CHRONIC STRESS AND AGEING: EFFECTS ON IMMUNE FUNCTION

by

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ABSTRACT

The research in this thesis is concerned with the effect of chronic stress, caregiving and bereavement, and ageing on immune and endocrine parameters. First, there was no difference in serum anti-CMV antibody titre between younger caregivers and controls, but CMV seropositive caregivers with negative health behaviours had higher CMV antibody titre. Second, there was no difference in neutrophil function between the groups in both younger and older cohort, while only younger caregivers showed a higher serum cortisol:cortisone ratio than controls. Further, those caregivers that reported higher anxiety and burden symptoms had lower neutrophil phagocytosis. Third, caregivers had more T cells expressing KLRG1 marker of senescence than controls, but comparable number of those expressing PD-1 marker of exhaustion and thymic output. Finally, young bereaved showed similar neutrophil function and serum cortisol and DHEAS levels as non-bereaved controls, whereas older bereaved had impaired neutrophil function and a higher cortisol:DHEAS ratio. These findings suggest that chronic stress can have differential effects on immune and endocrine parameters, but in some cases, presence of immunosenescence is required for immune decrements to be observed. Further, they emphasise the importance of focusing on the individual's response to chronic stress rather than their chronic stress status, *per se*.

DEDICATION

I dedicate this thesis to my mum, Gorana, and my dad, Stipe, for not implementing their own rules, but trusting in the choices I was making instead.

Mami Gorani i tati Stipetu, zbog toga sto nisu nametali svoja pravila, nego su verovali u moje izbore.

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LIST OF PUBLICATIONS

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3. Vitlic, A., Lord, J.M., Oliver, C. & Phillips, A.C. (2014) 'Bereavement reduces neutrophil oxidative burst only in older adults: Role of HPA axis and immunosenescence', *Immunity and Ageing*, 11:13. doi:10.1186/1742-4933-11-13
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The following papers are also in preparation and under review:

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LIST OF ABBREVIATIONS

ACTH - adrenocorticotropic hormone; ANO(C)VA - analysis of (co)variance; APC - allophycocyanine; AR - adrenergic receptor; ASD - Autism spectrum disorder; 11β-HSD - 11beta-hydroxysteroid dehydrogenase; BI - Zarit Burden Index; BMI - body mass index; BSA - bovine serum albumin; CA - cateholamines; CAS - caregiving activity survey; CBI - core bereavement items; CD - cluster of differentiation; CMV - Cytomegalovirus; CNS - central nervous system; CRH - corticotrophin releasing hormone; CRP - C reactive protein; DC - dentritic cells; DHEAS - dehydroepiandrosterone sulphate; DHR - dihydrorhodamine; DNA - deoxyribonucleic acid; EBV - Epstein Barr virus; ELISA - enzyme linked immunosorbent assay; *Escherichia coli* - *E. coli*; FITC - fluorescein isothiocyanate; FOXO3a - forkhead box class O; GC - glucocorticoids; HADS - hospital anxiety and depression scale; HIV - human immunodeficiency virus; HPA - hypothalamic-pituitary-adrenal; HSV - herpes simplex virus; Ig - immunoglobulin; IES - item event scale; IFN-γ - interferon gamma; IL - interleukin; KLRG1 - killer lectin-like receptor G1; LC-MS/MS - liquid chromatography tandem mass spectrometry; MFI - mean fluorescence intensity; MHC - major histocompatibility complex; MTBE - methyl terc-butyl ether; NF-κB - nuclear factor kappa B; NHS - National Health Service; NK - natural killer; NKG2D - natural killer G2 family of C-type lecti; OD - optical density; PBMC - peripheral blood mononuclear cells; PBS - phosphate buffered saline; PD-1 - programmed death; PE - phycoerythrine; PHA - phytohemagglutinin; PI - phagocytic index; PPB - Pearlin behaviour problems; PSS - perceived stress scale; ROS - reactive oxygen species; RT - room temperature; SDQ - strength and difficulties questionnaire; SEM - standard error of the mean; SFS - support function scale; SIRT1 - sirtuin 1; TCR - T cell receptor; Th - T helper cells; TNF-α - tumour necrosis factor alpha; TREC - T cell receptor excision circle; VCA - virus capsid antigen.

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

Ageing is a physiological process that emerged as a side-product of normal development and the metabolic processes involved in the reproductive potential of the species (Cutler 1982). It has been developed most likely as a non-adaptive phenomenon with no biological function (Partridge and Gems 2002) and allowed to evolve through a trade-off mechanism termed antagonistic pleiotropy (Williams 1957), reviewed in (Partridge and Barton 1993). According to Thomas Kirkwood's modification of Orgel's (1963) theory, ageing can also be linked to the attempt of the body to protect germ line cells at the expense of somatic cells, the so-called "disposable soma theory" (Kirkwood 1977). Therefore, ageing is a phenomenon that evolved as a negative by-product of positive traits that were beneficial to organisms, such as fat deposition or sexual hormones, oxygen metabolism (generation of the reactive oxygen species (ROS)), but also stress and glucocorticoids (GC), as part of the fight or flight response. Among these, development and oxygen metabolism are constant and necessary involuntary forces, and hence unchangeable, but the stress response in humans and other primates has changed over time, gaining another characteristic; the capacity to be chronic and detrimental (Sapolsky 2007).

In this chapter, the mechanisms by which stress can influence different aspects of immunity will be presented, as well as the interaction between ageing and stress and the consequences of this for immune health. The content will first present stress as an adaptive phenomenon, and explain its relationship with the immune system generally. Second, the focus will be on how psychological stress affects the immune system. In the latter part of the introduction, the influence of ageing will be discussed, firstly describing the interplay between ageing and stress and then their additive effect on immunity. Finally, the pathophysiological consequences of the interaction between stress, ageing, and immunity will be discussed.

Although the focus is mainly on humans, where applicable, studies using small animal models are included.

1.2. STRESS

A challenging life demands tough reactions and has led to the development of a number of physiological changes that constitute the stress response in animals and humans. In a hostile environment, more complex organisms like vertebrates develop a response that Walter Cannon (1929) first introduced as 'fight or flight'. The main function of this response is maintaining body homeostasis. The key site involved in this process is the hypothalamus (Barrett 2005), a part of the brain that communicates by sending nerve impulses to other parts of the body. In this way, the hypothalamus acts within seconds and via sympathetic nervous system stimulates medulla of the adrenal gland to release catecholamines, CA (adrenalin and noradrenalin). In addition, the hypothalamus also produces chemical messengers that act more slowly and in minutes travel through the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al. 2000). Chemical messengers in this pathway include corticotrophin releasing hormone (CRH) which stimulates the anterior pituitary gland to release another hormone, adrenocorticotropic hormone or ACTH. The target organ of ACTH is again the adrenal gland, but this time it is the cortical cells that synthesise and release species-specific GC into the blood. The tight control of these GC (mainly cortisol in humans) is sustained via negative feedback that controls and, in the end, terminates the release of CRH (Griffin and Ojeda 2004).

Clear distinction is made today between two types of stress, acute and chronic stress. From an evolutionary point of view, the acute component is beneficial in that it provides organisms

with the mechanisms for protection from a changeable and threatening environment. In that context, an interaction such as one between a lion and a zebra, even though there is no common interest for the outcome between these two animals, will trigger the same cascade of events in the body of both. Adrenalin and noradrenalin mainly, but with GC potentiating the effects, will increase arousal, alertness and vigilance, focus attention and elevate core temperature. In addition, they are responsible for the increase in the pain threshold (Kulkarni 1980), cardiovascular output, respiratory rate (Coles et al. 1956), and blood flow to the brain and skeletal muscle (Brown et al. 1979, Charmandari et al. 2005, Coles et al. 1956, Dowd et al. 2009, Kulkarni 1980). Skeletal muscle will gain the supply of the energy from the adipose and hepatic cells stores, whereas all other activities that are inessential in that moment, such as digestion, reproduction, feeding and growth, will be decreased through the action of GC (Cannon 1929, Selye 1956, Sorrells and Sapolsky 2007). Although acute stress and the events that follow have evolved as an adaptation, and are therefore beneficial, too great or too long of a response can be detrimental to the body. For example, synaptic plasticity in adult rats was negatively affected by prolonged stress exposure (Trentani et al. 2002). In humans, continuous or repeated psychological stress is strongly associated with detrimental effects on the cardiovascular system, and through it, with obesity and hypertension (McEwen 1998, Phillips et al. 2012, Sedova et al. 2004). However, despite the seemingly direct relationship between stress and different physiological functions, the general rule regarding the particular effects of GC alone on other organ systems is not sufficient to explain their action on the immune system, as described below. In other words, the negative effect of stress on the immune system is actually a non-adaptive phenomenon, as, using the analogy of Sapolsky, an injured animal would not survive and propagate the species if it escaped a predator only to then die of sepsis soon afterwards (Sorrells and Sapolsky 2007).

1.3. STRESS AND THE IMMUNE SYSTEM

1.3.1. GC and CA effects of acute and chronic stress

It was previously thought that the stress response, through the action of GC, strictly suppresses immunity. There are several sources of evidence for this anti-inflammatory action of GC, such as: involution of the thymus explained by Selye in 1930s (Selye 1936, Viner 1999); the shift from a cellular T helper cells (Th)1 to a humoral Th2 phenotype and inhibited production of the pro-inflammatory cytokines (interleukin (IL)-12, interferon gamma, IFN-γ, tumour necrosis factor alpha, TNF-α) (Franchimont et al. 2000, Hu et al. 2003, Steer et al. 2000), accompanied by the increased secretion of anti-inflammatory cytokines (IL-4 and IL-10) by Th2 cells (Elenkov and Chrousos 2002, Mozo et al. 2004, Wu et al. 1991); limitation of the capacity of dendritic cells (DC) to interact with immature T cells by preventing up-regulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules (Akdis et al. 1997, DeKruyff et al. 1998, Franchimont 2004, Ramirez et al. 1996). It is now known, however, that the way stress impacts immunity is not always straightforward, and can be highly influenced by the duration of the stressor (Sorrells and Sapolsky 2007).

Initially in the acute stress response, the immune system is activated rather than suppressed (Sorrells and Sapolsky 2007), and only after this first reaction, in the following stages of the stress response, when levels of GC further rise, their anti-inflammatory effects come on the scene (Munck et al. 1984). Higher concentrations of GC will then help the organism to recover from the early phases of the stress response (Munck et al. 1984). However, it is certain that upon repeated stimulation by stress, or through prolonged, chronic stress, the immune system is suppressed, and this is, at least in part, mediated by the action of GC (Sorrells and Sapolsky 2007). The explanation for this dual behaviour of GC could be in the

way they transmit their effects through steroid hormone signalling (Sorrells and Sapolsky 2007). The actual mechanism by which GC exert these different effects is yet to be elucidated. However, a potential explanation is that mineralocorticoid receptors, which paradoxically have much higher affinity for GC than GC receptors, are first to bind GC. Therefore, during the initial acute stress phase, when GC are present in lower levels, they will predominantly bind mineralocorticoid receptors, which are thus, together with CA release, responsible for the initial stress-induced increase in the immune function (Sorrells et al. 2009). In the following stages of the stress response, when mineralocorticoid receptors get saturated, GC receptors become increasingly occupied and can govern some of the anti-inflammatory action. On the other hand, it is uncertain if the same explanation could apply to the immune system in the periphery. For example, even though expressed in macrophages, mineralocorticoid receptors do not seem to be involved in modulating immune cells function by GC. Instead, that action is strictly confined to the concentration-dependent binding of the GC to the GC receptors (Lim et al. 2007). Nevertheless, it seems likely that a similar type of concentration-dependent effect of GC could be responsible for the difference in the effect of acute and chronic stress on the peripheral immune system, too (Sorrells and Sapolsky 2007). It is important to reiterate here that GC are neither the only, nor the primary inducers of immune alterations during the chronic stress response (Moynihan 2003). For example, the suppression of mitogen responses by rat lymphocytes was dependent only upon the production of β-endorphin and without any influence by corticosterone levels (Panerai 1997). Similarly, Shi et al. (Shi et al. 2003), showed that the apoptosis of lymphocytes during restraint stress was dependent on the state of opioid receptors only. The adrenergic receptor (β_2-AR) after binding CA through two different signalling pathways triggers DNA (deoxyribonucleic acid) damage as well as degradation of an important tumour suppressor in

the brain of rats (Hara et al. 2011). Similar results were obtained after exposing rats to chronic restraint stress, whereas, the administration of propanolol, a β-blocker, diminished this effect. Finally, the mutual action of GC and CA during chronic restraint stress will simultaneously affect migration of mononuclear cells in the peripheral blood of a specific mouse strain (Hermann et al. 1995), largely reduce cytokine expression from the lymph nodes and spleen (Dobbs et al. 1996), delay cytokine gene transcription in the lungs and lymph nodes (Sheridan et al. 1998), as well as diminish activation of cytotoxic T lymphocytes in the lymph nodes (Dobbs et al. 1993, Sheridan et al. 1998). However, it was also shown that chronic stress-induced changes in the concentrations of GC alone can have different effects on different pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 (DeRijk et al. 1997). For example, daily fluctuations of these hormones were able to strongly affect TNF-α concentrations, whereas IL-1β responded only after exercise-induced increases in the levels of cortisol, and lowering IL-6 demanded pharmacological concentrations of GC (DeRijk et al. 1997). All three of these cytokines are predominantly produced by monocytes, but IL-6 can be produced by endothelial cells, fibroblasts and keratinocytes (Heinrich et al. 1990). Finally, while TNF-α, IL-1β are strictly pro-inflammatory, IL-6 can have both pro-inflammatory and anti-inflammatory effects (Scheller et al. 2011). Therefore, perhaps, the differential effects of stress hormones on TNF-α, IL-1β, and IL-6 are largely caused by the different roles of these cytokines.

1.3.2. Immuno-enhancing and immunosuppressive effects of acute and chronic stress

There is a well established difference in the effects that acute and chronic stress exert on the immune response, being immuno-enhancing and immunosuppressive, respectively. The acute stress response and its associated immunological changes closely resemble those related to infection, and involve both energy mobilisation, and activities of cytokines and

neurotransmitters (Maier and Watkins 1998). Acute brief restraint stress, applied before surgical sponge implementation into the rats has lead to more prominent increase in all blood leukocyte count comparing to the non-stressed group (Viswanathan and Dhabhar 2005). Similarly, the same type of acute stress administered before the immunisation increased the number of memory and effector Th cells following immunisation (Dhabhar and Viswanathan 2005). This also seems to influence more robust immune response upon repeated stimulation with the same antigen months later (Dhabhar and Viswanathan 2005). This can be beneficial in cases when increased immunoprotection is needed, but detrimental in the cases of immunopathology such as allergic conditions and autoimmune disease (Atanackovic et al. 2013, Dhabhar 2009, Dhabhar and Viswanathan 2005).

Chronic stress is generally accepted as being immunosuppressive. However, if it was strictly immunosuppressive, it would not be able to negatively influence disease outcomes in infectious and neoplastic disease (associated with inadequate immunity) on the one hand, and allergic and autoimmune disease (that emerge from an excessive immune response) on the other, as explained by Segerstrom and Miller (Segerstrom and Miller 2004). One possible explanation for these seeming mutually exclusive consequences of chronic stress was given by Marshall et al.(Marshall et al. 1998), who suggested that chronic stress drives the Th1-to-Th2 shift by altering patterns of cytokine expression. In that way, stress-induced suppression of Th1 cytokines (such as IFN-γ) involved in the defence against many kinds of infection and some neoplastic diseases, would lead to activation of Th2 cytokine production, involved in allergies and different autoimmune diseases, such as IL-10 (Marshall et al. 1998). In addition, it has been shown that chronic stress caused by immobilisation affects the number of lymphocytes in rats, but impacts on different subsets in different ways (Dominguez-Gerpe and Rey-Mendez 2001). The overall decrease in the number of circulating lymphocytes was

accompanied by the increase in the number of mainly immature T lymphocytes, suggesting one of the potential mechanisms by which stress associated immunosuppression can affect and exacerbate autoimmune diseases (Moroda et al. 1997).

In summary, acute stressors usually, with the exceptions of natural killer (NK) cell function (Cunnick 1988) and neutrophil superoxide production (Khanfer et al. 2010, Khanfer et al. 2012) boost the immune system, particularly its innate component which is the one able to act quickly (Bosch et al. 2003, Dhabhar and McEwen 1997, Sapolsky 1998). The majority of chronic stressors, on the other hand, are associated with global immunosuppression and have an impact on both innate and adaptive component of the immune system (Kiecolt-Glaser et al. 1991a, Phillips et al. 2006a, Segerstrom and Miller 2004, Thaker et al. 2006) to mention a few.

1.3.3. Beneficial and detrimental effects of acute and chronic stress

Both the immune response and the fight-or-flight response provide an adequate defence for survival and further protection against infection after the injury occurs. In that context, the relationship between acute stress and immune up-regulation can be viewed as an adaptive trait. On the other hand, chronic exposure to stress appears to have detrimental effects on immunity through continual activation of the same mechanisms. This overlap between the stress response and the immune response to infection could be the answer to some of the seemingly contradictory processes that arise as the consequence of different durations of the same stressor. For example, in response to acute stressors, T cells in the rat react by redistributing into the skin, which is the organ that is the most likely to be affected in a life threatening situation when attacked by the predators. On the other hand, prolonged action of the same type of stressor will lead to the progressive decrease in this stress-induced

redeployment of T cells, as well as to the suppression of delayed type hypersensitivity in the skin (Dhabhar and McEwen 1997). Similarly, surgical operation in cancer patients after oesophagectomy has been associated with an increase in peripheral blood lymphocytes apoptosis (Kono et al. 2001). The mechanism used to explain this rise was increased expression of Fas, a transmembrane protein that mediates apoptosis (Oka 1996), and changes in T lymphocyte signal transduction through down-regulation of T cell receptor ζ with a crucial influence of activated post-operative monocytes on the process overall (Kono et al. 2001). Negative effects on lymphocytes associated with surgical stress were also observed in combination with psychological (daily life hassles) and physiological (cold pressor) stress upon stimulation of lymphocytes with phytohemagglutinin (PHA) and pokeweed mitogen, respectively (Linn et al. 1988). Through altering IFN-γ production and the ability to respond to both interferons and pro-inflammatory cytokines, e.g., IL-2, chronic restraint stress affects the activity of NK cells, components of the innate immunity important in resolving viral infections such as infection with herpes simplex virus (HSV) (Bonneau et al. 1991).

1.4. PSYCHOLOGICAL STRESS AND IMMUNITY

A relationship between the central nervous system (CNS) and immune system was first discovered in early animal experiments where it was revealed that immunosuppression could be induced through classical conditioning (Ader 2003, Ader and Cohen 1975, Garcia et al. 1955). A large number of studies have emphasised the behavioural changes that accompany chronic stress situations (such as alcohol consumption (Nguyen et al. 2012, Silva and Madeira 2012), smoking (Lee et al. 2007), nutrition (Thompson et al. 2013), and sleep disturbances) that are already known to have direct and serious health consequences and could mediate the

negative effect of stress on health indirectly (Dallman et al. 2003, Hussain 2010). Other indirect effects might be via changes in social roles and social support associated with stress at the same time affecting health and quality of life (Baron et al. 1990, Pressman et al. 2005, Rutledge et al. 2004, Segerstrom and Miller 2004). However, many direct effects on immunity have also been demonstrated. Several studies reported changes in cytokine profile in students during an academic examination period. The general pattern seems to be the emphasis of Th2 response through the decrease in pro-inflammatory cytokines (TNF-α, IL-6, IL-1, INF-γ) and higher levels of anti-inflammatory cytokines (IL-10 and IL-4) (Kang and Fox 2001, Marshall et al. 1998). Similar to these studies, delayed wound healing and a decrease in IL-1β, a key interleukin involved in this process, has been demonstrated in young healthy students during an examination period compared to a non-stressful holiday period (Marucha et al. 1998). The opposite was the case in students with higher anxiety where levels of pro-inflammatory cytokines rose just before the important exam (Kamezaki et al. 2012, Maes et al. 1998). The explanation for this seemingly contradictory effect of examination stress is seen in its duration, as it can be divided into acute examination stress (i.e. stress immediately before the exam, as in (Kamezaki et al. 2012, Maes et al. 1998)), and its prolonged, chronic component (i.e. during the examination period, as in (Kang and Fox 2001, Marshall et al. 1998)), for a review see (Bosch et al. 2002). In one of the first studies that examined the relationship between psychological stress and the immune system in humans, the strong psychological stressor of bereavement was associated with the decreased function of T lymphocytes (Bartrop et al. 1977). In a similar way, neutrophil killing ability was suppressed in the bereaved, the effect that was accompanied by the increase in cortisol:DHEAS (dehydroepiandrosterone sulphate) ratio (Khanfer et al. 2011). This ratio has been previously used as a measure of the effect stress hormones have upon immune system

components (Butcher et al. 2005). Further, homeless people who reported higher stress levels had lower density of lymphocyte β-AR (Dimsdale et al. 1994). This could indicate either a down-regulation of receptors due to higher stress hormone levels (CA or GC) or simply a change in the lymphocyte subsets, both of which could be a consequence of prolonged exposure to a stressful lifestyle. In addition, in children with a history of recurrent colds and flu who demonstrated higher levels of psychological stress, salivary immunoglobulin (Ig) A/albumin ratio was lower, indicating a potential link between stress and colds and flu (Drummond and HewsonBower 1997).

