) for superoxide production against *E.coli* and PMA respectively for the bereaved versus non-bereaved were 141.3 (86.16) versus 201.6 (162.57), and 237.3 (153.15) versus 341.2 (158.70). However, for neutrophil phagocytosis of *E.coli*, there was no significant group difference between the bereaved and non-bereaved, $F(1,45) = 1.89$, $p = 0.18$, $\eta^2 = 0.040$, although the bereaved showed somewhat lower phagocytic ability; mean (SD) of raw values = 185.0 (74.18) versus 259.2 (172.74).
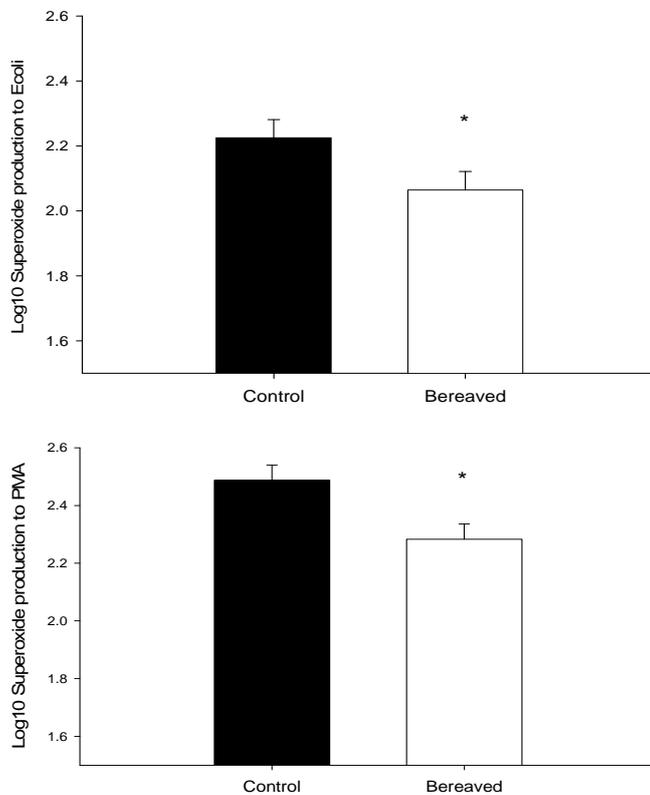
Figure 1: Neutrophil superoxide production on stimulation with *Escherichia coli* (top), or with PMA (bottom), between the bereaved and non bereaved groups, error bars are standard error of the mean.

Serum Cortisol and DHEAS

Analysis revealed a significant difference in the cortisol:DHEAS ratio, $F(1,46) = 5.17$, p =0.03, $\eta^2 = 0.101$, such that, as illustrated in Figure 2, there was higher cortisol:DHEAS ratio among the bereaved group than the non-bereaved (raw mean (SD) = 332.4 (442.80) versus 150.5

(102.8). The differences in cortisol or DHEAS alone did not reach statistical significance, although they indicated higher cortisol, mean (SD) = 120.0 nmol/L (65.2) versus 87.8 (44.4); p =0.051 and similar DHEAS 0.69 µmol/L (0.57) versus 0.79 (0.46); p=0.49 in the bereaved group. Correlations revealed that the cortisol:DHEAS ratio, or the individual hormone levels were not significantly associated with the magnitude of superoxide production to *E.coli* or PMA. Neither were depressive symptoms nor anxiety scores related to superoxide production. However, depressive symptoms (p =0.02) and anxiety symptoms (p <0.001) scores were significantly positively associated with the cortisol:DHEAS ratio, such that individuals with a higher ratio exhibited greater depressive and anxiety symptoms (see Figure 3).

Figure 2: The serum cortisol to DHEAS ratio compared between the bereaved and non bereaved groups, error bars are standard error of the mean.

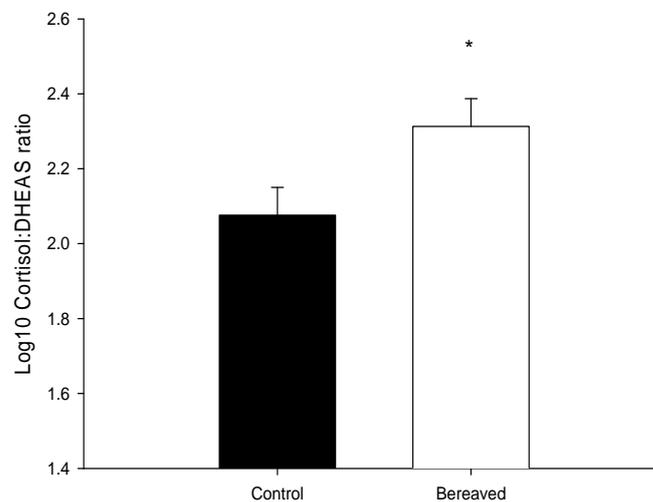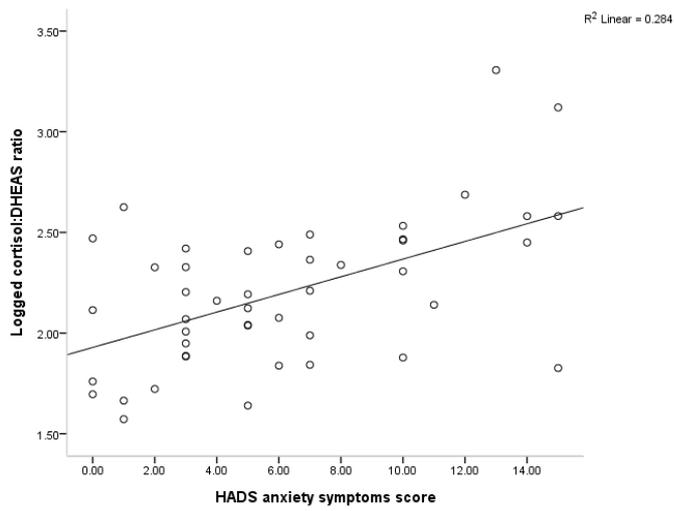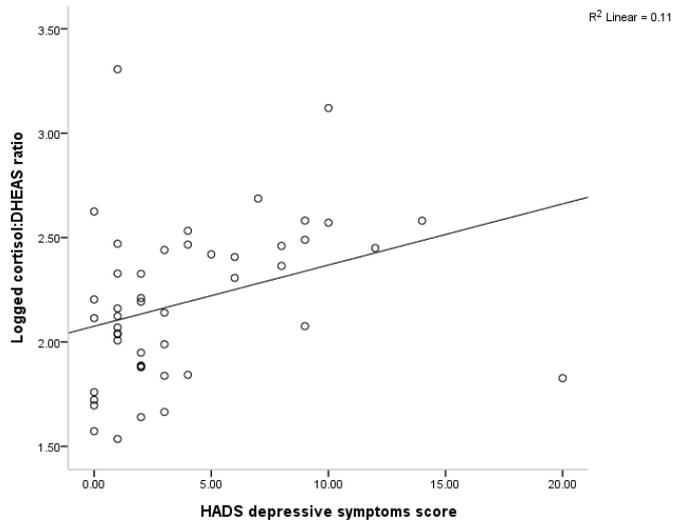Figure 3: Correlations between HADS scores and the cortisol:DHEAS ratio