Loneliness affects NK cell activity not only in psychiatric patients (Kiecolt-Glaser et al. 1984b), but also in young and healthy medical students indicating general importance of social relationships for individuals' wellbeing (Kang et al. 1998, Kiecolt-Glaser et al. 1984a). Marital quality and recent separation among young women were associated with depressive symptoms and poorer immune function, seen through poorer proliferation of lymphocytes after stimulation with different mitogens (concavalin A and PHA) (Kiecolt-Glaser et al. 1987a). More frequent marital concerns were associated with flatter cortisol profile (Barnett et al. 2005), an indicator of non-adaptive cortisol metabolism during chronic stress exposure.

One commonly studied model of the impact of stress on immunity is role of caring for someone, be it a spouse or child with a physical or mental illness or disability. Caregiving is now well established as having a serious effect on psychological well being, physical health and self-efficacy among caregivers when compared to matched non-caregiving individuals (Pinquart and Sorensen 2003). For example, parents of cancer patients when compared to control parents had a decreased sensitivity to the anti-inflammatory effect of GC which could potentially contribute to the development of asthma, different cardiovascular and autoimmune diseases, as well as indicate dysregulation of the immune system that may become incapable

of resolving infections (Miller et al. 2002). This is where we see the other side of stress effects on immune function where it exacerbates excessive immune response. Another study of adaptive immunity in mothers of children with developmental disabilities showed a lower T helper: suppressor ratio, indicating again potentially less effective adaptive immunity for fighting the pathogens among the older age cohort of caregivers (Pariante et al. 1997).

One well researched indicator of immune challenge in the context of psychological stress is immunity to latent viruses, with reduced immunity indicated by raised antibody titres to latent viruses. Generally, latency is the ability of a virus to lie dormant in the host cell after the initial infection, and emerge as an acute infection once the immune surveillance of the host weakens (Nowak 1991). Therefore, even though asymptomatic in the immunocompetent hosts, these infections could cause serious harmful and even fatal effects in the immunocompromised (Pawelec et al. 2005, Rasmussen 1991). In that context, separated women also had higher antibody titre against Epstein-Barr virus (EBV), indicating poorer control of the virus, as well as lower number of both NK cells and Th lymphocytes when compared to married women, with worse depression and immune outcomes seen in those with greater attachment to their ex-husbands (Kiecolt-Glaser et al. 1987a). In the case of married and separated men, those who went through divorce were more depressed, lonelier, and had a higher antibody titre against both EBV and HSV (Kiecolt-Glaser et al. 1988). Finally, more negative behaviour in marriage has been shown to adversely affect endocrine responses in women, and immunological activation, seen through antibody titres to EBV and the blastogenic response to T cell mitogens, in both genders (Kiecolt-Glaser et al. 1997). Another study also demonstrated higher antibody titres against cytomegalovirus (CMV) in a group of caregiving individuals when compared to the controls, indicating poorer latent virus control (Pariante et al. 1997).

Vaccination produces immune memory against specific pathogens, ready to respond to an infection. An inadequate response and failure to provide full protection after vaccination, measured in terms of antibody titre, is indicative of a poorer immune response in the recipient. Unmarried older adults and those who had poor marital quality had a weaker antibody response to the influenza vaccination than happily married older adults (Phillips et al. 2006a). Life events stress and perceived stress were also related to a lower antibody response after vaccination against flu and meningitis in students (Burns et al. 2003, Burns et al. 2002, Phillips et al. 2005), while greater social support enhanced antibody titres for some vaccine strains (Phillips et al. 2005). However, studies that have examined the antibody response to vaccination in younger caregivers have reported inconsistent results. The first study of this type compared hormonal and immune status between 41 partner of multiple sclerosis patients and 62 controls (Vedhara et al. 2002). Multiple sclerosis was chosen as serious chronic and degenerative illness that usually causes physical and cognitive complications (Barcellos et al. 2002), and as such thought to cause equivalent level of burden for caregivers as seen in spouses and partners of dementia patients. Despite reporting higher levels of stress, but not anxiety and depression, caregivers showed no difference in either antibody response to an influenza vaccination, nor their IFN-γ and IL-4 levels. Similarly, cortisol and DHEAS and their ratio was not different between the groups (Barcellos et al. 2002), supporting previous reports of preserved immune responses in stressed caregivers. In contrast, a study conducted by Gallagher et al. (Gallagher et al. 2009b) showed a poorer antibody response to pneumococcal vaccination in caregiving parents of children with developmental disabilities when compared to age and sex matched control parents. These findings were further supported in another study by Gallagher et al. (Gallagher et al. 2009a) which showed a reduced ability of caregiving parents to mount an adequate antibody response compared to

control parents after vaccination against the influenza virus. It was argued that the difference in immune response in these studies could be due to characteristics of the care-recipient such as challenging behaviours, rather than the caregiving role *per se*, and that perhaps the caregiving experience, reported as more challenging in case of parents of children with developmental disabilities, is what drives this change in immune function (Gallagher et al. 2009b). Indeed within the caregiving group, those parents who reported higher child problem behaviours had a poorer antibody response to the pneumococcal vaccine (Gallagher et al. 2009b).

Stressed individuals also show significant changes in the DNA repair process. Changes in DNA repair processes have been associated with different types of cancer such as cutaneous malignant melanoma (Wei et al. 2003), lung cancer (Wu et al. 2003), and breast cancer (Sharan et al. 1997). One of the theories of ageing suggests that the accumulation of DNA damage is a potential cause of gradual frailty in living organisms (Freitas and de Magalhaes 2011), emphasising the involvement of the repair mechanism in the ageing process (de Boer et al. 2002). Several studies have shown correlations between stress and the efficiency of DNA repair (Yang and Glaser 2003). This is also a demonstration of the complicated relationship between stress and body mechanisms, as it shows that in addition to the duration of the stressor, the consequences of its action depend also upon the capacity of the organism to adapt to change and maintain homeostasis. The effect of stress was different depending on the population tested, with a decrease seen in the DNA repair mechanism after X-irradiation in psychiatric compared to non-psychiatric patients (Kiecolt-Glaser et al. 1985), whereas in young healthy students, stress during an examination period influenced the increase in the extent of DNA repair after UV radiation (Cohen et al. 2000). As examination stress increases DNA damage and hence the need for its repair, it could be that the increase in the repair

process in young healthy students indicates their ability to meet this criteria, an ability that is not present in psychiatric patients who exhibit a decrease (Yang and Glaser 2003). Forlenza et al. (Forlenza et al. 2000) confirmed this in a study that showed an increased rate of nucleotide exchange repair in students during an examination period when compared to the low stress holiday period. Further evidence can be found in the rat studies where sister chromatid exchange, a process that is known to occur with higher incidence in the presence of agents with oncogenic potential (Banerjee and Benedict 1979), showed a doubling potential in the bone marrow cells of rats subjected to different types of behavioural stressors, such as swimming, white noise, and foot shock (Fischman and Kelly 1987). On the other hand, a decreased DNA repair capability was reported after carcinogen administration in rats exposed to the rotational stress compared to a non-stressed group (Glaser et al. 1985).

When considering factors, tightly regulated and controlled, and related to both maintaining and disturbing homeostasis on a cellular level, structures that emerge as one of the key controllers of the cell cycle are telomeres. Telomeres are complexes of repetitive DNA sequences (TTAGGG) located on the very end of each chromosome surrounded by a large number of proteins. They have several functions, but are mainly involved in maintaining chromosome stability (Dahse et al. 1997). Due to the nature of the DNA replication process, telomeres protect the core of DNA, shortening by approximately 50 base pairs with every cell cycle. Telomeres eventually become so short that the DNA is exposed, replication ceases and cells enter replicative senescence (Dahse et al. 1997). Telomeres thus act as a “biological clock” counting the number of cell divisions a cell has made (Dahse et al. 1997). Indeed, a large number of studies have suggested that telomere shortening is an important factor in the process of ageing, as well as diseases such as human immunodeficiency virus (HIV), hepatitis, Alzheimer's, inflammatory bowel disease, and cancer (Jiang et al. 2007). The

importance of adequate telomere length is a key to the immune system, particularly adaptive immunity, as cell division in lymphocytes is necessary for their response to antigenic challenge (Kaszubowska 2008). However lymphocytes express the enzyme telomerase, which can extend telomere length, giving them additional replicative capacity before they enter senescence (Kaszubowska 2008). Studies have indicated that chronic stress might in this case mimic or accelerate immunosenescence. For example, telomere length in peripheral blood mononuclear cells (PBMCs) was shorter in parents caregiving for a chronically ill child and experiencing higher stress levels compared to those in the low stress caregiver group, even though there was no difference in telomere length between caregiving parents generally and their age and sex matched controls (Epel et al. 2004).

Another very important mechanism inside the cell that needs to be carefully regulated and kept in balance in order for the cell to function normally is programmed cell death or apoptosis. Apoptosis is essential for proper embryonic development, functioning of the immune system, as well as maintaining of the homeostasis in response to different physiological and pathological stimuli (Elmore 2007). For example, apoptosis is an important mechanism in the regulation of neutrophil function. Neutrophils are key effector white blood cells in defending the host from bacterial infection by producing various cytokines and ROS (Lloyd and Oppenheim 1992). However, neutrophils are also important factors in regulating inflammatory processes, and the existence of an adequate regulatory mechanism is necessary to prevent these protective components becoming damaging to the host (Sendo et al. 1997). With a half-life of 10-12 hours, these cells are given enough time to perform their protective role and fight bacterial infection, but prevented from any deleterious effect on surrounding tissue by entry into apoptosis once they have engulfed bacteria (Sendo et al. 1997). Intense exercise stress prolongs the survival of neutrophils, whereas longer lasting examination stress

inhibits the process (Sendo et al. 1997). This extended lifespan could be useful at times of infection, but in its absence could be damaging, indicating the variety of ways in which stress can affect health and the complexity that lies behind that relationship.

1.5. AGEING, STRESS AND THE IMMUNE SYSTEM

One of the important components that should be taken into account when considering the effect of stress on immunity is age. Even in the cases where the effect of stress on the immune system is strong, such as in the case of caregiving, this might be even more evident or might change once the immune system is challenged by both ageing and stress simultaneously (Segerstrom and Miller 2004). Ageing is considered to weaken the body's ability to respond to stress, and with stress affecting organisms in a similar way as ageing, it may lead to accelerated ageing (Sapolsky 1999). It has been suggested that one of the factors contributing to this exacerbated effect of stress in the aged organisms is their inability to terminate the production of GC in response to stress (Sapolsky et al. 1986). According to the GC cascade hypothesis, failure of the control mechanism that should stop the production of GC after the effect of the stressor has ended is caused by the age-induced degeneration of the region of the brain responsible for communication with the endocrine system (Sapolsky et al. 1986). In that way, excessive amounts of GC further damages the target brain region, starting a positive feedback cascade (Sapolsky et al. 1986). Functional connections between the immune and neuroendocrine systems stem from the existence of the interplay between their components, cytokines and hormones, on various levels (Ottaviani and Franceschi 1997), thus the GC response to stress and ageing has a significant impact on immune function.

The way ageing and stress simultaneously act to affect the immune response seems to be influenced not only by the way organisms age, but it could be highly influenced by early life event experience (Graham et al. 2006). Long-term effects seem to emerge not only after negative maternal behaviours, such as poor prenatal nutrition, but also following external, psychological and environmental stress in mothers, reviewed in (Graham et al. 2006). For example, early life stress through the excess of maternal stress hormones, mainly GC (Painter et al. 2012) has been shown to relate to emotional problems and learning deficits, and it could lead to the conditions such as type 2 diabetes and general depression and anxiety symptoms in the adulthood (Weinstock 2008).

A theory of ageing known as the 'disposable soma' hypothesis emphasises the difference between the efficiency of the translational machinery in reproductive and somatic cells, where the latter have traded accuracy in order to save energy for other more important functions (Kirkwood 1977). In the same manner, this theory explains longevity through the presence of the 'more successful' alleles of the genes involved in the protective mechanisms of the cellular response to a variety of physical stressors such as oxidative stress, radiation, and heat (Kapahi et al. 1999). Further, it has been suggested that the immune system has been developed as a response to pathogens which are a specific type of stressors (i.e. antigenic stressors) (Ottaviani and Franceschi 1997). In that way, immunosenescence in more complex organisms such as vertebrates will be the product of the continuous accumulation of damage due to lifelong exposure to antigenic stress (Franceschi et al. 1999).

A typical consequence of ageing on immunity is involution of the thymus where T cells mature, but also changes in bone marrow stem cells that shift the number of circulating T cells from naive to a relative increase in memory T cells (Castle 2000, Miller 1996). NK cells show decreased activity per cell (Castle 2000), and dendritic cells show decreased ability to

reach T cells as their target and promote adequate production of cytokines such as IFN-γ and IL-10 by influenza-specific T cells (Castle 2000). Toll-like receptors, membrane proteins that recognise conserved structure from microbes, are present in lower levels on macrophages from aged mice than young mice (Renshaw et al. 2002). Further, neutrophils from elderly donors have poorer phagocytic function, and diminished ability to fight of infections caused by Gram positive bacteria, such as pneumonia, which is one of the major causes of death in the older population (Butcher et al. 2001). Ageing is also accompanied by the remodelling of the T cell profile and the increase in the number of T cells with a senescent and exhausted profile (Chou and Effros 2013, Wherry 2011). Senescent T cells are characterised by the loss of cluster of differentiation (CD)28 co-stimulatory molecules corresponding to the decreased antiviral function and ability to produce robust protective response after vaccination (Effros 2004, Strioga et al. 2011). Exhausted T cells are characterised by expression of the cell surface programmed cell death (PD)-1 marker (Wherry 2011). These cells not only show reduced proliferative responses upon stimulation through the T cell receptor, but also have a more pro-inflammatory response. In addition the CD28 negative cells also express receptors normally associated with NK cells, such as a member of NKG2 family of C-type lectins (NKG2D), and killer cell lectin-like receptor subfamily G member 1 (KLRG1), which allow them to response to self antigens and increase the risk of autoimmunity in older adults (Abedin et al. 2005, Tarazona et al. 2000).

The existence of compromised immunity in both younger stressed individuals (Cohen et al. 1997, Gallagher et al. 2009b, Gallagher et al. 2009a), and older adults (Butcher et al. 2000, Butcher et al. 2001, Duggal et al. 2013b, Hazeldine et al. 2012, Hazeldine and Lord 2013, Pawelec et al. 2005) suggests a potential common mechanism that may be shared between stress and ageing in relation to the immune system. Some forms of immunosuppression seen

in response to stressors are also present with age. One example would be the change in cytokine production in the elderly, with cytokines from the Th2 response, such as IL-10 and IL-4, taking over from those typical for Th1 response, such as IFN-γ and IL-12 (Rink et al. 1998). Others are stress induced thymus changes in both animals (Kioukia-Fougia et al. 2002) and humans (Gruver and Sempowski 2008), that are characteristic of normal ageing (Singh and Singh 1979), but also molecular changes seen as telomere shortening in chronically stressed (Epel et al. 2004) that also progressively occur with age (Cherif et al. 2003, Mikhelson 2008).

There is also an interesting association between age-related changes and stress in the pattern of adrenal steroid production (Arlt and Hewison 2004). Even though its exact effect on immunosenescence is yet to be established, DHEAS, an adrenocortical steroid, is considered to have immune enhancing capacity (Hazeldine et al. 2010, Phillips et al. 2007). Another characteristic of this hormone is that it reaches its peak in the third decade of human life and then gradually declines with age, termed adrenopause (Orentreich et al. 1984). On the other hand, cortisol, a GC with known immunosuppressive effects, does not change with age, thus the cortisol:DHEAS ratio increases with age even in the absence of stress, giving an overall immunesuppressive endocrine environment. In addition, the systemic tissue based availability of cortisol may increase with age. This idea comes from the fact that activity of the enzyme capable of converting cortisone to active cortisol, 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1), is increased with the higher pro-inflammatory status, which is considered typical in the ageing process (Tomlinson et al. 2004). The result of this is a higher cortisol:DHEAS ratio in tissues expressing 11β-HSD1 .

Many of the complex processes of ageing and the stress response remain unclear; nevertheless, research continues to suggest common pathways between them on all

organisational levels, from organ systems to intracellular pathways and their gene candidates. One component also involved in and frequently related to processes of ageing and the stress response is a transcriptional factor, nuclear factor-kappa B (NF-κB). NF- κB is a whole family of transcription factors (Gilmore 2006), involved in regulation of both innate and adaptive immunity. Its dysregulation leads to autoimmune diseases and cancer, but it is also studied as an ageing- and stress-related factor. The suggested mechanism through which psychological stress can be transferred to the intracellular level to affect NF-κB functioning and increase its binding activity, involves a signalling pathway that is activated through binding of increased concentrations of noradrenalin to α_1- and β-AR during stress (Bierhaus et al. 2003). Another possible link between NF-κB and stress response is its interaction with GC receptors in a way that is yet to be elucidated (De Bosscher et al. 2003). It is not surprising that a transcriptional factor that is involved in the response to so many key processes, such as oxidative stress, growth, immune function, DNA damage, like NF-κB, is also considered one of the components affected by ageing. Other factors with a role in ageing, immunity and resistance to stress are the transcription factor forkhead box class O (FOXO3a) (Adler et al. 2007) and the histone deacetylase sirtuin 1 (SIRT1) (Longo and Kennedy 2006). Interestingly, they both act by inhibiting activation of NF-κB, emphasising the significance of this factor in both ageing and stress (Adler et al. 2008).

1.6. CHRONIC STRESS, AGEING AND THE IMMUNE SYSTEM

The main danger to immunity, however, occurs with synergy of ageing and chronic stress. In that respect, it might be that chronic stress is one of the main threats in an already immune-compromised older age. As mentioned above, severe stressors with a long term effect such as

a loss after death of a close family member or friend have been shown to relate to changes in the ability of aged neutrophils to produce ROS through which they kill rapidly dividing pathogens (Khanfer et al. 2011). This detriment in neutrophil immunity was also accompanied by a higher cortisol:DHEAS ratio in the bereaved older adults relative to age-matched non-bereaved controls (Khanfer et al. 2011). Bereavement in older adults has also previously been associated with a poorer antibody response to vaccination against the influenza virus (Phillips et al. 2006a). Changes in the cortisol:DHEAS ratio with diminished immune function again suggest a potential mechanism through which stress could influence the body's defence mechanism against infection. For example, older adults who had suffered the physical stress of a hip fracture and gone on to develop a bacterial infection post-surgery, showed decreased neutrophil superoxide production accompanied by a higher serum cortisol:DHEAS compared to age-matched controls (Butcher et al. 2005).

Older caregivers have most commonly been studied in this context, using the model of family dementia caregiving (Gouin et al. 2008). The severity of the stress in these circumstances comes not only from the patient's progressive deterioration in performing daily activities that pose growing problem for caregivers (Potkin 2002), but also from the loss of cognitive function, such as the ability to recognise people around them, and changes in behaviour such as hoarding, anger, and repetitive behaviour (Grossberg 2002). Both innate and adaptive immunity are affected by chronic stress experienced by older adults, and both of these components are necessary for the protection against different pathogens that can damage the body. It was shown, for example, that wound healing was slower in older dementia caregivers when compared to age, sex and income-matched controls (Kiecolt-Glaser et al. 1995). Wound healing is a complex process comprised of various phases (immediate response, inflammatory response, proliferation, migration and contraction and resolution) that

activates many different cells and molecules (Shaw and Martin 2009). Cells such as neutrophils and macrophages, and high concentrations of cytokines are main players in the inflammatory phase with a role to protect from invading pathogens and set the conditions for the repair process such as angiogenesis regulation (Shaw and Martin 2009). Lower production of pro-inflammatory cytokines involved in the wound healing process such as IL-1α, IL-8 (Glaser et al. 1999), as well as IL-1β (Kiecolt-Glaser et al. 1995) seen in caregivers compared to the controls, indicates the possibility of a direct effect of stress on cytokine production in wound healing. NK cell activity between older dementia caregivers and controls showed no difference in the ability of these cells to kill K562 target tumour cells (Irwin et al. 1991), but in the presence of cytokine stimulation (recombinant IFN-γ and IL-2) this similarity between stressed individuals and controls was not preserved; NK cells from caregivers responded more weakly compared to those from the controls (Esterling et al. 1994). All this, together with the stress-induced reduction in IFN-γ production (Glaser et al. 1986), indicates cytokines as a common target during chronic stress exposure, and a potential effector through which much of the immune suppression may occur.

A further association between the chronic stress of caregiving was found for adaptive cell mediated immunity; elevated cortisol levels as well as poorer proliferation to PHA and lower IL-2 production was shown in the caregiving group (Bauer et al. 2000). As observed in younger stressed participants (Marshall et al. 1998), caregiving stress in older adults has also been shown to be associated with the Th1-to-Th2 shift in cytokine responses, with the difference that in the older stressed individuals this was driven purely by an increase in IL-10 production, with no difference in IFN-γ production by Th1 cells (Glaser et al. 2001).

Vaccination responses are affected in older adults due to immunosenescence, which makes them particularly vulnerable to frequent infections such as pneumonia and influenza, among

the top five causes of high morbidity and mortality in this age group (Thompson et al. 2003). It would be expected that this aspect of immune incompetence would be further exacerbated in older adults affected by the chronic stress of caregiving. This is indeed the case; a significantly lower percentage of older caregivers of dementia patients showed a four-fold increase in antibody titre in response to vaccination against the influenza virus, a response that is considered to be protective against infection (Vedhara et al. 1999). This was accompanied by higher salivary cortisol concentration in this group when compared to the controls, pointing again to the role of HPA axis in immune regulation among stressed individuals. Most microbial antigens, however, trigger both humoral, i.e. antibody response which is generated by B lymphocytes, as well as cellular responses, mainly mediated by cytotoxic $CD8^+$ T cells (Glaser et al. 2000, Kiecolt-Glaser et al. 1996, Siergist 2008). In addition, $CD4^+$ T cells are necessary as mediators between those two. It has been shown that both the antibody response to vaccination against the influenza virus, as well as IL-2 production in response to antigen stimulation, was lower in caregivers compared to controls (Kiecolt-Glaser et al. 1996). In the case of the pneumococcal pneumonia vaccine, even though caregivers managed to exert an adequate immune response initially, shown as a rise in IgG antibody titre, it declined over time more rapidly in this group than in the group of matched controls, likely either as a consequence of a decrease in number of antibody-specific B cells, or their ability to produce IgG (Glaser et al. 2000, Vedhara et al. 1999).

As with other psychological stressors, a frequently used approach for assessing the severity by which caregiving stress affects the immune system of older caregivers is that of studies of latent virus antibody titres. It is known, for example, that reactivation of latent viral infections, such as those initiated by Herpes viruses (HSV-1, EBV, CMV) is typical for

immunosuppressed patients such as HIV and transplant patients (Rasmussen 1991). Interestingly, older caregivers had higher IgG antibody titres against EBV virus capsid antigen (VCA) compared to matched controls, indicating poorer control of the latent infection in this group (Kiecolt-Glaser et al. 1991b). Together with the higher antibody titre to total viral antigen of HSV-1, caregivers also had a decreased virus-specific T cell response; another and very important component of immune system necessary for controlling the infection (Glaser and Kiecolt-Glaser 1997). Older parental caregivers have also been characterised by higher antibody titres against CMV when compared to the controls (Pariante et al. 1997).

Another concept that often occurs in the literature when discussing ageing of the immune system is inflammageing. Inflammageing indicates an imbalance between inflammatory factors (C-reactive protein (CRP), TNF, IL-1, IL-6) necessary to fight infection, and anti-inflammatory components (IL-10) which act to resolve inflammation. It has been suggested that the rate of ageing and age-related disease could be dependent on this balance (Franceschi et al. 2007). One consequence of this might be that chronically stressed older adults, such as dementia caregivers, could have elevated inflammatory markers even when compared to non-caregiving older adults who have immunosenescence. Indeed, not only did older caregivers show higher levels of IL-6 (von Kanel et al. 2006), but its rate of increase was four times higher than in non-caregiving controls, leaving them particularly vulnerable to IL-6 related diseases such as physical frailty, cardiovascular diseases, osteoporosis and others (Ershler and Keller 2000).

Finally, other molecular mechanisms in ageing appear to be exacerbated by chronic stress in older adults. Caregivers of dementia patients had shorter PBMC, T cell and monocyte telomere lengths, and this was not due to having a higher number of these cells with shorter telomeres (Damjanovic et al. 2007). On the other hand, they also showed an increase in basal

telomerase activity, which could indicate an attempt of these cells to compensate for the loss of their telomere length (Damjanovic et al. 2007).

In summary, both the chronicity of stress and the ageing process are detrimental to an organism's well being. The mechanisms of these effects are yet to be elucidated more fully. However, it is clear that many of the ways in which both ageing and stress affect the body are through shared mechanisms, with particular regard to the neuroendocrine and immune systems from the level of the tissues, cells and even intracellular components. Less is known about the additive impact of ageing and stress on the innate immune system with the exception of studies of NK cells. A better understanding of the processes by which stress and ageing affect health will lead to a greater capacity for intervention, be it behavioural or pharmacological.

1.7. AIMS OF THE THESIS

Given this three way relationship between ageing, stress and the immune system, the aim of this thesis was to examine the effects of both ageing and the chronic stress of caregiving and bereavement separately on different components and functions of the immune system, and to investigate the HPA axis stress hormones, cortisol and DHEAS, as potential effectors of any interactions seen. The thesis addressed four specific research objectives, to determine:

1. The influence of caregiving stress on the control of CMV by measuring serum anti-CMV antibody titres, testing the hypothesis that chronic caregiving stress negatively impacts on the adaptive immune system's ability to control latent virus infection. The study used serum samples of young parental caregivers and matched controls collected on two different occasions; one sample from a cross-sectional study of caregiving stress, ageing and immune function and the second,

from a previously published case-control vaccination response study from our group using only baseline samples from that study.

2. The effect of the chronic stress of caregiving and ageing on neutrophil function, serum cytokine levels and serum cortisol, cortisone and DHEAS concentrations, to test the hypothesis that stress and immunosenescence would interact to give greater neutrophil functional decline in the older stress group. This was achieved using four groups of participants; young parental caregivers of children with developmental disabilities and older spousal caregivers of dementia patients, as the model of chronic stress without and with immunosenescence, respectively, and corresponding age- and sex-matched controls for each group.
3. The relationship between caregiving stress, ageing and T cell phenotype, examining T cell senescence and exhaustion markers, thymic output assessed by levels of T cells bearing T cell Receptor Excision Circles (TREC) and CMV serum antibody titre using the same four groups of participants as in 2. The hypothesis tested was that the chronic stress of caregiving would be associated with decreased immune functioning relative to controls indicating that stress accelerates immune ageing, and in that respect would mimic (in younger stressed group) and potentially act additively to (in older stressed group) the effect of ageing.
4. The effect of different type of chronic stress, that of suffering bereavement, in a young population, and compared this to an older sample combined with data from a previously published study from our group on bereavement effects in older adults. It was hypothesised that the stress of bereavement, even in the absence of immunosenescence, might still exert a negative impact on neutrophil function.

CHAPTER 2

ANTI-CYTOMEGALOVIRUS ANTIBODY TITRES AND CAREGIVING BURDEN IN YOUNGER CAREGIVERS

2.1. ABSTRACT

The analyses presented in this chapter examined whether or not young caregivers, parents of children with developmental disabilities, differed from controls in terms of CMV seropositivity and CMV-specific antibody titre. Second, it examined whether any particular socio-demographics, health behaviours, or psychological/caregiving variables were associated with a higher CMV antibody titre among caregivers. Young caregivers and age- and sex-matched controls were compared with respect to their reported health behaviour and psychosocial status as well as latent virus control. 117 parents of children with developmental disabilities and 52 control parents completed standard measures of health behaviours, socio-demographics, perceived stress, depression and anxiety, caregiver burden, child problem behaviours. They also provided a blood sample assayed for presence of CMV-specific antibody. Caregivers were no more likely to be CMV positive than controls and did not have higher antibody titres against CMV. In addition, there was no association between CMV antibody titre in seropositive caregivers and any of the psychological/caregiving variables. However, higher CMV antibody titres were significantly associated with a higher body mass index (BMI), lower exercise levels, smoking and lower fruit and vegetable and fat intake among seropositive caregivers. These data suggest that in the absence of immunosenescence, the chronic stress of caregiving is not sufficient to compromise the immune response to persistent CMV infection. However, an indirect mechanism to poorer health in caregivers might be via adoption of disadvantageous health behaviours in response to stress.

2.2. INTRODUCTION

CMV is a ubiquitous β-herpes virus with prevalence rates of infection as high as 60% (Dowd et al. 2009). The means of transmission are through bodily fluids such as saliva, tears, urine and breast milk, and it can also be transmitted sexually. As with any virus from the Herpesviridea family, CMV has the ability to remain in the body in the latent phase, typically in myeloid and dendritic cell progenitors, where its activation is initiated by inflammatory factors (Hahn et al. 1998). Clinical symptoms and activation of CMV virus in general is highly unlikely in healthy adults. On the other hand, immunocompromised individuals, such as those with HIV infection and organ transplant patients (Riddell and Greenberg 1997), can display symptomatic CMV infections (Rasmussen 1991). CMV infection may also contribute to the age-related decline in immunity, immunosenescence (Olsson et al. 2000, Pawelec and Derhovanessian 2011), and thus to an increased susceptibility to infectious disease and risk of mortality, at least in the very old (Almanzar et al. 2005, Pawelec et al. 2005, Simanek et al. 2011, Strandberg et al. 2009). The link between CMV seropositivity and increased mortality was proposed to be due to an increase in pro-inflammatory cytokines (Forsey et al. 2003, Franceschi et al. 1999, Licastro et al. 2005, Trzonkowski et al. 2009), though recent data from own laboratory has refuted this by showing that inflammation increased with ageing irrespective of CMV serostatus in one large cohort study (Bartlett et al. 2012).

It has been shown that psychosocial factors, such as the chronic stress of caregiving, are associated with immune decrements. For example, older spousal caregivers of a person with dementia exhibit a range of immunological features such as a decreased percentage of T lymphocytes, and higher antibody titres to EBV (Kiecolt-Glaser et al. 1987b). In addition, caregivers showed lower levels of salivary antibodies compared to non-caregivers, but this

was observed only in the elderly cohort of three age groups (Gallagher et al. 2008), suggesting accelerated immune ageing in individuals experiencing periods of chronic stress.

Younger parental caregivers of a child with developmental disability, particularly those who experience greater child problem behaviours, have also been shown to have significantly lower responses to both influenza and pneumococcal vaccinations compared to sex- and age-matched controls (Gallagher et al. 2009a, Gallagher et al. 2009b). In only one other study were immune decrements shown in younger caregivers compared to controls; and caregivers had a lower T helper:suppressor cell ratio than controls (Pariante et al. 1997), an indicator of an early immunosenescence. This suggests that perhaps irrespective of the caregiver's age, caring for someone with severe cognitive and behavioural problems may compromise immunity. In addition, older spousal caregivers of dementia patients showed changes in health behaviours which include changes in diet, smoking behaviour and sleep patterns that can also contribute to, and further damage their health (Gallant and Connell 1998) and may be an indirect mechanism through which caregiving stress impacts on immunity.

What is unknown is whether stress in younger caregivers can specifically compromise the ongoing immune response to CMV. Anti-CMV antibody titre is a good marker of an ongoing immune response such that a higher titre would be indicative of compromised immune function. In one study, older but not younger caregivers had higher serum antibody titres against CMV compared to controls (Pariante et al. 1997). However, this in part might be driven by the increased likelihood of CMV infection with ageing or the response to episodes of sub-clinical viral reactivation in older adults (Pawelec and Derhovanessian 2011). In addition, even though CMV and risky health behaviour changes such as smoking, bad diet and inadequate sleep pattern have all been linked to adverse health consequences, the potential interplay between these factors, to our knowledge, remains unknown.

Given the negative effects of caregiving stress on other indices of immunity, this study aimed to examine the influence of caregiving stress on CMV antibody titre in younger caregivers. Further, the present study assessed associations between CMV antibody titre and various indices of health behaviours and other psychosocial variables within the caregivers group. It was hypothesised that caregivers of children with developmental disabilities, particularly those with the greatest caregiver burden or child behaviour problems, would have higher antibody titres against CMV than non-caregivers.

2.3. METHODS

2.3.1. Participants

117 parents caring for children with developmental disabilities and 52 parents of normally developing children were recruited to the study and provided a blood sample for CMV analysis. Developmental disability is the term used to describe conditions including but not limited to Autism spectrum disorders and Down syndrome (Eunice Kennedy Shriver National Institute of Child Health and Human Development - http://www.nichd.nih.gov/health/topics/idds/conditioninfo/Pages/default.aspx#f1). These parental caregivers were recruited via invitation letters distributed by their respective disease support group associations, by advertising in associated newsletters, or by direct contact with family support groups. Inclusion criteria for these parents were: caring for at least one child with Autism Spectrum Disorder (ASD), Angelman, Down, Cornelia de Lange, fragile X, or Smith-Magenis syndromes. These disorders evidence a range of problem behaviours, which are particularly common among children with syndromes other than Down (Arron et al. 2011, Blacher and McIntyre 2006, Chadwick et al. 2000).

Since the emotional reaction of parental caregivers is highly influenced by the diagnostic process (Graungaard and Skov 2007), this study aimed to avoid this particular event and focus on the parents' stressful experiences of caring *per se.* Thus, in keeping with existing research (Hastings et al. 2006), children with developmental disabilities had to be aged between 3 and 19 years and living at home during the school term. The majority of parents reported caring for a child with Angelman syndrome (28.3%) and Smith-Magenis syndrome (25.7%); followed by the parents of a child with fragile X syndrome (17.7%) and ASD (15%); the remainder were caring for a child with Cornelia de Lange (8.8%), or Down (4.4%) syndromes. Controls, i.e. parents of typically developing children who were in the same age range as the sample of children with disabilities, were recruited via posters in local schools, the university, and the local area (e.g. sports centres and clubs). None reported suffering from an ongoing chronic immune disease, being acutely ill, taking immunosuppressive medication, or reported being pregnant in case of female participants. Attempts were made to match the groups as closely as possible on age, sex, socioeconomic position, ethnicity, and marital status, by recruiting individual parents of normally developing children that matched as near as possible individual parents of children with developmental disabilities. The total time period for the recruitment of the controls was less than three years. All participants provided written informed consent and the studies had ethical approval from the appropriate local research ethics committees.

2.3.2. Study design and procedure

The current sample size was comprised of two separate groups of participants that were part of two separate younger caregiving studies focussing on different elements of immune function. The first was a part of a prospective case-control vaccination response

study involving three testing sessions; details elsewhere (Gallagher et al. 2009b). The analysis of CMV status and antibody titre and caregiving variables reported from that study involved baseline sampling only where parents completed questionnaires and then provided a blood sample. The second study was a part of a cross-sectional assessment of neutrophil function comprising one blood sampling session.

2.3.3. Questionnaires

Health behaviours

A questionnaire adapted from the Whitehall II study (Marmot et al. 1991) was used to assess the health behaviour in parents. This questionnaire has been consistently used in previous stress and immunity research (Burns et al. 2003, Phillips et al. 2005). Parents were asked about their sleep, smoking status, how much alcohol they drank, and simple categorical scoring system was used in all cases. They were also asked about their exercise engagement (if and how many hours are they involved in the mildly, moderate, or vigorous exercise). They also reported consumption of various food items, which gave a measure of how healthy (fruit and vegetable), or high in fat (snacks, chips, processed meat) their diet was. Categorically scored health behaviour variables with little overall variability were split at the median and converted to binary variables e.g. smoker versus non-smoker, > 20 alcohol units consumed per week. Fruit and vegetable and fat intake were scored from 0 ‘never’ to 6 ‘more than once per day’ for a range of foods consumed per week; fruit and vegetables listed were then summed along with fatty foods to produce two separate scores. Exercise frequency was scored categorically from 0 ‘none to 5 ‘11+ hours per week’ for mild, moderate, and vigorous activity. A total weighted score was then produced from mild + (moderate * 2) + (vigorous * 3).

All of the caregiver measures were also appropriate to parents of non-developmentally disabled children, as they cover the general parental caring role and child behaviours.

Sleep

Sleep quality was measured by the 19-item Pittsburgh Sleep Quality Index (Buysse et al. 1989). These 19-items are then combined to create seven component scores with scores ranging from 0, no problems in area, to 3, high problem area. Higher total scores indicated poorer sleep quality. Adequate internal consistency (Cronbach's α =.69) has been demonstrated previously (Spira et al. 2012). In the present sample, the Cronbach's α = .77.

Depression and anxiety

The Hospital Anxiety and Depression Scale (HADS) was used for measuring psychological morbidity (Zigmond and Snaith 1983). The scale consists of two subscales with seven items in each, one assessing anxiety and the other largely anhedonic aspects of depression. The answers are scored from 0 (not present) to 3 (considerable). Scores for both scales had a range from 0 - 21, with the scores 0-7 being classed as normal, 8-14 as moderate and 15-21 as severe depression and anxiety respectively. The HADS has good concurrent validity (Bramley et al. 1988, Herrmann 1997), and boasts good internal consistency, Cronbach's α of .90 for depression and .93 for anxiety (Moorey et al. 1991); and test-retest reliability, .85 for depression and .84 for anxiety (Herrmann 1997). The internal consistency in this sample was .78 for depression and .80 for anxiety.

Time spent caregiving

Amount of time spent caregiving was assessed using a modified version of the Caregiver Activity Survey (CAS) (Davis et al. 1997). Parents were asked how much time they

spend on five specific (transport, dressing, eating, bathing and supervision) caring activities. The total daily score for time spent caregiving was obtained by summarizing hours for each caring role.

Caregiver Burden

Parental caregiver burden was assessed using an adapted form of the Zarit Burden Interview (BI) (Zarit et al. 1980), originally designed to assess the burden experienced by family caregivers of elderly persons with dementia. Questions in the scale used in the current study had 'your relative' amended with 'your child', for example 'Overall, how burdened do you feel in caring for your child? Responses ranged from 0, never, to 4, nearly always, and the overall score ranges from 0 to 48. Internal consistency in current sample was .93.

Child behaviour difficulties and problems

Child behaviour difficulties were measured using the Strengths and Difficulties Questionnaire (SDQ) (Goodman 1997). Questions are rated as 0, not true, 1, somewhat true, or 2, certainly true, and higher score indicates more problem behaviour. Some items are reversed scored (e.g. generally liked by other children, has at least one good friend). The overall score for child problematic behaviours can vary from 0 to 50. The scale has been shown to be reliable (Cronbach's $\alpha = .76$) and effective at identifying behavioural problems in children (Goodman and Scott 1999) and has been used extensively in research with children with developmental disabilities (Hastings et al. 2006). Internal consistency for the scale in this study was .80.

Perceived stress

This 14-item perceived stress scale (PSS) assessed control over and overload with the daily life stress during the past month. It has been frequently used in caregiver research (Glaser et al. 2000, Vedhara et al. 2002), and measures both participants' subjective feeling of how much control they feel they have over daily events, as well as their inability to cope with things. The scale ranges from 0 to 4 and higher scores indicate higher perceived stress. The overall score can range from 0 to 56. The scale shows good test–retest reliability (r = .80) and internal reliability (Cronbach's α = .75). Internal reliability in the present sample was 0.79.

Social support

The 12-item Support Functions Scale (SFS) – short form (Dunst et al. 1988) was used to assess types of support available to parents. The support is assessed by 5-point Likert scale with 1 meaning support is not available and 5 that it is available quite often. The total score on this scale varies from 0 to 60. This scale has been shown to be reliable (Cronbach's α = .86) and has been used previously in developmental disability research (White and Hastings 2004). In the present study internal consistency was 0.88.

2.3.4. Blood sampling and CMV analysis

Venous blood was collected from an ante-cubital vein into a plain 6ml tube (BD Vacutainer, Oxford, UK). The samples were allowed to clot for at least one hour, were centrifuged at 1325xg (MSE Centaur 2, MSE (UK) Ltd, London, UK) for 10 min, and the separated serum was frozen at −20 °C until assayed. Serum was analysed for the presence of CMV infection using CMV a standardised enzyme-linked immunosorbent assay (ELISA) developed by the Antiviral Immunology lab, Cancer Sciences, University of Birmingham as previously

reported. The method consists of coating the plate with appropriate dilutions of CMV- and mock-lysate (negative control) on the first day, using carbonate/bicarbonate buffer (pH=9.6), then covering with parafilm and incubating over night at 4°C. On the day 2, serum samples were thawed and 600µl of 1:600 dilutions were made using phosphate buffered saline (PBS) + 1% bovine serum albumin (BSA) + 0.05%Tween20 as a dilution buffer. For standard curve mix of three healthy, CMV positive donors was used. For 7-point standard curve 1:4 dilution series were made up from initial 1:50 dilution. Previously coated plate was washed three times with PBS+0.05%Tween20, discarding supernatant each time to remove any unbound particles. 100µl of standards, blank and samples were added onto the plate, with testing samples present in one lysate and one mock well, and the plate was incubated for an hour at room temperature (RT). Plate was washed 3 times and supernatant discarded as before, after which 100µl of the secondary anti-human IgG-horsereadish peroxidise (HRP) antibody (goat anti-human IgG; Southern Biotech #2040-05), prepared in 1:8000 dilution in PBS+1%BSA+0.05%Tween20 was added, and left to incubate for 1 hour at RT. After washing the plate three times following the same procedure described above, 100µl of tetramethyl benzidine (TMB)-solution was added into each well and incubated for 10 minutes at RT in the dark, after which the reaction was stopped by adding of 100µl 1M HCl. The readings were done at 450nm on The Wallac 1420 VICTOR^{2}™ Multilabel Counter. The standard curve measured up to 1000 arbitrary units of IgG and those with more than 10 units were considered as CMV positive.