Discussion

In the present study bereaved older adults showed reduced neutrophil function, specifically lower neutrophil superoxide production, compared to the control group when neutrophils were challenged with either the physiologically relevant stimulus *E.coli* bacteria or the potent protein kinase C activator PMA. The bereaved group also had a significantly higher cortisol:DHEAS ratio compared to the controls although this did not relate to neutrophil function. For neutrophil phagocytosis, there was lower phagocytic ability among the bereaved group, but the difference did not meet statistical significance. The psychological questionnaire scores showed that the bereaved had significantly greater depressive and anxiety symptoms than the non-bereaved controls, and that this related to having a greater cortisol:DHEAS ratio.

These findings are consistent with the previous research into bereavement effects on other parameters of the immune system such as T-cell numbers and NK cell activity (Goforth et al., 2009; Goodkin et al., 1996; Irwin et al., 1987; Spratt and Denney, 1991), and are unique as far as we are aware in considering bereavement in the context of an aged immune system. Moreover, this is the first study to examine the effect of recent bereavement on neutrophil function in older adults, but the data have strong parallels with our previous work showing that neutrophil function is reduced as a result of physical stress (hip fracture) in seniors (Butcher et al., 2003). Tsukamoto and colleagues have also shown that older adults who have fewer stress coping factors, such as hobbies and close links with friends and family, had lower neutrophil superoxide production compared to those with greater numbers of these factors (Tsukamoto et al., 2002). Similarly, a study on the effect of stress and depression on neutrophil function among children, has shown that neutrophil superoxide production but not neutrophil phagocytosis was reduced among children with major depressive disorder (MDD) compared to controls (Bartlett et al., 1997). However, it should be acknowledged that the nature of the psychological distress and the population sampled in that study differed from the present research, and depressive symptoms did not relate to neutrophil function in our data presented here. It is possible that major depressive disorder but not depressive symptomatology can influence neutrophil function, although further research would be needed to test this possibility.

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The psychological stress of bereavement is considered relatively chronic compared to the acute psychological stressors lasting for minutes or hours. The present results support previous findings of an association between chronic stress and immune dysfunction among older adults (Gouin et al., 2008). For example, the chronic stress of caregiving for a spouse with dementia was found to relate to a lower influenza vaccination response among elderly caregivers (Kiecolt-Glaser et al., 1996). Our present findings of greater depressive and anxiety symptoms among the bereaved is also supported by previous research showing that older adults who reported higher anxiety and depression symptoms had delayed wound healing compared to those with fewer symptoms (Cole-King and Harding, 2001). In our previous study on the effects of hip fracture on neutrophil function in older adults,(Butcher et al., 2003) we observed that the cortisol:DHEAS ratio was increased compared to the healthy controls. Cortisol is known to suppress neutrophil superoxide generation (Bekesi et al., 2000), whereas DHEAS enhances this function (Radford et al., 2010). However, as this ratio was significantly affected by bereavement but not correlated with neutrophil function in the present study, the mechanism driving the observed neutrophil function changes remains unknown. It is possible that the altered cortisol:DHEAS ratio seen in this study contributes to the higher depressive and anxiety symptoms among the bereaved, given the observed significant positive correlations between the depressive and anxiety symptoms scores and the cortisol:DHEAS ratio and the reported opposing effects of cortisol and DHEAS upon mood (Duval et al., 2006; Morsink et al., 2007).

The present study has some limitations. First, the sample size was small. However, it should be noted that recruiting bereaved older participants with no co-morbidity and within two months of their bereavement is exceptionally difficult, and this sample size took more than two years to be achieved. Also the sample size is comparable to those in previous studies focused on bereavement and immunity (Gerra et al., 2003; Kemeny et al., 1995; Lindstrom, 1997; Spratt and Denney, 1991).

In conclusion the current study provides novel preliminary evidence that bereavement is associated with reduced neutrophil bactericidal function among older adults. Clearly the bereaved group showed lower neutrophil superoxide production to PMA and *E.coli*, and also

exhibited a higher cortisol:DHEAS ratio compared to non-bereaved controls. The current study is the first study we know of to examine the effect of bereavement on neutrophil function, particularly among older adults. Indeed, given the very small number of studies that focused on bereavement and innate immunity, the novel findings could help to explain the underlying mechanisms of the higher morbidity and mortality and poorer psychological state among bereaved older adults. In addition, a similar study in younger adults would be valuable in determining whether or not neutrophil effects extend to younger bereaved individuals or are specific to those with coexisting immunosenescence. Considering the decline in neutrophil function with ageing and the higher associated morbidity, we believe the current results are valuable and indicate a need to minimise factors that will further compromise the immune system in bereaved older adults, such as social isolation.

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CHAPTER SIX

GENERAL DISCUSSION

PNI research can elucidate the effects of psychosocial factors on immunity not only at the cellular but also at the molecular level (Gidron et al., 2010; Kurokawa et al., 2011; Li et al., 2011; Mathews and Janusek, 2011). The research in the current thesis makes a novel contribution to the PNI field. My studies are the first I know of to reveal the effects of psychological stress on human neutrophil function using sensitive assays via flow cytometry. The novelty of these findings emerges from two directions. First, most of the PNI studies which investigated the association between psychological stress and immune function have focused on different parameters of the adaptive immune system, whereas much less known about this association or effect of stress on the innate immunity, in which neutrophils play a major role. Second, the participants in the present studies were not only university students but also community-dwelling healthy older adults, including the difficult to reach group of the bereaved.

Thesis aims and findings

The **first study** in this thesis examined the effects of an acute laboratory mental stress task on human neutrophil function, specifically phagocytosis of *E.coli* and stimulated superoxide production. Participants were 40 university students; blood samples to determine neutrophil function by flow cytometry were taken at the end of resting baseline, during an acute stress task and during recovery. The stress task was a 10-minute time-pressured mental arithmetic challenge with social evaluation. The self report and cardiovascular data confirmed that the acute stress task provoked emotional and physiological responses. An acute stress-induced lymphocytosis and granulocytosis was also observed. The acute stress task also affected neutophil function; there was an acute increase in phagocytosis and reduction in neutrophil superoxide production when stimulated with *E.coli* during stress exposure. **Study two** aimed to explore the effects of acute stress on neutrophil function among older adults (n = 17), and compare this to the previous younger adult study. It also investigated levels of serum cortisol, DHEAS, and their ratio during acute stress as a potential mechanism underlying any neutrophil effects. Similar to the first study among university students, the data showed that the psychological stress task caused acute physiological changes among older adults, as manifest by changes in the cardiovascular parameters during the session. In addition, the stress task was associated with significant granulocytosis and lymphocytosis. Further, there was a significant reduction in neutrophil superoxide production when stimulated with *E.coli* in response to the

stress task, but no significant alteration in neutrophil phagocytosis. There was also a trend for an increase in cortisol level in response to the stress task, although the magnitude of the cortisol stress reaction was not associated with the size of the decrease in neutrophil superoxide production. Finally, comparison between studies one and two showed that the older adults had higher cortisol and cortisol:DHEAS ratio, and lower neutrophil superoxide production overall than younger adults. Nevertheless, the lack of association between neutrophil function and cardiovascular and cortisol/DHEAS responses to the acute stress task made it difficult to further elucidate the mechanisms underlying the observed acute stress effect on neutrophil superoxide production.

In **study three**, it was hypothesised that the emotional stress of bereavement among older adults would suppress immunity, specifically neutrophil bactericidal activity. Neutrophil function and levels of serum cortisol, DHEAS, and their ratio were compared between a bereaved group (n = 28) and the same number of sex and aged-matched control participants. The bereaved group had significantly more symptoms of depression and anxiety than the non-bereaved controls. Importantly the bereaved older adults showed significantly lower superoxide production, in response to *E.coli* and PMA, compared to the non bereaved group. Also the bereaved group showed somewhat lower phagocytic ability, although the group difference was not statistically significant. Although there were no significant differences in the levels of cortisol or DHEAS between the groups, there was a higher cortisol:DHEAS ratio among the bereaved group than the non-bereaved group. Again, neutrophil function in this study was not correlated with the concentrations of cortisol and DHEAS, nor with their ratio. Neither were depression nor anxiety symptoms related to superoxide production. Interestingly, depression and anxiety symptom scores were significantly positively associated with the cortisol:DHEAS ratio, such that individuals with a higher ratio reported more symptoms.

Acute versus chronic psychological stress effect on neutrophil function

The above novel findings provide clear evidence that human neutrophil function is sensitive to psychological stress across the life course. Indeed, the first two studies have shown that human neutrophil function is sensitive even to very short-term stress tasks, although it is neutrophil superoxide production which appears to be susceptible to stress rather than phagocytosis. The finding that neutrophils are sensitive to acute stress is consistent with previous work showing an effect of acute stress on neutrophil activation using a different short-term mental stressor, namely Raven`s Advanced Progressive Matrices task (Ellard et al., 2001). The increase in the neutrophil numbers as well as lymphocyte numbers seen in the first two studies is also consistent with the findings of previous human research on leukocyte numbers and distribution as a result of acute psychological stress exposure (Anane et al., 2009; Anane et al., 2010; Bosch et al., 2005; Maes et al., 1999). In these studies, flow cytometry was used to measure the changes in leukocyte number including neutrophils, although no neutrophil functional assays were conducted. Recent reports from animal studies have also shown evidence of neutrophil activation and diminished superoxide production, measured by flow cytometry and fluorescent microplate assays, as a result of acute psychological stress (Brown et al., 2008; Curry et al., 2010), including noise stress and stress caused by social disruption (social disruption -SDR -model). In the latter, an aggressive intruder mouse was allowed to attack and cause disruption among resident mice, it was found that a significant number of neutrophils infiltrated the lungs of the stressed mice and contributed to an inflammatory process; these neutrophils were also found to be highly activated (Curry et al., 2010).