2.3.5. Statistical analyses

Based on our previous caregiver research we attempted to detect a medium sized effect (f = 0.29) giving an overall sample size of N = 96, with at least 48 participants in each group. Chi-square and analysis of variance (ANOVA) were used to determine group

differences (caregiver versus control) in health behaviour, socio-demographics and psychosocial variables. Any significant group differences in socio-demographic variables and health behaviours were controlled for in subsequent analyses. Prior to statistical analysis of CMV data, these were checked for normality. Due to the skew of the data, antibody titres were subjected to $\log_{10}$ transformation. Four caregivers and two controls were removed from analyses due to outlying CMV antibody values or finding out post-consent that their child was < 3 or > 19 years. Chi-square was used to establish whether CMV status differed between the groups (caregiver versus control) or related to any socio-demographic, health behaviour, or psychological/caregiving variables. ANOVA was used to compare CMV antibody titre between the caregivers and controls among those who were seropositive only. For CMV titre, among the seropositive participants only, correlations were conducted within the caregiver group alone to determine if any of descriptive, health behaviour or psychological/caregiving variables related to antibody titre. The effect size (ϕ, η^2, r) was reported for all the analyses conducted, followed by observed power. Any variations in the degrees of freedom reflect occasional missing data.

2.4. RESULTS

2.4.1. Socio-demographic, childcare and psychosocial characteristics of parental groups

The demographic and summary childcare characteristics of the two parental groups are presented in Table 1. Caregivers and controls did not differ on key socio-demographics , but parents of children with developmental disabilities had a higher body mass index

(BMI), depression and anxiety symptoms, perceived stress, alcohol intake, fat intake, poorer sleep quality, and spent more time caregiving. As might be expected, parents caring for a child with a developmental disability had a greater caregiving burden, and their children had more child behaviour difficulties than parents of typically developing children (see Table 1). Further, social support questionnaires revealed poorer quality of support (SFS) than that received by controls.

2.4.2. CMV status

Of the group as a whole, 76 (47%) participants were CMV positive; the number CMV seropositive within each group is shown in Table 1. The percentage of CMV positive individuals among caregivers and controls were almost identical, $\chi^2(1) = .01$, $p = .92$, $\phi = .008$, <.26. Overall, CMV status was also not associated with any of the socio-demographic, health behaviour, or any of the psychological/caregiving variables.

2.4.3. Caregiving and CMV antibody titre

For the CMV positive subset (N = 76), socio-demographic, caregiving and psychosocial characteristics are presented in Table 2. This time, in addition to higher alcohol intake, higher BMI, depression and anxiety symptoms, higher perceived stress and lower social support, caregiving parents differed in occupational group (more likely to be manual), ethnicity (more likely to be White), and smoking behaviour (more likely to be smokers) compared to control parents, but showed similar fruit and vegetable and fat intake.

Although CMV positive caregivers had higher CMV antibody titres than controls, the difference was not statistically significant, even after controlling for the significant socio-demographic and health behaviour variables, $F(1,50) = 0.14$, $p = .71$, $\eta^2 = .003$, $1-\beta=.06$, and significant psychosocial variables, $F(1,60) = 1.73$, $p = .19$, $\eta^2 = .028$, $1-\beta=.25$.

2.4.4. Characteristics of all participants and CMV antibody titre

CMV antibody titre overall was also not associated with most of the socio-demographic, health behaviour, and psychological/caregiving variables, although women had significantly higher titres, $F(1,69) = 4.38$, $p = .04$, $\eta^2 = .060$, $1-\beta = .54$, as did those with higher BMI, $r(64) = .26$, $p = .03, .69$. Adjustment for sex and BMI as covariates did not alter the previous lack of group difference.

2.4.5 Characteristics within the caregiving group and CMV antibody titre

When seropositive caregivers were considered as a group, there was no association between CMV antibody titre and age, age of child, sex, ethnicity, occupational status, marital status, or sleep quality. However, there was a positive correlation between CMV antibody titre and BMI, $r(44) = .29$; $p = .05, .63$, as observed in the whole group, and smokers had higher antibody titre than non-smokers, $F(1,47) = 5.92$, $p = .019$, $\eta^2 = .112$, $1-\beta = .66$.

Table 1. Socio-demographics, childcare and psychosocial characteristics of caregiving and control parents.

	Mean (SD) / N (%)		***Analyses***
	Caregivers	**Controls**	
	(N = 113)	**(N = 50)**	
Socio-demographic characteristics			
Sex (Female)	70 (67)	30 (61)	$X^2(1) = .54$, $p = .46$, $\phi = .060, .23$
Marital status (Partnered)	90 (87)	40 (82)	$X^2(1) = .63$, $p = .43$, $\phi = .064, .23$
Ethnicity (Caucasian)	96 (92)	42 (86)	$X^2(1) = 1.64$, $p = .20$, $\phi = .103,.23$
Occupational status (non-manual)	66 (71)	38 (79)	$X^2(1) = 1.10$, $p = .29$, $\phi = .088, .22$
Age (years)	40.8 (6.83)	40.2 (4.72)	$F(1,151) = .301$, $p = .58$, $\eta^2 = .002$, $1-\beta = .17$

Health behaviour characteristics

Smoking (smoker)	37 (36)	19 (40)	$X^2(1) = 0.28$, $p = .60$, $\phi = .043, .23$
Alcohol (>20 units per week)	23 (22)	1 (2)	$X^2(1) = 9.67$, $p = .002$, $\phi = .253, .69$
Body Mass Index (kg/m^2)	26.7 (5.40)	24.3 (3.14)	$F(1,142) = 7.45$, $p = .007$, $\eta^2 = .050$, $1-\beta = .77$
Fruit and vegetable intake score	13.7 (3.92)	12.8 (3.38)	$F(1,149) = 2.21$, $p = .14$, $\eta^2 = .015$, $1-\beta = .32$
Fat intake score	19.9 (6.97)	17.0 (8.06)	$F(1,149) = 5.02$, $p = .03$, $\eta^2 = .033$, $1-\beta = .61$
Exercise score	10.1 (6.31)	10.4 (6.22)	$F(1,140) = 0.95$, $p = .76$, $\eta^2 = .001$, $1-\beta = .06$

Sleep total score	9.3 (3.21)	7.1 (2.83)	$F(1,140) = 17.09$, $p < .001$, $\eta^2 = .109$, $1-\beta = .98$
Number CMV seropositive	53 (47)	23 (46)	$X^2(1) = 0.01$, $p = .92$, $\phi = .008$, <.25
Raw CMV antibody titre	110.2 (162.97)	92.3 (142.98)	$F(1,161) = 0.45$, $p = .50$, $\eta^2 = .003$, $1-\beta = .10$
Psychosocial/caregiving characteristics			
Age of main care recipient (years)	9.1 (4.14)	7.9 (4.34)	$F(1,161) = 2.95$, $p = .09$, $\eta^2 = .018$, $1-\beta = .40$
Hours spent caregiving per day	12.5 (9.48)	6.8 (12.50)	$F(1,148) = 9.63$, $p = .002$, $\eta^2 = .061$, $1-\beta = .87$
HADS depression	8.5 (3.66)	4.0 (3.29)	$F(1,149) = 51.54$, $p = <.001$, $\eta^2 = .257$,

			1-β =1.00
HADS anxiety	10.3 (4.26)	5.9 (2.52)	F(1,151) = 45.13, *p* = <.001, η^2 = .230, 1-β =1.00
Perceived stress score (PSS)	30.3 (7.33)	23.6 (6.47)	F(1,151) = 30.00, *p* = <.001, η^2 = .166, 1-β =1.00
Caregiver burden score (BI)	27.3 (9.10)	13.0 (7.58)	F(1,151) = 53.43, *p* = <.001, η^2 = .261, 1-β =1.00
Child behaviour difficulties (SDQ)	19.3 (5.17)	7.7 (4.34)	F(1,150) = 182.41, *p* = <.001, η^2 = .549, 1-β = 1.00
Social support (SFS)	30.1 (9.01)	37.9 (10.48)	F(1,147) = 22.25, *p* = <.001, η^2 = .131, 1-β = 1.00

Table 2. Socio-demographics, childcare and psychosocial characteristics of CMV seropositive caregiving and control parents.

	Mean (SD) / N (%)		***Analyses***
	Caregivers (N = 53)	**Controls (N = 23)**	
Sex (Female)	35 (71)	13 (59)	$X^2(1) = 1.06$, $p = .30$, $\phi = .122$, <.20
Marital status (Partnered)	41 (84)	16 (73)	$X^2(1) = 1.15$, $p = .28$, $\phi = .127$, <.20
Ethnicity (Caucasian)	46 (94)	17 (77)	$X^2(1) = 4.19$, $p = .041$, $\phi = .243$, .39
Occupational status (non-manual)	24 (57)	18 (86)	$X^2(1) = 5.15$, $p = .023$, $\phi = .286$, .64
Age (years)	42.1 (5.99)	40.6 (5.05)	$F(1,69) = 1.00$, $p = .32$, $\eta^2 = .014$, $1-\beta = .166$
Smoking (smoker)	25 (51)	5 (24)	$X^2(1) = 4.44$, $p = .035$, $\phi = .252$, .39
Alcohol (>20 units per week)	9 (18)	0 (0)	$X^2(1) = 4.43$, $p = .035$, $\phi = .251$, .39
Body Mass Index (kg/m^2)	26.8 (3.85)	23.8 (2.87)	$F(1,64) = 9.51$, $p = .003$ $\eta^2 = .129$, $1-\beta = .859$
Fruit and vegetable intake score	12.9 (4.18)	13.6 (2.73)	$F(1,69) = 0.54$, $p = .46$, $\eta^2 = .008$, $1-\beta = .112$
Fat intake score	17.2 (7.67)	19.5 (7.21)	$F(1,68) = 1.40$, $p = .24$, $\eta^2 = .020$, $1-\beta = .214$

Exercise score	9.0 (6.71)	10.6 (4.92)	$F(1,64) = 1.00$, $p = .32$, $\eta^2 = .015$, $1-\beta = .166$
Sleep total score	9.6 (3.48)	6.8 (2.62)	$F(1,65) = 10.95$, $p = .002$, $\eta^2 = .144$, $1-\beta = .903$
Raw CMV antibody titre	234.1 (166.50)	199.5 (152.49)	$F(1,74) = 0.73$, $p = .40$, $\eta^2 = .010$, $1-\beta = .134$
Age of main care recipient (years)	9.7 (4.37)	7.8 (4.53)	$F(1,74) = 3.06$, $p = .08$, $\eta^2 = .040$, $1-\beta = .408$
Hours spent caregiving per day	12.1 (8.04)	10.1 (17.62)	$F(1,67) = 0.44$, $p = .51$, $\eta^2 = .006$, $1-\beta = .100$
HADS depression	8.7 (3.76)	3.5 (2.97)	$F(1,69) = 32.44$, $p < .001$, $\eta^2 = .320$, $1-\beta = 1.00$
HADS anxiety	10.2 (4.08)	5.5 (2.06)	$F(1,69) = 26.04$, $p < .001$, $\eta^2 = .274$, $1-\beta = .999$
Perceived stress (PSS)	30.3 (7.58)	22.7 (5.20)	$F(1,69) = 18.33$, $p < .001$, $\eta^2 = .210$, $1-\beta = .988$
Caregiver burden score (BI)	34.1 (14.44)	14.9 (7.94)	$F(1,69) = 34.19$, $p < .001$, $\eta^2 = .331$, $1-\beta = 1.00$
Child behaviour difficulties (SDQ)	20.4 (5.18)	6.8 (4.45)	$F(1,68) = 110.38$, $p < .001$, $\eta^2 = .619$,$1-\beta = 1.00$
Social support (SFS)	28.9 (8..93)	36.0 (10.90)	$F(1,67) = 8.22$, $p = .006$, $\eta^2 = .109$, $1-\beta = .807$

Other variables that showed significant associations with CMV antibody titre in the caregivers group were fruit and vegetable consumption, r(47) = -.28, p = .048, .63, fat intake, r(46) = -.34, p = .019, .63, and exercise score, r(42) = -.37, p = .013, .61, such that those who consumed fewer fruit and vegetables and fat, and undertook less exercise had higher CMV antibody titres. There were no associations between CMV antibody titre and psychological/caregiving variables, as shown in Table 3.

Table 3. Correlations between CMV titres and the caregivers' psychosocial characteristics

	r	**df**	***p***
Hours spent caregiving per day	-.14	45	.35
HADS depression	.03	47	.81
HADS anxiety	.03	47	.82
Perceived stress (PSS)	-.11	47	.44
Caregiver burden score (BI)	.13	47	.37
Child behaviour difficulties (SDQ)	-.04	47	.77
Social support (SFS)	.12	46	.42

2.5. DISCUSSION

Despite differing on all main psychological/caregiving variables but not on socio-demographics, parents caring for children with developmental disabilities were no more likely to be CMV seropositive than parents of typically developing children. Caregivers were also no more likely to have significantly higher antibody titres against CMV. Finally, although CMV antibody was not associated with any of the psychological/caregiving variables or most socio-demographics, women and people with higher BMI had higher antibody titres. This gender difference is consistent with previous research (McVoy and Adler 1989, Haarala et al. 2012), the exact reason being unknown. One possibility might be that hormonal changes, specifically the increase in oestrogen that occurs during pregnancy relates to reactivation of CMV, and consequently higher titres later in life (McVoy and Adler 1989, Kleinman et al. 1986). Higher CMV antibody titres in those with higher BMI is also in concordance with a previously reported findings in immunocompetent adults (Gkrania-Klotsas et al. 2013). This is perhaps due to associations noted between obesity and inflammation (Forsythe et al. 2008) and CMV and inflammation (Hummel and Abecassis 2002), although this has not been observed in all studies (Bartlett et al. 2012).

Within the seropositive caregiving parents, there was no association between CMV antibody titre and any of the psychological/caregiving variables. However, those with higher CMV antibody titres, potentially indicating poorer latent virus control, generally did have poorer health behaviours, including being more likely to smoke, have a higher BMI, intake less fruit and vegetables, and undertake less exercise.

This finding is consistent with that of Pariante et al. (Pariante et al. 1997), who did not find a greater likelihood of CMV infection or higher titres among the younger age range of their caregiving sample, as well as with studies in caregivers for a person with a

physical disorder which found no group differences in comparison to controls (Baron et al. 1990, Epel et al. 2004, Provinciali et al. 2004). These studies suggest that younger caregivers and those caregiving for a more physical than mental disorder are less vulnerable to immune decrements. However, the present findings contrast with the other caregiving and immunity studies in younger adults, which suggested poorer immunity among young caregivers, at least in response to vaccination (Gallagher et al. 2009a, Gallagher et al. 2009b) and lymphocyte proliferation to mitogen (Gennaro et al. 1997). This may reflect greater resilience of the $CD8^+$ T cell-mediated anti-viral immune response to stress, compared with the many components of the vaccination response which include macrophages and dendritic cells in the skin as well as T and B cell co-operation to generate antibody.

A similar percentage of CMV positive parents in both caregiving (47%) and control (46%) groups indicated no difference in infection rate consistent with the fact that disparities in CMV infection rate among parents are mainly attributed to ethnicity and income (Colugnati et al. 2007), which did not differ between the groups. In addition, other studies have shown that children in day care have a higher CMV prevalence (Pass and Hutto 1986), which might in turn lead to greater infection in their parents. However, information regarding day care or children's CMV titres was not available in the present study. Nevertheless, exposure to CMV-shedding children alone is not sufficient for infection in parents (Yamashita et al. 2003), thus parents exposed to children with developmental disabilities who may have a high burden of infection themselves (Arron et al. 2011, do Canto et al. 2000) are not necessarily more likely to be seropositive.

Caregiving and control groups also had comparable CMV antibody titres. The determinants of CMV-specific antibody titre are unclear but are likely to be related to the

frequency and magnitude of subclinical viral replication *in vivo*. In addition, a high level of circulating antibody will not prevent reactivation of the virus (Glaser and Kiecolt-Glaser 1994), but rather indicate that it has likely been reactivated. Viral reactivation events are believed to become more common with increasing age and it is of interest that an effect of caregiving stress and burden on CMV antibody titre was noted in a study of older adults (Pariante et al. 1997) in contrast to the present younger caregiving group. This perhaps indicates that in earlier life, caregiving *per se* is not sufficient to induce significant immune decrement seen here as greater viral reactivation, which is again consistent with some studies in young caregivers (Gallagher et al. 2008, Epel et al. 2004, Vedhara et al. 2002), but in contrast with others (Gallagher et al. 2009a, Gallagher et al. 2009b). Testing of the age x caregiver group interaction in this study did not reveal any significant interaction effect. Further, although Pariante et al. (Pariante et al. 1997) demonstrated a higher antibody titre in caregivers when compared to the controls in their eldest cohort, the sample size used for the analysis was only 18 female participants which was further reduced to 9 participants in each group after splitting by age. Therefore, it could be argued that the evidence for poorer control over this latent virus in older caregivers is only weak.

CMV is also suggested to have a role in the ageing of the immune system, and has been used to explain the difference in the various immune components between CMV infected and CMV non-infected elderly (Pawelec et al. 2009). In addition, older caregivers of Alzheimer's patients showed dramatic increase in antibody titre to EBV, another latent virus from Herpesviridae family, pointing to impaired control of cellular immunity towards virus replication (Kiecolt-Glaser et al. 1991b). This suggests that as caregivers age, they are at higher risk of poorer latent virus control and its consequences.

Among CMV seropositive caregivers, smokers had higher antibody titre than non-smokers. This is not surprising as the T and B cell response against different antigens is severely reduced by smoking (Sopori 2002, Holt and Keast 1977), which could influence susceptibility to infection (Evans et al. 2000), and perhaps in this particular case, control of latent virus reactivation. Other health behaviour characteristics that were associated with CMV titre were fruit and vegetable and fat intake and amount of exercise. To our knowledge there is no previous evidence of the effects of dietary intake on latent virus control. Thus, what we may be picking up on here are a range of negative health behaviours which have an impact on a several health outcomes and processes, including a flattening in the diurnal rhythm of cortisol similar to that observed in immunosenescence (Heaney et al. 2012) and now control of CMV reactivation. Further, an indirect mechanism between the stress of caregiving and the poor health reported in caregivers under high levels of stress and burden (Forbes et al. 2007) might be via engagement in negative health behaviours such as poor diet and lack of exercise, resulting in poorer immunity as well as worse health generally. However, longitudinal studies including markers of health and more thorough assessment of health behaviours in caregivers would need to be conducted in order to test this theory in depth. That these associations with CMV titre only emerged in the caregiver group rather than overall might be due to the increased importance of these behaviours in combination with existing stress. This argument has been made previously with regard to the emerging impact of a psychosocial/behavioural factor in combination with an existing source of stress which itself had no direct impact on immunity. For example, only older hip fracture patients who developed depression showed suppressed immunity, rather than those who had the stress of hip fracture alone (Duggal et al. 2013a). In younger non-stressed groups, although still detrimental in the long term, these

health behaviours might not demonstrate their impact on particular aspects of immunity until later in life.

The present study has a number of limitations. First, even though the initial sample size might be regarded as small, it was substantial when compared with the other caregiving studies (Gallagher et al. 2009b, Pariante et al. 1997, Vedhara et al. 2002). We also recruited fewer controls than caregivers, as our key interest lay in the potential associations between elements of caregiver stress and CMV titre which might have explained any caregiver-control group differences. However, once focusing on seropositive caregivers our sample size has significantly decreased and as such may have limited power, but, again, it is of similar magnitude or larger than other caregiver studies (Glaser et al. 2000, Pariante et al. 1997, Vedhara et al. 1999) and we have reported observed power throughout. Nonetheless, the number of tests might have increased the likelihood of a Type I error. Second, even though antibody titre has been commonly used as a measure of the adequacy of an individual's immune system to control latent virus, it is known that cellular immunity and the T cell response in particular are key components needed for suppression of CMV reactivation (Vasto et al. 2007, Pawelec and Derhovanessian 2011). However, such assays require large quantities of whole blood and for the present analyses only stored sera were available. Third, assessment of other antibodies besides IgG such as IgM would be useful in order to ascertain how recent infection and re-infection occurred in caregivers and controls as well as reactivation, but we did not have data on IgM levels. Fourth, this study was cross-sectional at one time point, and therefore unable to determine whether comparable CMV antibody titres between the groups were indeed a consequence of adequate latent virus control in the stressed group, or they were related to other factors such as different times of initial infection. Measuring burden and CMV antibody titre over several time points in future research would

help to clarify this. For example, in a study of antibody titres against EBV between older caregivers and controls, comparable titres were found at baseline but a greater increase was observed in the caregiver group over time (Kiecolt-Glaser et al. 1991b). Finally, there is an inevitable limitation in the method of recruitment for this study, as the contact with caregiving parents was made mainly via family support groups and on the particular syndrome conference days, thus might have been biased towards those caregivers who have an access to adequate support and therefore, at least in part, obtain relief from the burden of caregiving. However, in our experience, those who attend syndrome days in order to gain relief would not have this support available to them generally in their daily life, thus are still a very stressed group.

In conclusion, there was no evidence of an association between caregiving and CMV seropositivity or anti-CMV antibody titres. This suggests that caregiving in younger adults may not accelerate all elements of immunosenescence, but that such decrements are only observed in older caregivers. However, there was some evidence of an association between caregivers engaging in unhealthy behaviours (dietary intake, exercise and BMI) and CMV antibody titre, suggesting that an unhealthy lifestyle in response to stress in some individuals could be an indirect pathway through which health is affected in caregivers.