The reduction in neutrophil superoxide production observed in the present studies as a result of either short laboratory based mental stress task or the more chronic stress of bereavement is also consistent with a study examining the effect of academic stress on neutrophil superoxide production (Ignacchiti et al., 2011). In this recent study, neutrophil superoxide production was evaluated using the superoxide dismutase-inhibitable reduction of ferricytochrome C assay, blood samples from post graduate students subjected to the stress of completing their final thesis examination; the samples were taken one day before their viva. Experience of this stressor was associated with a significant reduction in neutrophil superoxide production in comparison to a non-stressed control group. However, it should be noted that the stress of an academic

examination or PhD submission and viva stress, persisting over several days or weeks, cannot be considered as acute psychological stress in the same way as laboratory stress exposures lasting minutes. Rather, they may be more sensibly considered to be relatively chronic stressors, more similar perhaps in chronicity and impact to the bereavement stress in the present study three which can last for several months. In contrast, the superoxide production in all three empirical studies comprising this thesis was significantly decreased as a result of the stress exposure. This contrasts with the findings to research showing that the stress of an academic examination period was associated with enhanced neutrophil fiunction including increased superoxide production (Kang et al., 1996; Kang et al., 1997). It should be noted, however, that the methods use to measure neutrophil function in these examination stress studies were different from the present techniques; further, some of the asthmatic students who participated in one of the above studies showed a reduction of neutrophil superoxide production compared to non-asthmatics as a result of examination stress (Kang et al., 1996). In addition, the Kang et al studies were conducted solely in young adults, whereas our own chronic stress study was in older adults. It is yet to be shown whether the chronic stress of bereavement is associated with neutrophil function down-regulation in younger samples using comparative assays.

The first study in this thesis also shows that, in young participants, psychological stress can cause a significant increase rather than decrease in phagocytosis along the decrease in superoxide production. Studies evaluating neutrophil function and its regulation have shown that phagocytosis and intracellular bacterial killing are independent activities of neutrophil-mediated antibacterial defense. Accordingly, it is quite reasonable that different indices of function can show different patterns of effect (Pechkovsky et al., 1996; Vollebregt et al., 1998). Moreover, an interesting animal study which investigated the correlations between neutrophil phagocytosis and neutrophil superoxide production, showed a significant negative correlation between these two indices of neutrophil function when rats were exposed to a psychological stress compared to a positive correlations during running exercise (Kuriyama et al., 1996). In this study, stress group rats (N = 10) were placed in a box and were exposed to the sight, sound, and smell of another group of rats being given 30 electric shocks, each lasting 30 minutes, which induced the painful jumping, crying, and the production of urine and faeces. What is not known from the present acute stress findings within this thesis in healthy younger and older adults is whether the

repeated down-regulation of superoxide production with acute stress is detrimental in terms of health. This is an important question for future research into acute stress and neutrophil function as longer term neutrophil function deficits are known to be detrimental to health. For example, patients who have an absent or reduced neutrophil oxidative burst are more susceptible to infections and in some cases those infections can be life threatening. One example of this is the occurrence of infections in patients after chemotherapy when their neutrophil numbers are significantly reduced (neutropenia). Inherited neutropenias, caused by mutations in proteins used by neutrophils during the killing mechanisms, such as cyclic neutropenia (CN) and severe congenital neutropenia (SCN) can cause serious infections too. Reduced or absent neutrophil oxidative burst activity is also observed in innate defects such as chronic granulomatous disease (CGD). Patients with CGD suffer from recurrent infections as a result (Hager et al., 2010; Nathan, 2006).

Further, whether the granulocytosis we show in this thesis as a result of acute stress is clinically significant also remains to be answered. A recent review has shown that neutrophils have the ability to produce anti-inflammatory cytokines such as IL-10 and TGF-β (Mantovani et al., 2011), but whether neutrophils produce these anti inflammatory cytokines during acute psychological stress is not known. Perhaps future *in vitro* neutrophil functional assays could include focusing particularly on anti-inflammatory cytokines secretion from stressed and non-stressed individuals. Exploring such an aspect of neutrophil function will enrich the PNI literature which generally supports the concept of immune enhancement in response to acute stressors.

It is also notable that the acute stress-induced increase in granulocyte and lymphocyte numbers observed in study two of older adults is somewhat larger than the increase observed in the study of younger adults, although the magnitude of the cardiovascular perturbations did not vary between age groups. Perhaps this is not surprising, as the baseline neutrophil numbers and percentages from older adults in study two were relatively higher than those found for the younger students in study one. What is notable is the difference in overall neutrophil function

between the age groups. Indeed, as indicated, the older adults in study two showed lower superoxide production overall compared to the younger adults in study one. Also, older participants showed a more pronounced decline in superoxide production in response to acute stress. Several studies have reported a decline in superoxide production with age (Christy et al., 2010; Di Lorenzo et al., 1999; Ginaldi et al., 1999; Ginaldi et al., 2001; Hajishengallis, 2010; Lord et al., 2001; Mishto et al., 2003; Panda et al., 2009; Shaw et al., 2010; Tortorella et al., 2001; Weiskopf et al., 2009). Further, recent reports confirm that although during immunosenscence the majority of the immune system parameters decline, findings of a decline in neutrophil phagocytosis are controversial (Wessels et al., 2010), and there is evidence of stable neutrophil numbers and phagocytosis with ageing (Agarwal and Busse, 2010; Füöp et al., 1984; Niwa et al., 1989; Plackett et al., 2004; Wessels et al., 2010).Given that limited research has focused on psychological stress effects on innate immunity in older adults, replication with greater numbers is necessary to confirm the current findings.

Study three is the first study, we are aware of, to examine the effects of bereavement on neutrophil function, particularly among older adults. The stress of bereavement is rightly considered to be relatively chronic compared to acute laboratory stress. The present results from study three provide additional support for the general findings of an association between chronic stress and immune dysfunction among older adults (Gouin et al., 2008). For instance, the chronic stress of caregiving for a spouse with dementia was found to relate to a lower influenza vaccination response among elderly caregivers (Kiecolt-Glaser et al., 1996). However, the present findings add to this literature by providing new information about a key but much neglected aspect of innate immunity. A related study in Japan has investigated the correlations between life style factors and neutrophil function in an elderly population, showing that older adults with no stress coping strategies have lower neutrophil superoxide production than elders with one or two stress coping resources (Tsukamoto et al., 2002). These stress coping factors included; having hobbies, keeping pets and having close relationships with family and friends. The results of study three are also consistent with the results of previous studies on the effects of bereavement on other parameters in the immune system such as T-cell numbers and NK cell activity (Goforth et al., 2009; Goodkin et al., 1996; Irwin et al., 1987; Spratt and Denney, 1991).

Responsible potential mechanisms

One of the key questions raised from the papers comprising this thesis is: what is the responsible mechanism behind the reported neutrophil function changes? There has been speculation about role of the neuroendocrine system, specifically the HPA axis and the sympathetic-adrenal-medullary axis (SAM). In the first two studies of this thesis, stress, as expected, induced significant increases in cardiovascular activity (a proxy for SAM activation). However, these reported changes did not appear to be correlated with neutrophil function or numbers in the present studies. Similarly, despite examining potential neuroendocrine mediators such as cortisol and DHEAS activation with stress, changes in these parameters were not associated with the observed neutrophil function changes. Thus, the specific underlying mechanisms which are driving the changes in neutrophil function as a result of psychological stress remain unknown. This is surprising given that studies from human and animal models have shown significant correlation between stress hormones like cortisol and neutrophil function. Indeed, *in vitro* studies have shown that neutrophils are able to respond to cortisol and display suppressed superoxide production. Also neutrophils are the only leucocytes to express the cell membrane transporter for DHEAS (Hazeldine et al., 2010). Also there is evidence that steroids can cause granulocytosis by shifting the PMNs from the marginated to the circulating pool, in addition to bone marrow release. However, the regulation of neutrophil function is under the control of many different receptors for a variety of cytokines as well as HPA-axis hormones (Pechkovsky et al., 1996), which are also differentially affected by stress (Steptoe et al., 2001; Yamakawa et al., 2009). Further, neutrophils themselves can secrete many cytokines (Scapini et al., 2000), and can affect and be affected by other immune cells (Kumar and Sharma, 2010). Indeed, it is well known that granulocytes production is controlled and affected by different cytokines like granulocyte colony stimulating factor G-CSF (major cytokine for neutrophil proliferation and survival) and others like macrophage colony stimulating factor M-CSF, GM-CSF, interleukin (IL)-6, IL-3, IL-17 and, most recently, IL-22 (Von Vietinghoff et al 2008;Mantovani et al., 2011). Whether the granulocytosis we see in as a result of acute stress driven by specific cytokines this remains unknown. IL-6 is a pro inflammatory cytokines which found to increased with physical and psychological stress(Carpenter et al., 2010). There is evidence that raised plasma G-CSF and IL-6 after exercise may play a role in neutrophil

mobilization into the circulation (Yamada et al., 2002), whether this is also the case in after acute stress remains unknown.

   Nevertheless, the present null associations with cardiovascular reactivity were somewhat unexpected given that neutrophils have been shown to have both α and β- adrenergic receptors (Abraham et al., 1999; Gosain et al., 2009; Harvath et al., 1991; LaBranche et al., 2010), and changes in adrenalin and noradrenalin have been shown to relate to circulating neutrophil numbers and function (Abraham et al., 1999; Trabold et al., 2007; Trabold et al., 2010). A recent report has shown that human neutrophil superoxide production and some of its surface proteins (CD11b, CD62 I) are sensitive to different catecholamines: specifically, adrenaline, noradrenalin and dopamine (Trabold et al., 2007). In this study, a high concentration of epinephrine suppressed the production of neutrophil superoxide *in vitro,* and its above surface protein expressions were inhibited when neutrophils were activated with fmlp. In clinical settings, the same research group, in a more recent study, tried to investigate the above findings in conjunction with using α or β- adrenergic receptor antagonists; they showed that neutrophil function changes (superoxide production) as a result of catecholamines and particularly epinephrine could be mediated by β2-adrenergic receptors (Trabold et al., 2010).   Unfortunately, in the studies comprising this thesis, catecholamines or neutrophil protein expression were not measured during testing due to ethical and time constraints. It would be interesting for future research to measure these parameters along with neutrophil function in the context of psychological stress in larger scale studies in order to elucidate these mechanisms in more detail.

   A group in Russia has investigated the role of adrenergic mechanisms on immune cell phagocytic ability during the stress response in rats, finding that neutrophil phagocytosis was increased as a result of psychological stress; importantly, rats exposed to stress but injected with a β-adrenergic receptor blocker, failed to show a significant increase in neutrophil phagocytosis (Shilov and Orlova, 2003). However, whether the above findings extend to humans neutrophil function during psychological stress is not clear, and requires further investigation. Designing studies which can investigate specifically α- and β-adrenergic receptor expression changes on neutrophils during psychological stress would significantly advance this developing area of

stress effects on neutrophils in humans, and would help in exploring some of the potential mechanisms behind the reported changes.