CHAPTER 3

NEUTROPHIL FUNCTION, STRESS HORMONES AND CAREGIVING STRESS IN YOUNGER AND OLDER ADULTS

3.1. ABSTRACT

The aim of this chapter was to examine the combined effects of caregiving stress and ageing on neutrophil function and stress-related hormones (cortisol, cortisone and DHEAS) in young and older individuals. As a model of caregiving in young adults, parents (mean age 38) of children with developmental disabilities were recruited and compared to older spousal dementia caregivers (mean age 69 years). Age- and sex-matched controls for both groups were also assessed. Participants completed a questionnaire pack assessing health behaviour, psychosocial, and caregiving characteristics, and provided a blood sample for neutrophil function (phagocytosis of *Escherichia (E.)coli* and generation of ROS), and serum hormone cortisol, cortisone and DHEAS concentrations as potential mechanisms. Caregivers in both age groups scored worse on the majority of psychosocial and caregiving variables than matched controls. Despite this, neutrophil function was comparable to that in controls and was unexpectedly higher in older adults when compared to younger adults overall. However, those caregivers who reported higher psychological morbidity and more burdensome caregiving showed poorer neutrophil phagocytosis. Further, there was no effect of caregiving in either group on cortisol and DHEAS concentrations or their ratio. However, the cortisol:cortisone ratio was higher in young caregivers compared to controls. Overall, neutrophil function and stress hormone levels were preserved in caregivers and neutrophil phagocytosis was only compromised in those with the highest levels of distress. This suggests that more attention should be paid to individual differences among caregivers rather than caregiving status *per se*.

3.2. INTRODUCTION

There is now substantial evidence that chronic psychological stress can impair immunity (Glaser and Kiecolt-Glaser 2005, Kiecolt-Glaser et al. 2002, Vedhara and Irwin 2005). For example, the experience of a large number of negative life events has been associated with reduced antibody responses to vaccination (Burns et al. 2003, Phillips et al. 2005) and lower levels of sIgA (Phillips et al. 2006b). Specific chronic stress exposures, such as bereavement, have been related to poorer NK cell function (Irwin et al. 1987), reduced neutrophil superoxide generation (Khanfer et al. 2011), and a reduced response to the influenza vaccination (Phillips et al. 2006a).

Caregiving for a sick or disabled relative has frequently been employed as a model for examining the effects of chronic stress on immune function (Cohen and Pollack 2005, Vedhara et al. 1999, Vitaliano et al. 1998). For example, in comparison to matched controls, caregivers mounted a poorer response to influenza and pneumococcal vaccinations (Glaser et al. 2000, Vedhara et al. 1999), had reduced NK cell cytotoxicity (Cohen and Pollack 2005)**,** took longer to heal from punch biopsy wounds (Kiecolt-Glaser et al. 1995), and displayed poor control of latent viruses (Kiecolt-Glaser et al. 1987b). However, the majority of these studies have been conducted in older adults. This raises the issue as to whether effects of caregiving stress are only observed in the context of immune ageing. For example, sIgA secretion rates were found to be lower in non-routine caregivers relative to controls, but only for the oldest of three distinct age cohorts (Gallagher et al. 2008). Further, the study found that young caregivers of patients with multiple sclerosis had similar antibody responses to influenza vaccination as control participants (Vedhara et al. 2002). In contrast, more recently, parents caregiving for children with developmental disabilities showed lower antibody responses to influenza and pneumococcal vaccinations

relative to matched control parents (Gallagher et al. 2009a, Gallagher et al. 2009b), and those parental caregivers reporting more child conduct problems mounted a poorer antibody response (Gallagher et al. 2009a, Gallagher et al. 2009b). This suggests that the behavioural characteristics of the care-recipients may be a key determinant of whether or not immunity is compromised, and that an ageing immune system is not a pre-requisite for a poor response to vaccination or other immune response in caregivers. Nevertheless, older caregivers tended to have a poorer antibody response to one of the influenza vaccine strains (Gallagher et al. 2009a), suggesting that we cannot dismiss the hypothesis that chronic stress and immune system ageing may have additive effects (Graham et al. 2006).

The effects of ageing *per se* on the immune system, termed immunosenescence (Pawelec 2007), are well established (Gruver et al. 2007). In terms of adaptive immunity, lower thymic output of naive T cells (Gruver and Sempowski 2008) and a shift from naive T cells towards memory $CD8^+$ T cells (Pawelec 2007) are some of the changes commonly observed in older age. These alterations are considered to underlie the age-associated decline in antibody response to vaccination (Kovaiou et al. 2007). In innate immunity, NK cells (Hazeldine et al. 2012, Mocchegiani and Malavolta 2004) and macrophages (Plowden et al. 2004) are also affected by age. Neutrophils show diminished phagocytosis and superoxide production (Butcher et al. 2001, Panda et al. 2009). This leaves them less able to fight bacterial infections, such as pneumonia, one of the major causes of morbidity and mortality among the elderly (Solana et al. 2006). To date, the effects of chronic stress have not been examined on younger and older samples simultaneously. In addition, research into caregiving stress has mainly focussed on adaptive immunity, with the exception of NK cells (Esterling et al. 1994, Kiecolt-Glaser and Glaser 1999). Bereavement, another source of chronic stress, was

seen to reduce neutrophil superoxide production in older adults relative to matched controls (Khanfer et al. 2011).

Potential mechanisms underlying stress and neutrophil function associations include stress hormones known to modify immunity: cortisol (Bekesi et al. 2000) and DHEAS (Radford et al. 2010). Cortisol is mainly immunosuppressive, whereas DHEAS is considered immune-enhancing (Phillips et al. 2007). An imbalance between these two hormones, resulting in a higher cortisol:DHEAS ratio, can arise in response to stress (McCraty et al. 1998), and ageing (Orentreich et al. 1984), and has negative implications for immunity. The ratio between serum cortisol and cortisone is a good indicator of systemic 11β-HSD1 activity such that a higher ratio could indicate increased activity of this enzyme (Homma et al. 2001), as is seen with ageing and increased pro-inflammatory status (Tomlinson and Stewart 2005). Finally, psychological and caregiving-specific characteristics have been given less attention in previous studies, but may be responsible for any caregiving effect on neutrophil function.

The present study aimed to elucidate the associations between stress, ageing, and neutrophil function through studying four participant groups: parents of children with developmental disabilities; age- and sex-matched parents of typically developing children; older dementia caregivers; and age- and sex-matched healthy older adults with immunosenescence. This allowed comparison of effects of caregiving stress on immunity with and without concomitant immunosenescence. Potential endocrine and psychological mechanisms were also examined.

3.3. METHODS

3.3.1. Participants

57 young parental caregivers and 34 matched parental controls, and 40 older caregivers and 42 matched older controls participated. Young caregivers had at least one child with a developmental disability, defined as described in chapter 2; older caregivers were aged 60+ years and full time spousal dementia carers. Parental caregivers were mainly recruited at national syndrome support group conferences; their children were aged between 3 and 18 years and living at home during the school term. The caregiver group consisted of 27 parents of a child with Smith-Magenis syndrome (47%), 22 parents with at least one child with fragile X syndrome (39%), seven parents of a child with Cornelia de Lange syndrome (12%), and one parent of a child with ASD (2%). Control parents of typically developing children were recruited locally and from media campaigns and newspaper advertisements. Older caregivers were recruited from different NHS trusts (Birmingham and Solihull Mental Health NHS Foundation Trust, Lincolnshire NHS Trust, North Staffordshire NHS Trust and Bradford District Care Trust). Older controls were recruited through the Birmingham 1000 Elders group (http://www.birmingham.ac.uk/research/activity/mds/centres/healthy-ageing/elders.aspx). Demographic characteristics are shown in Table 1. Exclusion criteria were: taking medication known to alter immune function and/or steroid synthesis and metabolism; an ongoing chronic immune-related disease (e.g. cancer, glandular fever, diabetes, rheumatoid arthritis); and, for the younger group, pregnancy.

3.3.2. Study design and procedure

This was a cross-sectional single session study with participants completing a questionnaire pack, and providing a blood sample in order to determine neutrophil function and levels of cortisol, cortisone and DHEAS in their serum.

3.3.3. Questionnaires

Health behaviours

A questionnaire adapted from the Whitehall II study (Marmot et al. 1991) was used to assess health behaviours. Subjective sleep disturbances were reported by participants through the Pittsburgh Sleep Quality Index (Buysse et al. 1989), where higher overall score indicates poorer sleep quality. Adequate internal consistency (Cronbach's α =.69) has been demonstrated previously (Spira et al., 2011) and in the present sample, α = .68.

Depression and anxiety

HADS (Zigmond and Snaith 1983) was used to measure current anxiety and depressive symptomatology in participants. The HADS has acceptable internal consistency (.80 to .93 for anxiety and .81 to .90 for depression) (Herrmann, 1997; White & Hastings, 2004), and in the present study, .86 and .81, respectively.

Perceived stress

PSS was used to assess how unpredictable and overwhelming daily life was during the previous month (Cohen et al. 1983a). The scale shows good internal reliability (Cronbach's α = .75); in the present study.86.

Caregiver burden

In order to assess the stress caused by a caregiving role, a short form version of BI was used (Bédard et al., 2001). This version has a Cronbach's alpha of .88. For younger group, the questions were amended replacing ‘your relative’ with ‘your child’, while 'spouse/partner' was used in older caregivers' questionnaire pack. Examples of items include ‘Do you feel that because of the time you spend with your child/young spouse/partner that you don’t have enough time for yourself?’ Internal consistency in the current sample was .91.

Time spent caregiving

The amount of time spent caregiving was assessed using a modified version of the CAS (Davis et al. 1997). Hours for each caring role (e.g., dressing, eating, transport) were summed together to yield a total daily score for time spent caregiving.

Social Support

The 12-item SFS (Dunst et al. 1988) was used to assess types of support available to participants in both younger and older group. In the present study the internal consistency was .89.

In the older group only, perceived social support was measured using The Medical Outcomes Study Social Support Survey (MOS) (Sherbourne and Stewart 1991). Overall functional support was assessed as a summary of four different categories of support: emotional/informal (e.g 'someone to confide in or talk about problems'), tangible ('someone to help you if confined to bed'), affectionate (e.g. 'someone who hugs you'), and positive social interactions (e.g. 'someone to do something enjoyable with'). The questions were assessed using 5-poing Likert-type scale ranging from 1-none of the time

to 5-all of the time. High internal consistency of .91 has been reported previously, as well as test-retest variability from .72 to .78 for all four categories (Goodman 1997, Sherbourne and Stewart 1991). The scale has been used previously in chronic stress research (Phillips et al. 2005, Phillips et al. 2006a). Internal consistency in the current sample was .97.

Care-recipient problem behaviour

SDQ (Goodman 1997) was used to screen for child behaviour difficulties. In the current sample the internal consistency for problem behaviour was .86.

In the older adults, the Pearlin Problematic Behaviour (PPB) subscale was used which is a part of longer model developed to assess the stress of the caregivers of people with dementia (Pearlin et al. 1990). The subscale focuses on frequency certain behaviours occur in a patient and demand caregiver's attention. Participants were asked to mark the number of days they had to deal with certain situations weekly from 1- '0 days' to 4 - '5 or more days'. Total score was calculated by adding the scores from the individual questions. This subscale alone has been previously used in the dementia research (Gaugler et al. 2000, Teel and Press 1999) and has a good internal consistency (Cronbach's α=.88) (Teel and Press 1999). Internal consistency in the current sample was .78.

3.3.4. Blood sampling and assays

Venous blood sample were collected in the morning, into one heparin tube and one plain tube (BD Vacutainer, Oxford, UK). The heparin tube was used to assess neutrophil function in whole blood, in particular bacteria-induced phagocytosis and ROS production. The samples in the plain tubes were allowed to clot for one hour, after which they were centrifuged at

1600xg (Eppendorf Ltd Centrifuge 5804/5804R, Stevenage, UK) for 10 min, and the separated serum was frozen at −20 °C until assayed.

Neutrophil functional assays

Phagocytosis of *E.coli* was measured using a fluorescence-based kit (Phagotest, Orpegen Pharma GmvH, Heilderberg, Germany) following the manufacturer's protocol. 100µl of the whole blood at the 0°C was incubated with 20µl of fluorescein isothiocyanate (FITC)-labelled bacteria, and then incubated at 37°C for 10 minutes with the negative control left on ice. After the incubation period, the test tube was transferred on ice to stop the reaction and FITC-quenching solution was added to the both tubes to extinguish the fluorescence of all the bacteria that were left un-phagocytosed. After two washing steps in PBS, the lysing solution was added to the tubes and left for 20min at the room temperature to allow lysing of erythrocytes and fixation of lymphocytes. The cells were then washed twice and dsDNA was stained with a nuclear counter-stain for 10min on ice. The assays were analysed using a three-laser Dako Cyan High Performance flow cytometer (Dako, Carpinteria, California), with Summit v 4.3 software. Neutrophils were distinguished from other leukocytes by gating on forward scatter and side scatter and any remaining bacterial aggregates were excluded using red fluorescence staining to identify cells having diploid DNA content. 10-15000 leucocytes per sample were collected and the phagocytosis was presented as phagocytic index (PI), which is number of bacteria ingested per cell, seen as mean fluorescence intensity (MFI) multiplied by the percentage of neutrophils that had phagocytosed *E.coli*.

Neutrophil oxidative burst in response to *E.coli* was measured using a commercial kit (Phagotest, Orpegen Pharma GmbH), the assays were performed according to the manufacturer's protocol. In summary, 100µl of the whole blood was mixed with 20µl of opsonised *E.coli*, and incubated at 37°C for 10 minutes together with another tube without

stimulus that served as a negative background control. Formation of ROS was monitored by addition and oxidation of dihydrorhodamine (DHR) 123 that served as a fluorogenic substrate. The reaction was stopped by adding lysing solution in order to remove erythrocytes and achieve partial fixation of leukocyte in the samples. The samples were then washed in PBS, and DNA staining solution was added. ROS generation was evaluated using flow cytometry using the same gating technique as described above, with MFI used as a measure of the oxidative burst activity of neutrophils.

Serum steroid hormone analysis

Steroid hormone analysis in serum was conducted by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Briefly, 200 µl of serum was mixed with internal standard cortisol-d4, and after protein denaturation at 55°C, samples were extracted using methyl t-butyl ether (MTBE) as a solvent. The mixture was then vortex-mixed, centrifuged at 1200rpm (MSE, Centaur 2, DJB Labcare, England) for 5 minutes and the MTBE layer was removed and evaporated under nitrogen at 40-55°C. The dried residues were resolved in 100µl 50/50 mixture of methanol and water, and transferred into a 96-well plate for analysis by LC-MS/MS.

For DHEAS, a protein crash method was used for extraction following the protocol described previously (Haring et al. 2013). Briefly, 20µl serum, internal standard DHEAS-d2, and $ZnSO_4$ each and 100µl of acetonitrile were combined, vortex mixed for 1 minute, and then centrifuged for 5 minute at 14000rpm (MSE, Centaur 2, DJB Labcare, England). Supernatant (100µl) was then transferred into a clean glass test tube, evaporated to dryness and reconstituted in 50/50 MeOH/H_2O for analysis.

A Waters Xevo mass spectrometer with Acquity UPLC local console was used. An electrospray ionisation source was used in a positive mode. Steroids were quantified by comparison to a calibration series with respect to the internal standard used, and quantification was completed using TargetLynx software version 4.1 (MassLynx 4.1). Intra-assay reproducibility was <10%. The calibration range was 1.38 - 1380 nmol/l for cortisol and cortisone, and 0.026 - 26 μmol/l for DHEAS.

3.5. Statistical analyses

Comparisons between the caregivers and control groups on socio-demographics and questionnaire scores were conducted by ANOVA and chi-square. Tests of the main effects of age and caregiving status as well as caregiving * age interaction effects were examined using 2x2 ANOVA; with effect sizes reported as η^2. Significantly different demographic or health behaviour variables between the groups were controlled for in further ANCOVAs. Correlations were used within the caregiver participants to determine whether or not psychosocial and caregiving variables were associated with neutrophil function. Where they were, hierarchical linear regression was conducted to further examine this association with age entered at step 1 and any significantly associated caregiving/psychosocial variable at step 2.

3.4. RESULTS

3.4.1. Demographic characteristics and health behaviours

The demographic and health behaviour variables for each group are summarised in Table 1. In the younger sample, caregiver and control parents did not differ in their demographic characteristics. Health behaviours were also similar, with the exception of

caregivers being more likely to drink alcohol daily (p=.005). In both caregivers and controls, a few participants reported that they suffered from chronic illness (mainly asthma and high/low blood pressure). Consequently, some of the parents in both groups reported taking medication, mainly non-steroidal asthma treatments and anti-hypertensives. In the older group, caregivers and controls were comparable on all of the demographic and health behaviour variables except for exercise scores where controls were more active than caregivers. Similar numbers of participants reported suffering from chronic disease, mainly from those typical for older age such as high blood pressure and mild arthritis.

3.4.2. Caregiving and psychosocial characteristics of each group

Caregiving parents reported spending more time caring for their child than control parents (Table 2). Other psychosocial characteristics were also significantly different between the groups and in the expected direction; caregivers reported higher depression and anxiety, perceived stress and child problem behaviours. They also reported poorer sleep quality, higher caregiving burden and poorer quality of social support. Table 2 shows that depression and anxiety symptomatology, perceived stress scores, sleep quality, and general social support were significantly worse in the older caregiver group compared to older controls. Caregiving burden reported by older caregivers was in the high range, according to the previously reported cut off of 17 (Bedard et al. 2001).

Problematic behaviour on the Pearlin scale was higher than previously reported for samples of caregivers (Roepke et al. 2008). Interestingly, younger caregivers reported higher depression and anxiety symptoms, perceived stress and caregiving burden than the older caregivers (Table 2).

Table 1. Demographic characteristics and health behaviours of young and old, caregivers and controls.

	Young			Older		
	Caregivers (N = 53)	**Controls (N = 33)**		**Caregivers (N =40)**	**Controls (N =42)**	
	N (%) / Mean (SD)		***p***	**N (%) / Mean (SD)**		***p***
Age (years)	38.3 (4.78)[a]	40.1 (5.44)	.10	69.3 (5.81)	72.4 (5.42)	.06
Age of child/spouse (years)	7.4 (3.70)	7.2 (4.54)	.85	72.3 (8.06)	73.1 (6.04)	.63
Gender (Female)	38 (69)	21 (62)	.55	26 (65)	19 (45)	.07
Marital status (Partnered)	47 (89)[a]	30 (88)	.95	40(100)	42(100)	-
Ethnicity (Caucasian)	50 (93)	27 (79)	.07	38 (97)	39 (93)	.33
Occupational status (non-manual)	40 (85)[a]	31 (94)	.22	22(63)	32 (80)	.10

In full time work	21 (47)[a]	19 (63)	.16	3 (8)	1 (4)	.53
Taking medications	5 (9)[a]	7 (21)	.14	33 (80)	29 (74)	.51
Alcohol intake (daily or more)	14 (26)	1 (3)	.005	10 (28)	12 (30)	.83
Smokers	10 (19)[a]	2 (6)	.10	1 (3)	3 (7)	.36
Body Mass Index	26.4 (4.89)	24.5 (3.58)	.06	26.3 (3.08)	26.1 (4.37)	.82
Exercise score	5.5 (4.99)[a]	6.4 (5.44)	.45	3.5 (3.16)	5.2 (3.71)	.04
Fruit and vegetable consumption score	8.2 (2.83)[a]	8.8 (2.36)	.25	9.7 (2.46)	10.3 (2.85)	.31
Fat consumption score	11.1 (3.31)[a]	10.9 (3.72)	.76	9.5 (3.62)	10.2 (3.91)	.46

[a]$p < .05$ between younger caregivers and older caregivers

Table 2. Caregiving and psychosocial characteristics of young and old, caregivers and controls.

	Young			Older		
	Caregivers (N = 53)	**Controls (N = 33)**		**Caregivers (N = 40)**	**Controls (N = 42)**	
	Mean (SD)		***p***	**Mean (SD)**		***p***
Hours spent caregiving per day minus supervision	4.8 (4.15)	2.9 (3.23)	.03	3.6 (3.28)	-	-
Sleep quality score	8.4 (3.13)	6.7 (3.15)	.02	8.1 (3.47)	6.2 (3.80)	.02
HADS anxiety score	10.4 (3.52)[a]	6.4 (2.85)	<.001	7.7 (4.96)	4.1 (3.93)	<.001
HADS depression score	8.6 (3.04)[a]	4.2 (3.67)	<.001	6.0 (3.94)	2.8 (2.61)	<.001
Perceived stress score (PSS)	30.8 (5.56)[a]	23.5 (6.59)	<.001	24.0 (8.21)	16.4 (7.83)	<.001

Social support score (SFS)	31.9 (7.99)	38.6 (9.62)	.001	33.6 (11.18)	-	-
Caregiver burden score (BI)	26.3 (7.67)[a]	13.7 (6.69)	<.001	21.0 (8.62)	-	-
Child behaviour problems (SDQ)	18.8 (4.64)	7.2 (3.99)	<.001	-	-	-
Pearlin problematic behaviour (PPB)	-	-	-	22.9 (5.89)	-	-
Social support score (MOS)	-	-	-	63.1 (18.66)	86.0 (11.34)	<.001

[a] $p < .05$ between younger caregivers and older caregivers

3.4.3 Neutrophil function across all groups

For neutrophil phagocytic ability, there was a main effect of age, $F(1,167) = 49.81$, $p < .001$, $\eta^2 = .230$, such that older adults had a higher PI , but no main effect of caregiving, $F(1,167) = 3.62$, $p = .06$, $\eta^2 = .021$, and no caregiving * age interaction effect, $F(1,167) = .53$, $p = .47$, $\eta^2 = .003$. These data are shown in Figure 1A. Repeated analyses with adjustment for health behaviours that differed between the groups (alcohol intake in young; exercise level in the older group) revealed the same significant main effect of age, $p < .001$.