Further, given the significant granulocytosis shown in the acute stress studies, the question remains as to where the neutrophils are coming from. This increase in circulating granulocytes is perhaps due to the demargination of neutrophils already in the vasculature (Von Vietinghoff et al 2008). However, whether the changes observed in the present study in neutrophil function is a result of the arrival of demarginated neutrophils which might have different functional capacity is not yet known. Mature peripheral blood neutrophils have been shown to localize to the liver, bone marrow and, to a lesser extent, the spleen; younger marrow-derivedcells home back mainly to the bone marrow, these post-migratory neutrophils are highly activated and this distribution might have different physiological effects.

There still remains a possibility of the HPA axis being involved in the present neutrophil function changes with stress given that in the third study there was a significant increase in the cortisol:DHEAS ratio in the bereaved group compared to the controls; in addition, study two shows trend towards cortisol increase after the stress task relative to baseline. Limited power may underlie the lack of significant correlations observed between these hormones and neutrophil function in the present studies. Some studies have shown some inhibitory effect of cortisol on neutrophil superoxide production *in vitro* (Bekesi et al., 2000). Others have reported an association between a raised cortisol:DHEAS ratio and reduced neutrophil superoxide generation in older adults after hip fracture and also showed that DHEAS was able to counteract the inhibition of superoxide generation by cortisol *in vitro* (Butcher et al., 2005). However, it should be noted that the physical stress of hip fracture may be very different from psychological stress. With regard to molecular mechanisms underlying the effects of cortisol and DHEAS, the same group has recently shown that DHEAS is able to directly enhance neutrophil superoxide generation via activation of protein kinase C and phosphorylation of NADPH oxidase subunits (p47) which are required for enzyme activation (Radford et al., 2010). Similarly, another study showed that serum cortisol levels were elevated among stressed students compared to controls, and when neutrophils from healthy controls were co-cultured with cortisol *in vitro*, reduced superoxide production was observed accompanied by a reduction in the above mentioned

NADPH oxidase components, p47. The authors concluded that glucocorticoids could be the underlying mechanism for the impact of stress on superoxide production change (Ignacchiti et al., 2011). It has been shown *in vitro* that dexamethasone can decrease superoxide production in cats (Hoffmann-Jagielska et al., 2003), and can suppress L-Selectin, the chemotaxis important neutrophil protein (Weber et al., 2004).

   Thus, findings from human and animal studies have shown that neutrophil function is not only sensitive to exogenous steroids (Langereis et al., 2011; Weber et al., 2004), but also that there may be an association between endogenous steroids and neutrophil function (Subandrio et al., 2000).   Further research is needed to replicate these findings, specifically with more focus on stress hormones and neutrophil function at the molecular level to confirm the involvement of the HPA axis hormones and neutrophil function during psychological stress exposure. It is clear that confirming the specific underlying mechanisms behind the observed stress-related changes in neutrophil function is a major challenge for this research field. Indeed, the well documented complex interactions which take place in the human body, at different levels during psychological stress, keep the doors open for many other mediators or confounders.

Limitations

   The studies comprising this thesis suffer from some limitations. First, the sample sizes were relatively modest in each empirical study, although for the first two studies sample size was of the same order of magnitude as previous studies of stress and human neutrophil function (Ellard et al., 2001; Kang et al., 1996; Kang and Fox, 2000) and larger than studies examining the effects of acute psychological stress on other immune outcomes (Bosch et al., 2005; Burns et al., 2008). Sample size inevitably reflects the demands of the current protocol: cannulation, early morning testing session, and same-day flow cytometry assays. Also it should be noted that recruitment of an older group to perform a 1.5 hour session in the laboratory with cannulation and stress proved difficult. For study three, it is important to highlight that recruiting bereaved older adults participants with no co-morbidity and within two months of their bereavement is exceptionally difficult, and this sample size took more than two years to be achieved. Further, the sample size is comparable to those in previous studies focused on bereavement and immunity (Gerra et al.,

2003; Kemeny et al., 1995; Lindstrom, 1997; Spratt and Denney, 1991). Third, despite the advantages of the flow cytometry assays, they are sensitive to diurnal and day-to-day variation (Hirt et al., 1994; Kampen et al., 2004). However, such effects were minimized by testing at the same time of day, and the use of internal controls for each assay. Assessing specifically different aspects of neutrophil function by flow cytometry has significantly increased nowadays and proved to be one of the most sensitive techniques (Elbim and Lizard, 2009).

Conclusions and future directions

It is now a common conclusion that acute stress is immune enhancing while chronic stress negatively affects immunity (Segerstrom and Miller, 2004). From the findings in the current thesis, this does not appear to be the case. Neutrophil superoxide production was reduced in response to acute stress as well as chronic bereavement stress. It is highly likely that such a reduction reflects a negative change, i.e., a reduction in the ability to fight disease, although the longer term clinical relevance of such changes requires further investigation. Evaluating the morbidity and mortality rates among older adults with varying levels of neutrophil function would be useful, given the documented involvement of neutrophils in the initiation and persistence of different inflammatory diseases such as asthma, COPD, bronchitis, cystic fibrosis, and many others (Cowburn et al., 2008; Downey et al., 2009; Muller et al., 2009; Saffar et al., 2011). The pro-inflammatory burden of neutrophils in some disorders, has caused neutrophils to be a target of many studies which have tried to used exogenous glucocorticoids to control these disorders (Saffar et al., 2011). However, the present results suggest that a decrease in neutrophils' ability to produce superoxide might also reflect dampened immunity and a reduced capacity to fight infection. Future research could include evaluating participants' infections rates when studying neutrophil function changes in the context of psychological stress in order to address the question of clinical relevance.

Findings from the studies comprising this thesis could contribute toward explaining the increased risk of infection in older adults, particularly those subject to frequent stress exposures or bereavement, especially considering the decline in some aspects of neutrophil function with ageing and the higher associated morbidity. Given that only a small number of studies have focused on psychosocial factors and innate immunity in older adults, the present findings must

necessarily be regarded as preliminary and replication is necessary. Finally, there is no doubt that psychological stress and its different associated stress hormones are implicated in a variety of diseases and disorders (Bose et al., 2009; Godbout and Glaser, 2006; Tosevski and Milovancevic, 2006; Yang and Glaser, 2002). However, the specific effect on health and disease in the context of stress and neutrophil function changes is still unknown.

Future research questions and directions which emerged from the findings of this thesis include; the need to explore the potential specific underlying mechanisms which are driving the neutrophil function changes as a result of psychosocial factors, particularly the effects of catecholamines on neutrophil function during stress. Also, the changes in α- and β- adrenergic receptors, as well as other important cell surface proteins such as (CD11b, CD62I) on neutrophils as a result of psychological stress should be explored. Extending the study of bereavement and neutrophil function to include a younger group, to see whether the changes seen are present in younger population without immunosenescence, and what the consequences are for infection rates, would be interesting to explore. Investigating potential delayed responses in neutrophil function post acute psychological stress to include longer time points, for instance; 1-4 hours post stress, in both old and young populations could be important in evaluating neutrophil function as a result of psychological stress, and more generalisable to real life, and could link the consequences of the present findings with participants' risks of infections and developing diseases.

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APPENDICES

APPENDIX I

UNPUBLISHED DEMOGRAPHIC/HEALTH BEHAVIOUR QUESTIONAIRES

The University of Birmingham

Neutrophil Study

Questionnaire Pack

Participant Number_____

Date:_____

General Information

Date of Birth ....
(Please circle the appropriate answers)

Sex   Male   Female
Age ....
Ethnicity   Asian   Black   White   Other
If other, please state ....

Have you suffered from an immune disorder in the past year?
Yes   No

Do you have any chronic illness/disease/condition(s) e.g. arthritis, high blood pressure etc.

Yes   No
If yes, please state what …………………………

Are you on any continuous medication?
Yes   No
If yes, please state what……………………………
How would you best describe your occupational status?

- If you are a student, please refer to the occupation of the parent who is the main breadwinner (if parents not living together put occupation of parent who has highest occupational status).

Professional e.g. doctor/ lawyer	Managerial/ Technical	Skilled non-manual e.g. nurse, PA	Skilled manual skilled e.g. craftsman	Partly- skilled e.g. e.g. buslabourer driver	Unskilled e.g.	Armed Forces

What is your/ your parents' postcode? ....(put postcode of main breadwinner if you are a student and if your parents are living separately)

Health Behaviour

Please circle the appropriate answer

Over the last year, how many cigarettes, on average, did you smoke per day?	None	1-5	6-10	11-20	21+	40+
Over the last year, on average , how often have you taken an alcoholic drink?	Never	Special Occasions only	1-2 per month	1-2 per week	Almost daily	2 per day or more

For the following question, please base your answers on the following:
1 unit = ½ pint of beer, 1 small glass of wine, 1 measure of spirit
Remember that home poured measures are likely to be larger
1 bottle of wine = 6 glasses, 1 average bottle of spirits = 27 measures

Over the last year, on average, how many units did you drink per week?	None	1-5	6-10	11-20	20-40	41+
Over the last year, how many hours, on average did you sleep per night?	0-3	4-5	6-7	8-9	10-11	12+
Over the last year, how often have you taken vitamin/mineral supplements?	Never	Once a month	Once a week	A few per week	Every day	More than one per day

Over the last year, how many hours per week on average, have you spent participating in activities which are:

Mildly energetic e.g. walking?	0	1-2	3-5	6-8	9-10	11+
Moderately energetic e.g. leisurely swimming, golf	0	1-2	3-5	6-8	9-10	11+
Vigorously energetic e.g. running, squash	0	1-2	3-5	6-8	9-10	11+

Please answer the following questions with reference to your diet over the last year. Answer as honestly and accurately as possible.

Are you on a special diet of any sort?	No	Vegetarian	Vegan	Weight-loss	Other

If other, please state……………………………………………………………….

How often do you eat breakfast?		Every day	Most days (3-6)	Once or twice a week	Less than once a week	Never

Apart from breakfast, how many main/cooked meals do you usually have during the day?   ....

How many cups/cans of caffeinated drink (coffee/tea/cola) do you usually drink in a day?   ....

For the following questions, please place a tick under the appropriate answer
Please indicate how often you have eaten each of the following foods over the past year.

	Never	Less than once a week	1 or 2 a week	Most days (3-6)	Once a day	2-3 times a day	4 or more times a day
Fresh fruit/salad/raw veg.							
Cooked veg. (not potatoes)							
Chips/fried food							
Potatoes/pasta/rice							
Bread (2 slices=one portion)							
Crisps/similar							
Tea							
Sweets/Chocolate							
Breakfast cereal							
Biscuits/cakes/puddings							
Low fat snack bars							
Full fat dairy products							
Reduced fat dairy products							
Fish/seafood (not fried)							
Poultry (not fried)							
Processed meat (e.g. pasties, pies, burgers, sausages)							
Beef/lamb/pork/ham/ bacon							
Soft drinks (non-caffeinated)							
Pure fruit juice							

APPENDIX II

ACUTE STRESS TEST

PASAT explanation

- **Read Stress Task explanation before practice test**

"The tape will read out a series of numbers between one and nine, at fixed intervals apart. You have to listen to the numbers and add them together. So if you hear a 4 then a 5, you say 9 out loud. However, you must remember the 5, the second number, in order to add it to the next number read out by the tape. So if the tape says, 4…..5 you say 9, but remember the 5, then if the tape says …3…you say 8, but remember the 3 and so on. You are always adding the number you just heard to the last number you heard before that, never to the number you have said out loud. Is that OK? (Answer any questions)

- Practice PASAT

PASAT instructions

- **Read instructions before running test**

Your performance on this task is assessed, recorded and you are in direct competition with your fellow students. It is very important that you perform to the best of your ability. You will start the test with 1000 points. For every number you miss, 5 points will be deducted from your score. Your score can decline rapidly if you do not concentrate or if you perform badly. The experimenter will be judging your performance. Performance tables will be produced and displayed to compare you to other participants in the study.