For neutrophil superoxide production, there was a main effect of age, $F(1,164) = 12.23$, $p = .001$, $\eta^2 = .069$, and caregiving, $F(1,164) = 4.98$, $p = .03$, $\eta^2 = .029$, such that older participants and caregivers had significantly higher superoxide production. However, there was no caregiving * age interaction effect, $F(1,164) = 0.18$, $p = .67$, $\eta^2 = .001$, see Figure 1B. Repeated analyses with adjustment for covariates revealed only a main effect of age, $p = .001$.

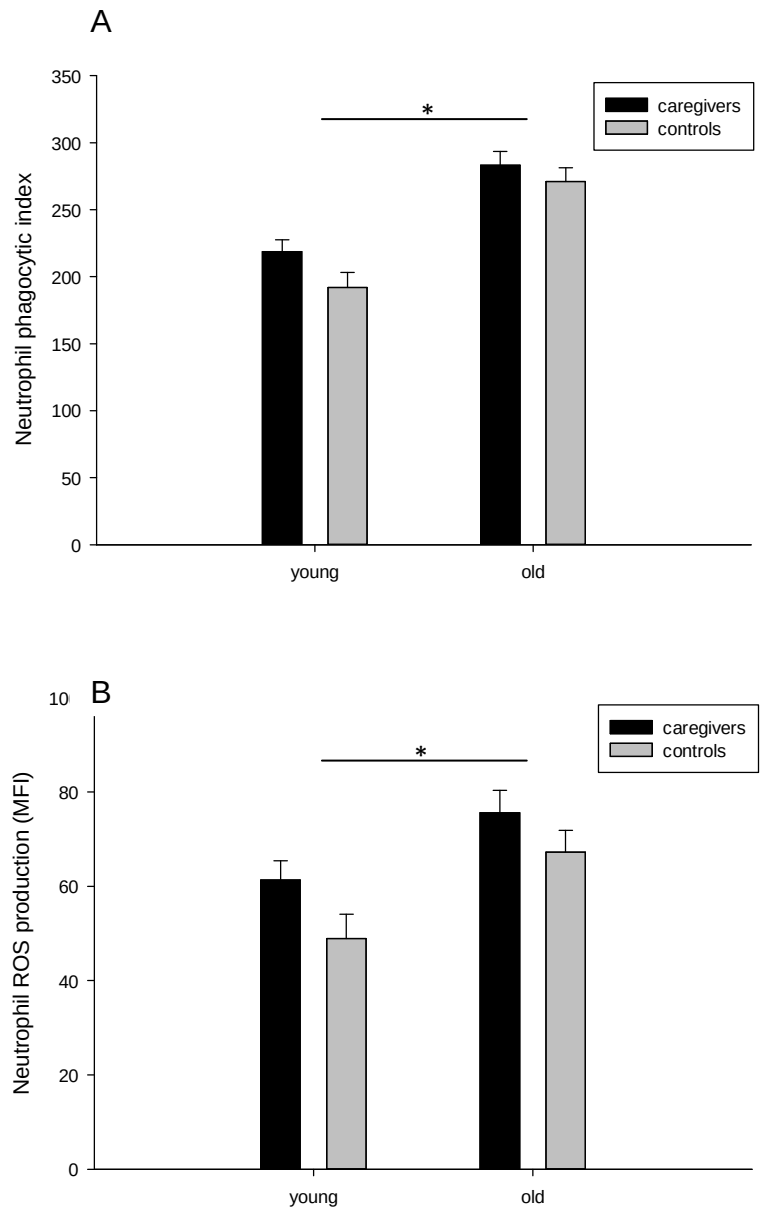

Figure 1. Neutrophil function in response to bacteria *E.coli* in young and old, caregivers and controls. A. Neutrophil phagocytosis of *E.coli* presented as phagocytic index (PI, number of bacteria ingested per neutrophil). B. Neutrophil reactive oxygen species (ROS) production presented as mean fluorescence intensity (MFI). Error bars are standard errors of the mean (SEM) and * indicates $p < .05$.

3.4.4. Serum steroid hormone concentrations

Due to the skew of the data for serum hormones and their ratios, these were log transformed for the analyses, but their raw values are presented in the figures. For cortisol, there was a main effect of age, $F(1,160) = 5.65$, $p = .02$, $\eta^2 = .034$, such that participants in the older group had higher concentrations of this hormone. However, there was no main effect of caregiving, $F(1,160) = .021$, $p = .88$, $\eta^2 = .000$, nor a caregiving * age interaction effect, $F(1,160) = .516$, $p = .47$, $\eta^2 = .003$, as shown in Figure 2A. Repeated analyses with covariate adjustment also revealed an age effect, $p = .03$. For DHEAS, as expected, there was a main effect of age, $F(1,156) = 109.52$, $p < .001$, $\eta^2 = .412$, with older adults showing lower levels. There was no main effect of caregiving, $F(1,156) = 1.15$, $p = .28$, $\eta^2 = .007$, nor caregiving * age interaction, $F(1,156) = 3.14$, $p = .08$, $\eta^2 = .020$ (Figure 2B). After covariate adjustment the main effect of age remained significant, $p < .001$. The cortisol:DHEAS ratio showed a significant effect of age, $F(1,154) = 120.44$, $p < .001$, $\eta^2 = .440$, with older adults having a higher ratio, but no main effect of caregiving, $F(1,153) = .16$, $p = .25$, $\eta^2 = .009$, nor a caregiving * age effect, $F(1,153) = .181$, $p = .21$, $\eta^2 = .010$, as presented in Figure 2C. Repeated subsequent analyses including the covariates for both young and old showed a similar main effect of age, $p < .001$. For the cortisol:cortisone ratio, there was no age effect, $F(1,160) = 1.51$, $p = .22$, $\eta^2 = .009$, but both a main effect of caregiving, and a significant caregiving * age interaction effect were present, $F(1,160) = 15.48$, $p < .001$, $\eta^2 = .088$, and $F(1,160) = 19.88$, $p < .001$, $\eta^2 = .111$, respectively, Figure 2D. Pairwise comparisons revealed significant differences between caregivers and controls in the younger group, $p < .001$, with higher ratio present in younger caregivers, but no

difference between older caregivers and controls, $p = .34$. Covariate analyses revealed analogous main, $p < .001$, and interaction effects, $p = .001$.

3.4.5. Psychological factors and immunity and hormones within caregivers

Analysis within all caregiving participants showed that caregivers with higher anxiety, $r(87) = -.34$, $p = .001$, depression, $r(87) = -.31$, $p = .003$, perceived stress, $r(87) = -.34$, $p = .001$, and caregiving burden, $r(87) = -.37$ $p \leq .001$, exhibited significantly lower neutrophil phagocytic ability. No such relationships emerged for neutrophil superoxide production, but caregivers with poorer sleep quality had lower oxidative burst activity, $r(85) = -.23$, $p = .03$. Correlations between questionnaire scores and hormones within the caregiving participants revealed that among older caregivers lower DHEAS was recorded in those with higher anxiety symptomatology ($r(35) = -.42$, $p = .01$) and perceived stress ($r(35) = -.33$, $p = .05$), while higher cortisol:DHEAS ratio was seen in those who reported higher anxiety symptoms ($r(35) = .34$, $p = .04$).

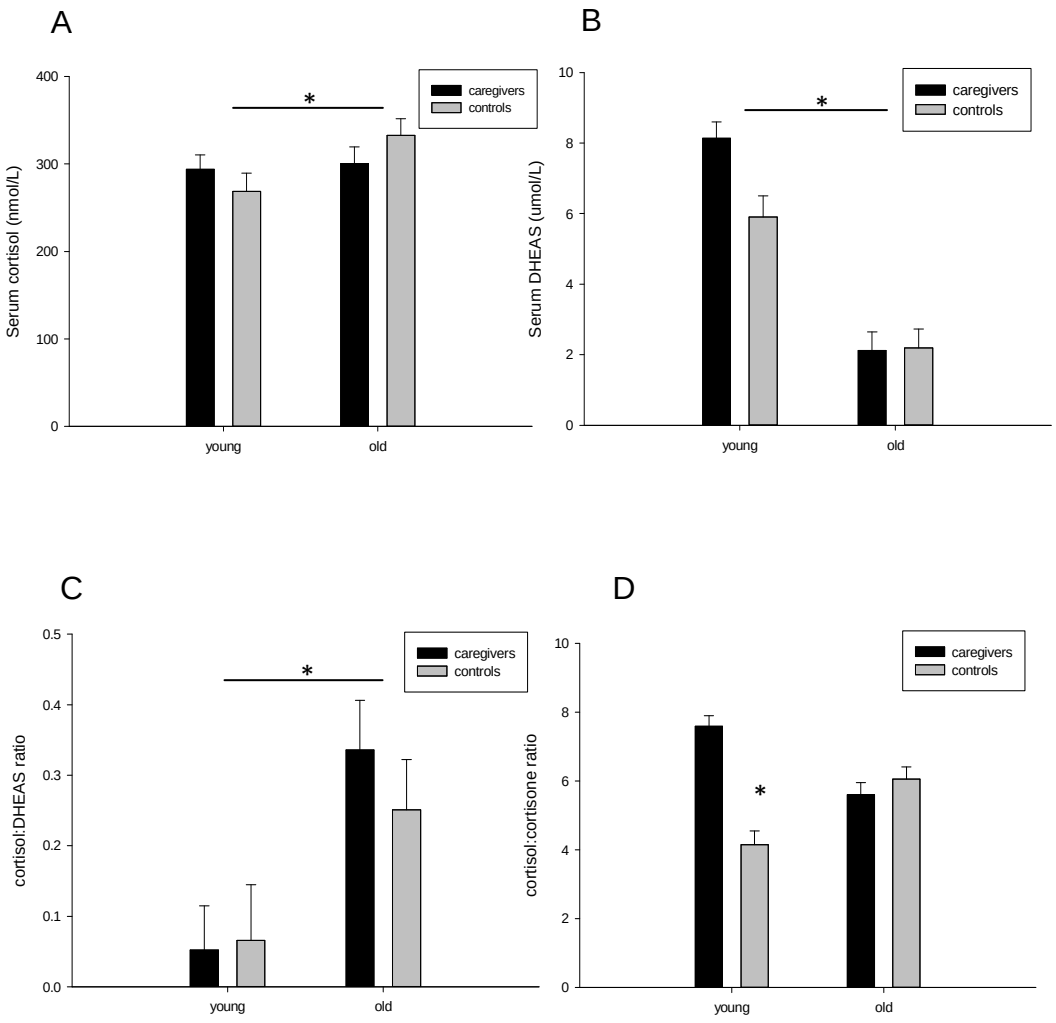

Figure 2. Serum stress hormone concentrations in young and old, caregivers and controls. A. Serum cortisol concentrations in nmol/l. B. Serum DHEAS concentration in μmol/l. C. Cortisol:DHEAS ratio. D. Cortisol:cortisone ratio. Error bars are SEM and * indicated $p < .05$.

3.4.5. Sensitivity analysis

Given the lack of effects of caregiving status *per se*, but the correlations with several of the psychological variables, regression analyses were conducted within the caregivers alone, predicting neutrophil functions from the significant psychological variables that emerged in the correlation analyses with adjustment for chronological age to use the full range of age data. For phagocytic ability, anxiety, $\beta = -.21$, $p = .036$, $\Delta R^2 = .038$, and caregiver burden, $\beta = -.24$, $p = .014$, $\Delta R^2 = .052$, were significant predictors, whereas depression, $\beta = -.14$, $p = .16$, $\Delta R^2 = .017$, and perceived stress, $\beta = -.15$, $p = .14$, $\Delta R^2 = .019$, were not, following adjustment for age. For oxidative burst activity, sleep quality adjusted for age significantly predicted neutrophil oxidative burst activity, $\beta = -.24$, $p = .025$, $\Delta R^2 = .055$.

3.5. DISCUSSION

In the present study, caregivers and controls differed, as expected, on the majority of psychosocial and caregiving variables. Despite this, neutrophil function of caregivers was comparable to that of the controls. The significant effects observed were for age; unexpectedly, older adults had higher phagocytosis and oxidative burst activity than the participants from the younger groups. This ageing effect was contrary to what might be expected, as previous research including from our own group, mainly reports a decrease in neutrophil function due to immunosenescence (Butcher et al. 2001, Panda et al. 2009, Wenisch et al. 2000). However, it is consistent with the previously reported increase in oxidative burst activity associated with ageing in other studies (Cannizzo et al. 2011, Tortorella et al. 2001). It is possible, that the present older sample, in particular older

caregivers, was predominantly comprised of the participants who have very effective immune function which provides resilience and increased likelihood of survival into older age, despite the additional stress of their caregiving role. In other words, what we are observing here could be a robustness of innate immunity with healthy ageing, and a continuing capacity to resist illnesses in general with the caregiving role. This has previously been observed in reductions in cancer incidence in the oldest old, where phenotypic changes in NK and T cells as well as age-related changes in the production of pro-and anti-inflammatory cytokines, such as IFN-γ and IL-4, create unsuitable surroundings for neoplastic transformations (Bonafe et al. 2001). Thus, our older sample may have comprised individuals whose genetic and epigenetic predisposition have provided them with a robust immune response, giving them a chance to live to old age (Davey Smith 2011). In addition, if we are observing a robustness of neutrophil function in the current older sample, then this is accompanied by a similar psychological resilience, as the overall scores for perceived stress, depression and anxiety symptomatology reported by the older adults were lower than those of the younger sample who were perhaps exhibiting a worse psychological response to their caregiving role (see Table 2). Such psychological robustness in individuals aged over 64 years compared to younger groups has previously been reported (Gooding et al. 2012).

Alternatively, the present results could also be interpreted as a support for the hypothesis that the innate immune system dominates in older age, and that immunosenescence is more evident in adaptive immunity (Franceschi et al. 2000). However, caution is warranted here, as the observation that innate immunity is predominant in older age was observed among the oldest old and centenarians, whereas the mean age in the present older sample overall was 71 years.

The absence of a caregiving status effect on neutrophil function was not anticipated, but it is possible that this aspect of innate immunity, unlike adaptive immune function such as the response to vaccination (Gallagher et al. 2009a, Gallagher et al. 2009b), is not diminished by the chronic stress of caregiving. It is also possible that the general immune integrity observed in the current caregiving sample is due to the method of assessing immunity. Previous studies showing caregiving effects have used *in vivo* challenge of the immune response, e.g., vaccination (Gallagher et al. 2009a, Gallagher et al. 2009b, Glaser et al. 2000), and wound healing (Kiecolt-Glaser et al. 1995). These *in vivo* methods demand an integrated immune response. The present study, on the other hand, examined *in vitro* stimulation, without a systemic pathogenic challenge. The negative impact of caregiving may be more readily observed in models where the immune system is challenged with pathogens *in vivo*.

Interestingly, analysis within the caregiving participants indicated that those individuals with anxiety and caregiver burden had poorer neutrophil function, specific to phagocytosis. For superoxide production, poorer sleep quality predicted poorer function. A negative association between chronic stress and neutrophil superoxide generation has been reported previously in the bereaved (Khanfer et al. 2011), who also showed high levels of depression and anxiety. Among elderly hip fracture patients, only those who reported high levels of depression showed poorer neutrophil function, though again this was restricted to superoxide generation (Duggal et al. 2013a). Similarly, poor sleep has been associated with alterations in immunity in a number of studies (Besedovsky et al. 2012). This suggests that while no overall detrimental effect of caregiving was seen in this study, individuals reporting higher psychological distress and suffering poorer sleep have poorer innate immune function.

Serum steroid hormone analyses revealed higher cortisol, lower DHEAS and higher cortisol:DHEAS ratio in the older sample overall, which is consistent with previously reported changes in hormone status with increasing age (Buford and Willoughby 2008). Comparable cortisol and DHEAS levels between caregivers and controls are perhaps surprising, as it is commonly accepted that chronic stress leads to the increase and decrease in these hormones, respectively (Epel et al. 2007). However, exception to these findings, such as those that showed increased DHEA levels in depressed patients (Heuser et al. 1998), as well as those that suggested differential effects of stress on DHEAS dependent on the level of anxiety (Boudarene et al. 2002), suggest greater caution when studying the relationship between stress response and these hormones. Further, the lack of increase in cortisol levels in caregivers could be the consequence of allostatic load, when due to a higher frequency of chronic stress exposure the body is shutting of endocrine parameters and preventing expected responses (McEwen 1998), increase in cortisol levels due to chronic stress of caregiving in this particular case.

Younger caregivers also had a relatively high cortisol:cortisone ratio. This ratio is an indicator of systemic 11β-HSD1 activity which in turn can be increased if pro-inflammatory status is raised (Tomlinson and Stewart 2005). An increased serum cortisol:cortisone ratio has also been reported in surgical patients, probably as a consequence of the continuous HPA axis stimulation during the stress response (Vogeser et al. 2003).

The study has a number of limitations. First, sample size in each group can be regarded as small, but is of a similar magnitude to other caregiving studies (Gallagher et al. 2009a, Gallagher et al. 2009b, Vedhara et al. 2002). Second, it could be argued that due to the high burden of the caregiving role, those worst affected by this type of chronic stress

would be less likely to participate in the study, making the current sample biased towards those that are healthier and cope better. However, the attempt to minimise such bias was through organising home visits and accommodating the testing session to best suit individual caregivers' needs even when they had high caregiver burden. Further, the scores on the psychological measures suggested that our sample did have high caregiver stress and burden similar to previous caregiver studies where immune effects were seen. Third, steroid hormones are known to show diurnal variations in secretion and therefore it could be argued that single time point blood collections will not give the accurate representation of their concentration. However, an attempt was made to overcome this issue by taking fasted morning blood samples. Finally, inclusion of a further measure of innate immunity, such as NK cell function, might have strengthened the study although effects of stress on this have already been demonstrated in older caregivers.

In conclusion, the neutrophil function in caregivers compared to controls overall was preserved, with the main differences emerging between young and old participants. Nevertheless, it should be noted that those caregivers who reported higher anxiety and burden levels, as well as poorer sleep quality, demonstrated poorer neutrophil function. Implications for future research are that studies should focus less on caregiving status in general, and more on individual differences among caregivers, specifically those with high levels of psychological morbidity.

CHAPTER 4

T CELL IMMUNITY AND CAREGIVING STRESS IN YOUNGER AND OLDER CAREGIVERS

4.1. ABSTRACT

Caregiving stress is a well established model for examining the impact of chronic stress on immunity. The present study aimed to examine the impact of stress and ageing on parameters of T cell immunity and anti-CMV antibody titre using caregivers and controls across two age cohorts. 79 young and older caregivers (parents mean age 38 years of children with developmental disabilities and spousal dementia caregivers, mean age 70 years, respectively) were compared to 76 age- and sex-matched non-caregiving controls. Participants completed questionnaires on a range of socio-demographic, health behaviour, psychosocial and caregiving variables, and provided a blood sample. PBMCs were stored for assessment of T cell senescence and exhaustion markers and thymic output, and serum was used to measure anti-CMV antibody titre. Despite greater psychological morbidity, seen as the greater depression, anxiety, and perceived stress than controls, caregivers showed robust immunity for most T cell parameters with the exception of the $KLRG1^+$ (marker of T cell senescence) T cell pool, where caregivers and older participants showed higher numbers of T cells expressing this molecule. In addition, amongst older caregivers, those who reported higher behavioural problems in care-recipients showed a greater senescent profile. A higher percentage of $KLRG1^+$ T cells in caregivers could explain their previously reported poorer immune response. These data also suggest that the impact of caregiving *per se* on immunity is not uniform and greater attention should be paid to those with greater psychological morbidity.

4.2. INTRODUCTION

Numerous adverse effects of chronic stress (e.g., caregiving or bereavement) on adaptive immunity have been reported in the past decades, see e.g. (Gouin et al. 2008, Glaser and Kiecolt-Glaser 2005, Powell et al. 2011, Cohen et al. 1991). The immune system correlates of the negative effects of stress include reduced vaccination response (Vedhara et al. 1999, Glaser et al. 2000, Phillips et al. 2006a, Gallagher et al. 2009a, Gallagher et al. 2009b), poorer latent virus control (Pariante et al. 1997, Glaser and Kiecolt-Glaser 1997), thymic involution indicated by reduced output of naive T cells (Selye 1956), the shift from a cellular Th1 to a humoral Th2 phenotype by changes in cytokine expression (Marshall et al. 1998), and increased blood leukocytosis (Franchimont 2004).

The adaptive immune system undergoes dramatic changes during the physiological ageing process. These changes, termed immunosenescence, have been well documented, for reviews see (Gruver et al. 2007, Miller 1999, Weng 2006), and include both a decrease in the number of newly produced naive T cells (Fagnoni et al. 2000, Hale et al. 2006, Gruver and Sempowski 2008), as well as a shift from naive T cells towards a higher number of longer lived memory T cells, in particular $CD8^+$ T cells as the peripheral memory T cell pool expands to maintain homeostasis (Pawelec 2007). The established means of quantifying thymic output and naive T cell production is through the number of cells expressing TREC, stable extrachromosomal DNA fragments created during T cell receptor (TCR) formation (Kong et al. 1998) in the process of somatic rearrangement during T cell maturation (Abbas and Janeway 2000, Janeway et al. 2001). In addition, ageing is accompanied by many phenotypic and

functional alterations in T cell subsets. For example, expression of CD28 antigen, a co-stimulatory molecule necessary for T cell activation (Lenschow et al. 1996), is decreased during ageing (Boucher et al. 1998), with implications for longevity of vaccination responses and maintained resistance to latent infection such as *Varicella zoster*. Further, an increase in the subsets of senescent $CD28^{-}CD57^{+}$ lymphocytes with normal ageing has been noted (McNerlan et al. 1998, Merino et al. 1998, Onyema et al. 2012). CD57 is present on terminally differentiated T lymphocytes and is considered as a marker contributing to T cell senescence (Brenchley et al. 2003) and indicative of the increase in activation-induced apoptosis (Focosi et al. 2010). It has been shown that oligoclonally expanded $CD8^{+}$ T lymphocytes are predominantly enriched with the $CD28^{-}CD57^{+}$ subset (Wood et al. 2009, Morley et al. 1995). PD-1, a receptor that plays an inhibitory role during T cell activation (Jin et al. 2011), and is important in T cell exhaustion (Wherry 2011) is also present more frequently on aged T lymphocytes (Lages et al. 2010). Finally, another inhibitory receptor that is commonly considered a marker contributing to T cell senescence, co-inhibitory cadherin KLRG1 (Grundemann et al. 2006, Henson and Akbar 2009) also shows increased expression in T cells from older donors (Vasto et al. 2007). Interestingly, it has been shown that blockade of KLRG1 leads to restoration of proliferative function by a mechanism that involves the phosporylation and activation of Akt protein kinase (Henson et al. 2009), suggesting KLRG1 as a regulator involved in both exhaustion and senescence-related pathways (Akbar and Henson 2011). The implications of these changes in both cell senescence and cell exhaustion are a negative impact on the functional capacity of memory T cells, weakening at the same time the integrity of the immune response overall and thus resistance to infection (Akbar and Henson 2011).

Another important contributor to immunosenescence is CMV (Pawelec et al. 2005). CMV is a latent virus belonging to the Herpesviridae family with the ability to remain silent until inflammatory factors and weakened immune surveillance trigger its activation (Hahn et al. 1998). This virus is believed to direct the restructuring of the lymphoid subsets in the periphery, seen as the increase in the effector memory T cell pool and the reduction in the naive T cells in those who are CMV-seropositive (Chidrawar et al. 2009). As a result, the presence of chronic viral infections such CMV leads to the accumulation of T cells with a senescent and exhausted profile namely a $CD28^{-}CD57^{+}KLRG1^{+}$ phenotype (Weng et al. 2009, Derhovanessian et al. 2009).

Until recently, there has been a consensus that caregivers, regardless of their age, experience poorer immunity than non-caregiving age-matched controls (Kiecolt-Glaser et al. 1991b, Esterling et al. 1994, Kiecolt-Glaser et al. 1996, Glaser and Kiecolt-Glaser 1997, Vedhara et al. 1999, Gallagher et al. 2009a, Gallagher et al. 2009b). In the previous chapter, however, it was demonstrated that caregivers, both young and older, displayed robust neutrophil function, compared to matched controls, observed as the ability to phagocytose bacteria and produce ROS. Indeed, these functions of the innate immune system were only diminished in those caregivers with higher psychological morbidity, as reported in chapter 3. The present analysis extends our previous observations in innate immunity by examining associations between caregiving stress, ageing, and T cell immunity using four different participant groups: younger parental caregivers of children with developmental disabilities and age- and sex-matched parental controls of typically developing children; older spousal dementia caregivers,

and age- and sex-matched healthy non-caregiving older adults. This four-group comparison allows us to examine the effects of caregiving stress on T cell immunity with and without the effect of ageing.

Focussing on adaptive immunity and specifically T cell immunity, this study examined TREC expression as a marker of thymic output and expression of markers that contribute to the senescent and exhaustion profile of T cells. It also aimed to determine whether any of the previously described psychological or caregiving-specific variables, such as depression, anxiety, caregiving burden, social support or behaviour of care-recipients were related to a more immunosenescent profile within the caregiver group. It was expected that caregivers, and particularly older caregivers would show the greatest evidence of immunosenescence, and that those with worse psychological morbidity would have greater expression of the molecules that are part of the T cell senescence and exhaustion pathways as well as reduced TREC frequency. Finally, by measuring CMV serostatus and anti-CMV antibody titre, the study aimed to ascertain if any group differences were driven by the presence of this chronic latent viral infection.

4.3. METHODS

4.3.1. Participants

The data presented here are from 39 young parental caregivers and 34 age- and sex-matched control parents, and of 40 older spousal caregivers and 42 matched controls. Inclusion criteria for the younger parental group were to have a child aged between 3 and 18 years, living at home. The young caregiver group consisted of 11 parents of a child with Smith-Magenis syndrome (28%), 21 parents with at least one child with

Fragile X syndrome (54%), and seven parents of a child with Cornelia de Lange syndrome (18%). Parents of children with a developmental disability were mainly recruited via syndrome group events. Control parents of typically developing children were recruited via local advertisements. Older caregivers were aged 60+ years and full time carers of a spouse/partner with a diagnosis of dementia, recruited via NHS trusts across England; older controls were recruited through the Birmingham 1000 Elders group of healthy older adults (http://www.birmingham.ac.uk/research/activity/mds/centres/healthy-ageing/elders.aspx). Exclusion criteria were: taking immunosuppressive drugs, suffering from an ongoing chronic immune-related disease (e.g. cancer, diabetes, rheumatoid arthritis); and pregnancy in the younger group.

4.3.2. Study design and procedure

This was a cross-sectional study where participants attended a one off session at which they completed a questionnaire pack, and provided a blood sample to determine markers of T cell exhaustion and senescence, and thymic output, as well as serum CMV antibody titre. Informed written consent was obtained prior to study participation, and the study was approved by the local NHS ethics committee.

4.3.3. Questionnaires

Health behaviours were assessed using a questionnaire adapted from the Whitehall II study (Marmot et al. 1991), as described in chapter 2. HADS (Zigmond and Snaith 1983), PSS (Cohen et al. 1983b), and BI (Bedard et al. 2001), were used to determine psychological morbidity while social support availability was examined using the SFS (Dunst et al. 1988). Finally, children's challenging behaviour was assessed through SDQ

(Goodman 1997) in the younger group, while the PPB (Pearlin et al. 1990) subscale was administered to older caregivers to report on the frequency of dementia-related behaviours in their spouse/partner.

4.3.4. Blood sampling and assays

Blood sampling

Venous blood was collected between 9-11 a.m. pre-prandially, from an ante-cubital vein into two 6ml heparin tubes. PBMCs were isolated by the standard density gradient centrifugation using Ficoll-Paque^{TM} PLUS (GE Healthcare, Upsala Sweden), following the supplier's protocol, aliquoted and stored in a freezing medium consisting of heat inactivated foetal calf serum (Sera Laboratories International, Sussex, UK) and 10% (v/v) dimethyl sulfoxide (DMSO), to allow recovery of frozen PBMCs that are functional.

Immunostaining for senescent and exhausted profile of T cells

Immunostaining was conducted by staining isolated PBMCs with a combination of fluorochrome-conjugated anti-human antibodies and consequent multicolour flow cytometry analysis, using $CyAn_{ADP}^{TM}$ cytometer (Dako, Cambridgeshire, UK). Optical alignment, sensitivity and linearity of the instrument were performed once per week. Appropriate isotope controls were used for setting the gates and compensation was conducted before each experiment electronically by Summit software using single stained cells. Anti-human antibodies used for staining were as follows: CD3 PE-Cyanine7 (clone UCHT1, ebiosciences, Hatfield, UK), CD4 eFluor® 450 (clone OKT4, ebiosciences, Hatfield, UK), CD8 PE (clone UCHT-4, ImmunoTools GmbH, Friesoythe, Germany), for defining $CD3^+CD4^+$ and $CD3^+CD8^+$ cells as appropriate; and CD28 APC (clone CD28.2, BD Pharmingen, UK), CD57 FITC (clone HCD57, BioLegend, Cambridge BioScience, UK), PD-1 APC (clone eBioJ105

(J105), ebiosciences, Hatfield, UK), and KLRG1 FITC (clone 2F1/KLRG1, BioLegend, Cambridge BioScience, UK) for further characterisation of senescence/exhaustion-related markers. Cells were then washed and re-suspended in PBS/BSA for flow cytometric analysis. At least 15000 events were acquired within the lymphocyte gate for each sample and appropriate gating was used to distinguish between live and dead cells. Further phenotyping was conducted on $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. T cell senescent profiles were characterised by the absence of CD28 and/or presence of CD57 marker, and exhaustion profile was assessed by PD-1 expression, in $CD4^+$ and $CD8^+$ T-cells separately. In addition, $KLRG1^+$ T cells were separately examined as potentially involved in both senescence- and exhaustion-related pathways. Data are presented as the percentage of antigen positive cells. Data analyses were conducted using Summit v4.3 software (Dako, Fort Collins, CO, USA).

Thymic output analysis

Thymic output was assessed through the TREC analysis, where low TREC expression ratio indicates lower thymic output of naïve T-cells (Douek et al. 1998, Hazenberg et al. 2001). DNA was isolated from PBMCs using a QIAamp DNA Blood Mini Kit (Quiagen) following the supplier's protocol and eluted in 50µl of DNA elution buffer. DNA concentration and purity was subsequently measured using a NanoDrop™ spectrophotometer and only those samples with 260/280 and 260/230 ratio above 1.8 were used. Aliquots of 200ng of DNA were used for quantitative (q)PCR analysis. QPCR was performed on a Real Time PCR Roche LC480 sw1.5 light cycler using TaqMan probes. Reaction volume consisted of forward and reverse primers in final concentration of 0.5 µM and probes in final concentration of 0.2 µM for both TREC and control gene, 2xTaqMan Master Mix from LightCycler 480 Probes Master Mix (Roche, UK) in final reaction volume of 20 µl per sample made using nuclease-free water

(Roche, UK). The primer sequences for TREC were 5′-CACATCCCTTTCAACCATGCT′ for forward and 5′-GCCAGCTGCAGGGTTTAGG-3′ for reverse primer (Mitchell et al. 2010), and ACACCTCTGGTTTTTGTAAAGGTGCCCACT for fluorescent sequence-specific probe. Primers were dissolved in the quantity of provided buffer to yield 100pmol/µl, then aliquoted and stored at -20°C. For normalisation, a referent gene was used which represent the constant region of the T cell receptor with 5'-CCTGATCCTCTTGTCCCACAG-3' forward and 5'-GGATTTAGAGTCTCTCAGCTGGTACA-3' reverse primer as well as 5'-ATCCAGAACCCTGACCCTGCCG-3' probe. Samples were run in triplicate and average Ct value was calculated for both TREC and reference gene. Rather than calculating exact number of copies of TREC per number of T cells for every sample, the method for relative quantification of TREC was developed which determines TREC status comparing to the calibrator (a 23 year old male) using the formula for the Pfafl method: Ratio = $(E_{\text{target gene = TREC}})^{\Delta Ct,\ TREC\ (calibrator - sample\ of\ interest)}/(E_{\text{referent gene=gene for a constant T cell receptor region}})^{\Delta Ct,\ referent\ gene\ (calibrator - sample\ of\ interest)}$, where E is amplification efficiency of particular DNA fragment, and C_t is the threshold cycle or cycle number at which enough amplified product accumulates to produce measurable fluorescence signal (Pfaffl 2001). The lower the expression ratio, the higher the decrease of the TREC gene in the test sample when compared to the calibrator.

CMV serum antibody titre

For the evaluation of CMV antibody titre, a standardised CMV ELISA developed by the Antiviral Immunology lab, Cancer Sciences, University of Birmingham was used as previously reported (Savva et al. 2013, Wall et al. 2013), and described in detail in

chapter 2. As before, the standard curve measured up to 1000 arbitrary units of IgG and those with more than 10 units were considered CMV positive.

4.3.5. Statistical analyses

Where data for the absolute numbers, percentage of T cells and TREC expression ratio used in the analyses were skewed, these values were log transformed. Tests of the main effects of age and caregiving status as well as caregiving * age interaction effects on T cell markers related to the senescence (CD28, CD57 and KLRG1) and exhaustion (PD-1), TREC and serum CMV antibody titre were examined using ANOVA, with partial eta-squared as the measure of effect size throughout. Demographic, health behaviour variables, and in the case of absolute numbers and percentage of T cells used in the analyses, a number of $CD3^+$ cells that were significantly different between the groups (determined by chi-square and ANOVA), were controlled for in further ANCOVAs run for each immune outcome. In addition, analyses were re-run using CMV status as a covariate to ascertain if any of the effect that emerged was driven by the presence of CMV infection. CMV seropositive subset of participants was then divided into two groups using median split of anti-CMV antibody titre to examine the relation to the parameters of T cell immunity. Finally, correlations were used within the caregiver group to ascertain whether or not psychosocial and caregiving variables were associated with immune outcomes.

4.4. RESULTS

4.4.1. Demographic, health behaviour and psychosocial characteristics

Descriptive statistics for demographic and health behaviour variables for each group are presented in Table 1. Briefly, caregivers and controls in the younger and sample were comparable on all demographics and health behaviours, with the exception of the ethnicity (p = .02), and the frequency of the alcohol consumption (p=.01) in young, with caregivers more likely to be White and to drink alcohol on a daily basis than control parents (p = .03). The only difference between the older groups was the exercise score (p = .04), with controls being more active than caregivers.

Psychosocial characteristics between caregivers and controls in both groups were significantly different in the expected direction; caregivers reported higher depression, anxiety, perceived stress, and lower social support. Caregiving burden was also higher in younger caregivers than controls, while older caregivers scored high on this scale according to the reported cut off of 17 (Bedard et al. 2001). This questionnaire was not administered to older controls as they were not caregiving for their spouse/partner. For problematic behaviour of care-recipients, younger parental caregivers reported more problems than control parents while older spousal dementia caregivers reported high scores on the Pearlin scale higher than those previously reported in caregivers (Roepke et al. 2008).

Table 1. Demographic characteristics, health behaviours and psychosocial variables of each group.

	Young			Older		
	Caregivers (N = 39)	**Controls (N = 34)**		**Caregivers (N =40)**	**Controls (N =42)**	
	N (%) / Mean (SD)		***p***	**N (%) / Mean (SD)**		***p***
Age (years)	38.7 (4.78)	40.1 (5.44)	.26	69.3 (5.81)	72.4 (5.42)	.06
Age of child/spouse (years)	7.6 (3.63)	7.2 (4.54)	.66	72.3 (8.06)	73.1 (6.04)	.63
Gender (Female)	25 (64)	21 (62)	.84	26 (65)	19 (45)	.07
Marital status (Partnered)	33 (89)	30 (88)	.90	40(100)	42(100)	-
Ethnicity (Caucasian)	36 (97)	27 (79)	.02	38 (97)	39 (93)	.33
Occupational status (non-manual)	30 (88)	31 (94)	.41	22(63)	32 (80)	.10
In full time work	15 (48)	19 (63)	.24	3 (8)	1 (4)	.53
Chronic illness (no)	35 (95)	29 (85)	.19	21 (55)	17 (42)	.26
Taking medications	2 (6)	7 (21)	.06	33 (80)	29 (74)	.51
Alcohol intake (daily or more)	10 (27)	1 (3)	.01	10 (28)	12 (30)	.83

Smokers	5 (14)	2 (6)	.30	1 (3)	3 (7)	.36
Hours of sleep (> 7 hours)	6 (17)	3 (9)	.38	7 (19)	8 (21)	.86
Body Mass Index	25.8 (4.65)	24.5 (3.58)	.21	26.3 (3.08)	26.1 (4.37)	.82
Exercise score	5.2 (4.30)	6.4 (5.44)	.29	3.5 (3.16)	5.2 (3.71)	.04
Fruit/vegetable consumption score	8.6 (2.75)	8.8 (2.36)	.70	9.7 (2.46)	10.3 (2.85)	.31
Fat consumption score	11.5 (2.94)	10.9 (3.72)	.44	9.5 (3.62)	10.2 (3.91)	.46
HADS anxiety score	10.4 (3.85)	6.4 (2.85)	<.001	7.7 (4.96)	4.1 (3.93)	<.001
HADS depression score	8.5 (3.18)	4.2 (3.67)	<.001	6.0 (3.94)	2.8 (2.61)	<.001
Perceived stress score	30.2 (5.68)	23.5 (6.59)	<.001	24.0 (8.21)	16.4 (7.83)	<.001
Social support score (SFS)	31.6 (7.17)	38.6 (9.62)	.001	33.6 (11.18)	-	-
Caregiver burden score (BI)	26.0 (7.82)	13.7 (6.69)	<.001	21.0 (8.62)	-	-
Child behaviour problems (SDQ)	19.6 (4.62)	7.2 (3.99)	<.001	-	-	-
Pearlin problematic behaviour	-	-	-	22.9 (5.89)	-	-
CMV seropositive	13(34)	19 (59)	.04	24 (63)	27 (64)	.92
CMV antibody titre	82.2 (178.75)	158.7 (223.34)	.63	200.2 (261.08)	169.9 (190.07)	.45

4.4.2. Immunostaining for exhausted and senescent profile of T cells

For $CD3^+$ cell numbers, there was no main effect of age, $F(1,140) = 3.08$, $p = .08$, $\eta^2 = .022$, no caregiving * age interaction effect, $F(1,140) = 0.325$, $p = .57$, $\eta^2 = .002$, but there was a main effect of caregiving, $F(1,140) = 7.97$, $p = .005$, $\eta^2 = .054$, such that caregivers had higher numbers (Figure 1B). Subsequent analyses with the covariate adjustment (ethnicity, alcohol intake, and exercise) confirmed this main effect of caregiving ($p = .005$). Therefore, $CD3^+$ cell number was treated as a covariate in subsequent analyses.

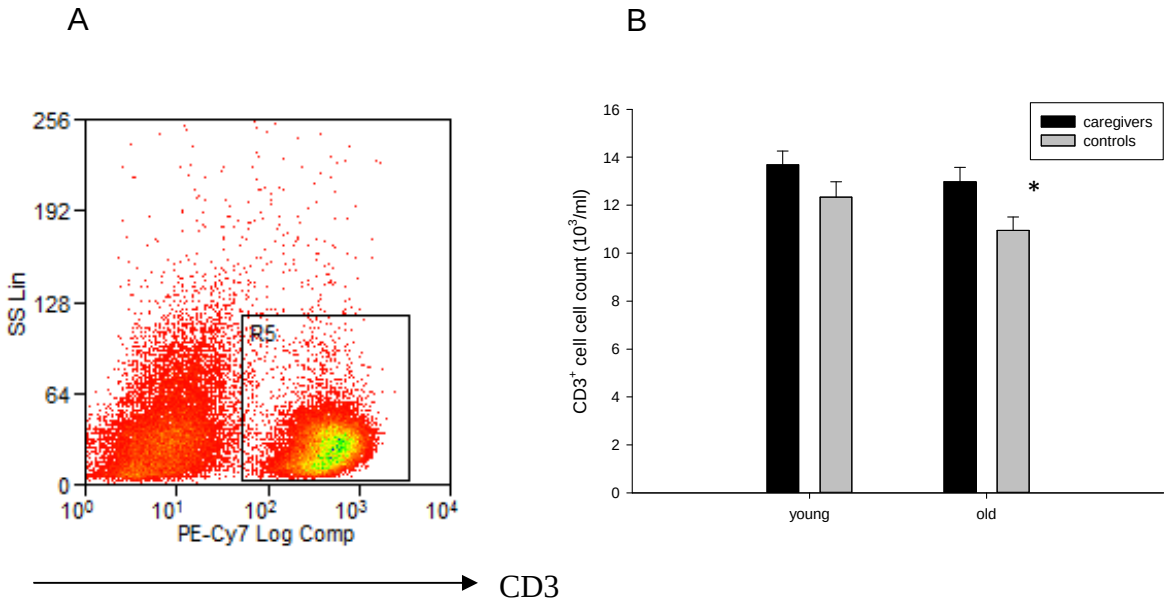

Figure 1. $CD3^+$ cell count. A) Representative flow cytometry scatter plot of $CD3^+$ cells. B) $CD3^+$ cell count in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls.