During this task, you will also be videotaped. Two independent body language experts will assess the videos in order to judge how much you blush, fidget or stammer during the task as a measure of your anxiety levels. You will also be able to see yourself live on the television during the task. You must watch the television at all times.
The experimenter will sound a buzzer every time you:
1. Give an incorrect answer
2. Look away from the television screen
3. Stutter, mumble or hesitate.

The numbers on the tape will get faster as the tape goes on; you must keep up with the numbers. If you miss a number, you must pick up the pattern again as soon as possible so that your score does not decline too much.

Now, make sure that you are looking at the television screen. The test will begin in 30 seconds.

PASAT SCORING SHEET

4	Answr	6	8	9	18	2	3
6	10	6	12	3	12	9	11
3	9	2	8	8	11	2	11
1	4	4	6	1	9	9	11
7	8	6	10	3	4	1	10
3	10	3	9	2	5	2	3
3	6	3	6	5	7	4	6
2	5	5	8	3	8	5	9
2	4	1	6	5	8	4	9
4	6	5	6	7	12	7	11
4	8	8	13	4	11	9	16
9	13	5	13	6	10	8	17
5	14	9	14	6	12	3	11
7	12	4	13	4	10	6	9
1	8	8	12	8	12	2	8
8	9	9	17	5	13	1	3
8	16	7	16	7	12	6	7
6	14	7	14	8	15	1	7
9	15	3	10	3	11	3	7
5	14	4	7	9	12	6	9
5	10	8	12	6	15	7	13
4	9	3	11	6	12	4	11
5	9	1	4	1	7	2	6
6	11	8	9	4	5	9	11
7	13	6	14	3	7	8	17
3	10	7	13	7	10	4	12
3	6	8	15	9	16	7	11
6	9	8	16	9	18	2	9
8	14	3	11	3	12	3	5
9	17	7	10	5	8	7	10
1	10	7	14	2	7	3	10
5	6	9	16	2	4	7	10
3	8	5	14	3	5	8	15
7	10	4	9	7	10	8	16
2	9	1	5	4	11	2	10
9	11	1	2	8	12	2	4
2	11	6	7	1	9	3	5
8	10	2	8	8	9	6	9
1	9	4	6	8	16	5	11
9	10	2	6	5	13	4	9
8	17	5	7	6	11	1	5
7	15	4	9	7	13	2	3
1	8	3	7	2	9	5	7
6	7	6	9	4	6	5	10
4	10	7	13	3	7	2	7
2	6	8	15	7	10	9	11
4	6	2	10	3	10	7	16
1	5	3	5	7	10	7	14
7	8	6	9	8	15	2	9
1	8	1	7	3	11	7	9
9	10	5	6	4	7		
2	11	8	13	5	9	Total	
2	4	9	17	1	6	10min	

APPENDIX III

NEUTROPHIL FUNCTIONAL ASSYAS

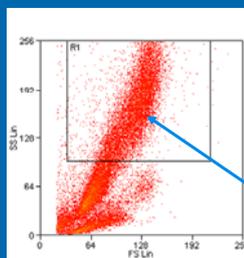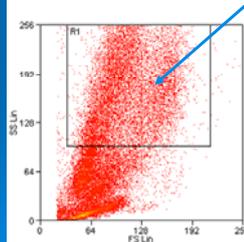Phagotest and Phagoburst Assays
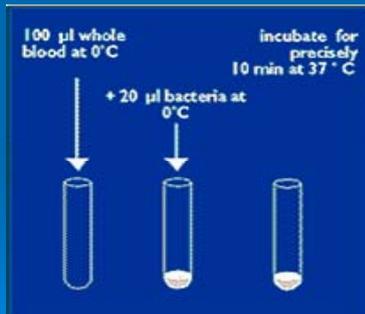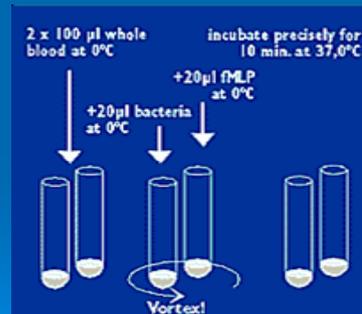
FLOWCYTOMETRIC ANALYSIS
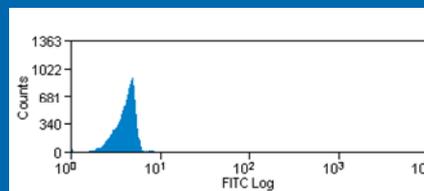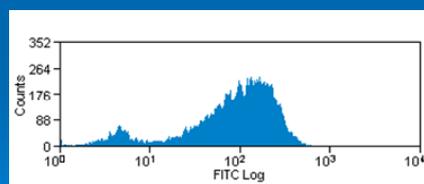
Phagotest Analysis

Control Tube

Granulocytes gated

Test Tube

MFI measured

MFI measured

Phagotest Analysis , Mean Fluorescent Intensity (MFI) compared