$CD3^+$T cells that do not express/express the CD28 co-stimulatory molecule

For $CD4^+CD28^-$ T cell count there was no main effect of age, $F(1,140) = 1.37$, $p = .24$, $\eta^2 = .010$, or caregiving, $F(1,140) = 1.16$, $p = .28$, $\eta^2 = .008$, nor a caregiving * age interaction effect, $F(1,140) = 0.56$, $p = .46$, $\eta^2 = .004$ (Figure 2C). Repeated subsequent analyses with the covariate adjustment ($CD3^+$ cell count, ethnicity, alcohol intake and exercise) confirmed this. For the percentage of $CD4^+CD28^-$ T cells, there was no main effect of age, $F(1,140) = 1.05$, $p = .31$, $\eta^2 = .007$, no main effect of caregiving, $F(1,140) = 2.07$, $p = .15$, $\eta^2 = .015$, nor a caregiving * age interaction effect, $F(1,140) = 0.16$, $p = .75$, $\eta^2 = .001$ (Figure 2D). Repeated analyses with the adjustment for covariates did not alter these results.

For $CD8^+CD28^-$ T cell counts there was a main effect of age, $F(1,140) = 12.69$, $p = .001$, $\eta^2 = .083$, such that older participants had higher numbers of these cells, but no main effect of caregiving, $F(1,140) = 1.80$, $p = .18$, $\eta^2 = .013$, nor caregiving * age interaction effect, $F(1,140) = 0.72$, $p = .79$, $\eta^2 = .001$, see Figure 2E. Repeated analyses with adjustment for covariates confirmed the main effect of age ($p < .001$), but revealed the main effect of caregiving, such that caregivers unexpectedly had lower numbers of these cells than controls ($p = .03$). For $CD8^+CD28^-$ T cell frequency there was a main effect of age, $F(1,140) = 22.94$, $p < .001$, $\eta^2 = .141$, and caregiving, $F(1,140) = 5.27$, $p = .02$, $\eta^2 = .036$, such that participants in the older group and controls had a higher percentage of $CD8^+CD28^-$ cells. However, there was no caregiving * age interaction effect, $F(1,140) = 0.36$, $p = .55$, $\eta^2 = .003$. Pairwise comparisons revealed that the difference within the younger group was the main driver of the caregiving effect, Figure 2F. Repeated analyses with adjustment for covariates revealed the same significant main effect of age ($p < .001$), and caregiving ($p = .02$).

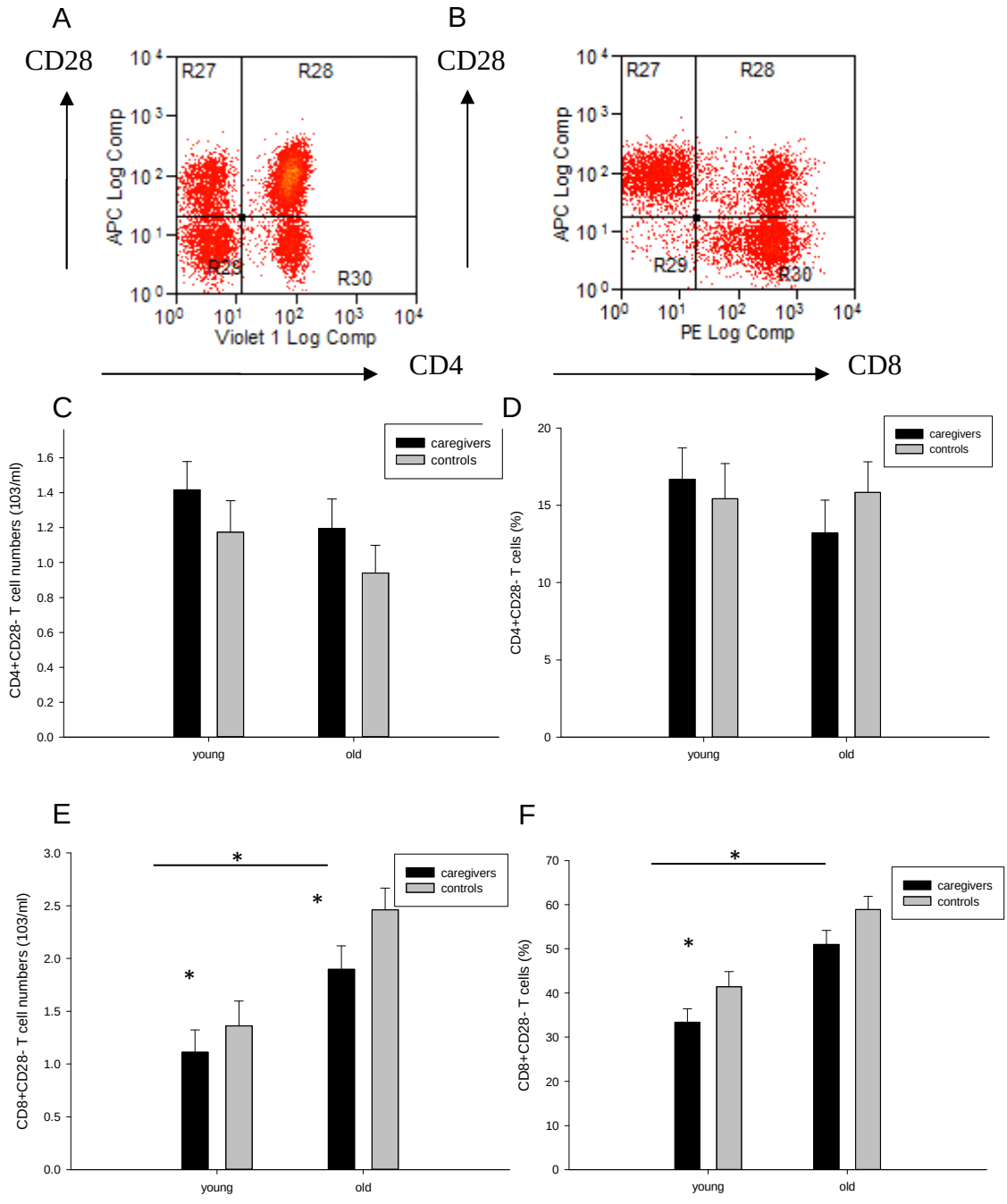

Figure 2. $CD3^+$ cells that do not express CD28 marker. Representative flow cytometric plots demonstrating the gating pattern used for identification of: A) $CD4^+$ and B) $CD8^+$ cells with and without CD28. C) Absolute numbers of $CD4^+$ $CD28^-$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls presented in 10^3/ml. D) Corresponding percentages of these cells in old and young, caregivers and controls. E and F) Absolute numbers (presented in 10^3/ml) and percentage, respectively, of $CD8^+$ $CD28^-$ T cells in old and young, caregivers and controls. Data are presented as mean and SEM of the raw values and * indicates $p < .05$.

For the numbers of $CD3^+$ $CD4^+$ cells that express the CD28 co-stimulatory molecule, there was no main effect of age, $F(1,140) = 0.29$, $p = .59$, $\eta^2 = .002$, but there was a main effect of caregiving, $F(1,140) = 11.55$, $p = .001$, $\eta^2 = .076$, and caregiving * age interaction effect, $F(1,140) = 4.63$, $p = .03$, $\eta^2 = .032$ (Figure 3A). Repeated subsequent analyses with the covariate adjustment revealed only a significant caregiving * age interaction effect ($p = .001$), such that older caregivers had highest numbers. For the percentage of these cells, there was no main effect of age or caregiving, nor caregiving * age interaction effect, $F(1,140) = 0.87$, $p = .35$, $\eta^2 = .006$, $F(1,140) = 1.99$, $p = .16$, $\eta^2 = .014$, $F(1,140) = 1.73$, $p = .19$, $\eta^2 = .012$, respectively, and this was further confirmed after covariate adjustment analyses (Figure 3B).

For $CD8^+$ $CD28^+$ cell numbers, there was a main effect of age, $F(1,140) = 49.79$, $p < .001$, $\eta^2 = .262$, and caregiving, $F(1,140) = 6.61$, $p = .01$, $\eta^2 = .045$, such that younger participants and caregivers had more of these cells, but no caregiving * age interaction effect, $F(1,140) = 0.17$, $p = .90$, $\eta^2 = .000$ (Figure 3C). Repeated subsequent analyses with the covariate adjustment revealed only the main effect of age ($p < .001$). For the percentage of these cells, again main effects of age, $F(1,140) = 35.35$, $p < .001$, $\eta^2 = .202$, and caregiving, $F(1,140) = 10.68$, $p = .001$, $\eta^2 = .071$ were observed, such that younger adults and caregivers had higher percentage of these cells, but no caregiving * age interaction effect, $F(1,140) = 0.74$, $p = .39$, $\eta^2 = .005$ (Figure 3D). Repeated subsequent covariate analyses confirmed both main effects of age and caregiving ($p < .001$ and .03, respectively).

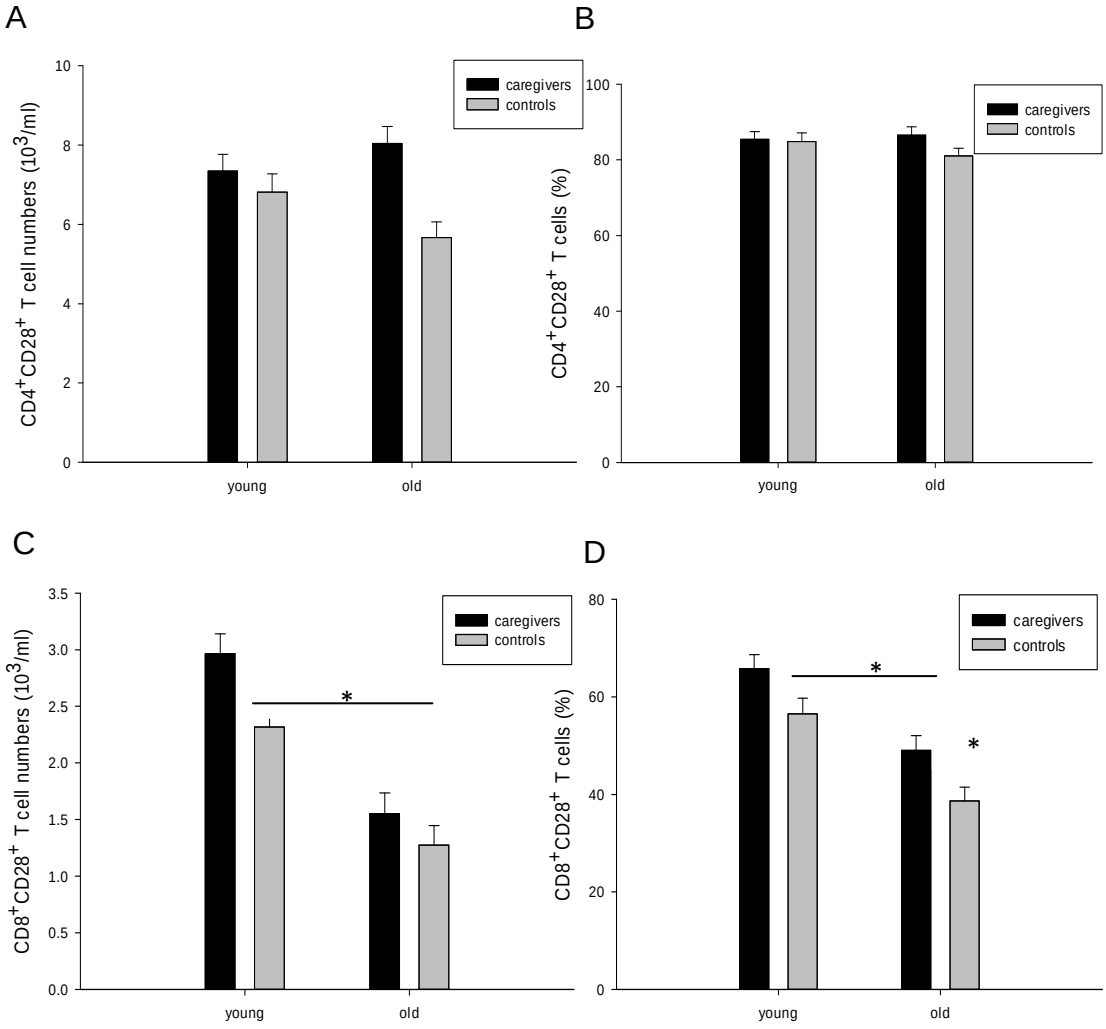

Figure 3. $CD3^+$ cells that express CD28 marker. $CD4^+CD28^+$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls presented in A) Absolute numbers (10^3/ml) and B) percentages. $CD8^+CD28^-$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls presented as C) Absolute numbers (10^3/ml) and D) percentages. Data are presented as mean and SEM, and * indicated $p < .05$.

$CD3^+$ cells that express the CD57 marker of T cell senescence

For the $CD4^+CD57^+$ T cell count, there was a main effect of age, $F(1,140) = 11.86$, $p = .001$, $\eta^2 = .078$, such that older adults had higher numbers of these cells, but no main effect of caregiving, $F(1,140) = 0.09$, $p = .92$, $\eta^2 = .000$, nor caregiving * age interaction effect, $F(1,140) = 0.168$, $p = .68$, $\eta^2 = .001$, as shown in Figure 4C. Repeated ANCOVAs confirmed the main effect of age ($p = .001$). For the $CD4^+CD57^+$ T cell percentage, there was a main effect of age, $F(1,140) = 13.56$, $p < .001$, $\eta^2 = .088$, and caregiving, $F(1,140) = 5.50$, $p = .02$, $\eta^2 = .038$, such that older participants and controls had a higher frequency of $CD4^+CD57^+$ cells, but there was no caregiving * age interaction effect, $F(1,140) = 0.55$, $p = .46$, $\eta^2 = .004$, as shown in Figure 4D. Repeated analyses with adjustment for covariates ($CD3^+$ cell count, alcohol intake, ethnicity and exercise) confirmed only the main effect of age ($p = .003$).

For $CD8^+CD57^+$ T cell numbers, there was a main effect of age, $F(1,140) = 12.69$, $p = .001$, $\eta^2 = .083$, such that participants from the older cohort had higher numbers of these cells, but no main effect of caregiving, $F(1,140) = 1.80$, $p < .18$, $\eta^2 = .013$, nor caregiving * age interaction effect, $F(1,140) = 0.72$, $p = .79$, $\eta^2 = .001$. Repeated covariate analyses confirmed the main effect of age, and revealed a main effect of caregiving ($p < .001$ and .03, respectively), see Figure 4E. For the percentage of these cells, there was a main effect of age, $F(1,140) = 38.70$, $p < .001$, $\eta^2 = .2815$, such that older participants had higher percentage of $CD8^+CD57^+$ cells, but no main effect of caregiving, $F(1,140) = 0.94$, $p = .34$, $\eta^2 = .007$, nor caregiving * age interaction effect, $F(1,140) = 0.91$, $p = .34$, $\eta^2 = .006$ (Figure 4F). Repeated analyses with the adjustment for covariates revealed the same main effect of age ($p < .001$).

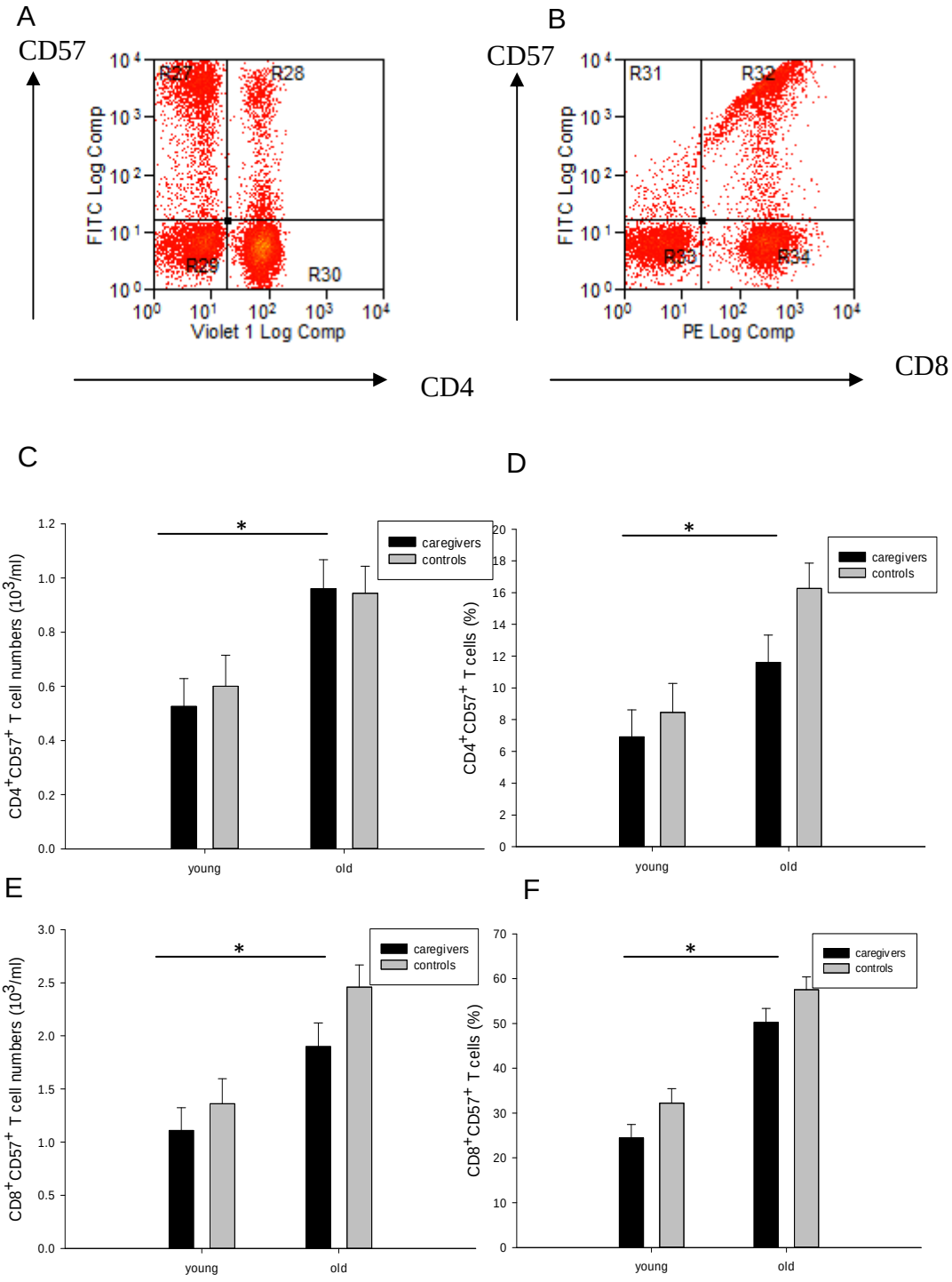

Figure 4. $CD3^+$ cells that express CD57 marker of T cell senescence. Representative flow cytometric plots demonstrating gating pattern for identification of $CD3^+$ A) $CD4^+$ and B) $CD8^+$ $CD57^+$ cells. $CD4^+$ $CD57^+$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 41) and young (n = 31) controls, presented in C) Absolute numbers (10^3/ml) and D) percentages. E and F) present corresponding histograms for $CD8^+CD57^+$ T cells. Data are presented as mean and SEM, and * indicated $p < .05$.

KLRG1^{+}T cells

For CD4^{+}KLRG1^{+} T cell numbers, there was no main effect of age, F(1,140) = 0.14, p = .71, η^2 = .001, nor caregiving * age interaction effect, F(1,140) = 0.06, p = .81, η^2 = .000, but there was a main effect of caregiving, F(1,140) = 6.69, p = .01, η^2 = .047, such that caregivers overall had higher percentage of cells expressing this marker (Figure 5C). Subsequent covariate analyses confirmed these findings (p = .04). For the percentage of CD4^{+}KLRG1^{+} T cells there was no main effect of age, F(1,140) = 1.04, p = .31, η^2 = .007, no effect of caregiving, F(1,140) = 3.15, p = .08, η^2 = .022, and no caregiving * age interaction effect, F(1,140) = 0.94, p = .33, η^2 = .007 (Figure 5D). After covariate adjustment a main effect of caregiving emerged (p = .03) such that caregivers had a higher percentage of these T cells.

For CD8^{+}KLRG1^{+} T cell numbers, there was no main effect of age, F(1,140) = 3.02, p = .08, η^2 = .021, nor caregiving * age interaction effect, but there was a main effect of caregiving, F(1,140) = 6.07, p = .02, η^2 = .042, such that caregivers overall had a higher numbers of these cells. After repeated analyses with covariate adjustment, the main effect of age emerged (p = .03), and the main effect of caregiving was confirmed (p = .04). These details are presented in Figure 5E. For the percentages of these cells, there was a main effect of age, F(1,140) = 5.93, p = .02, η^2 = .041, and caregiving, F(1,140) = 5.36, p = .02, η^2 = .037, with older adults and caregivers having the higher percentage of CD8^{+}KLRG1^{+} T cells, but no caregiving * age interaction effect, F(1,140) = 0.02, p = .87, η^2 = 0.000. These data are presented in Figure 5F. Covariate adjustment confirmed both main effects of age and caregiving (p = .01 and p = .02, respectively).

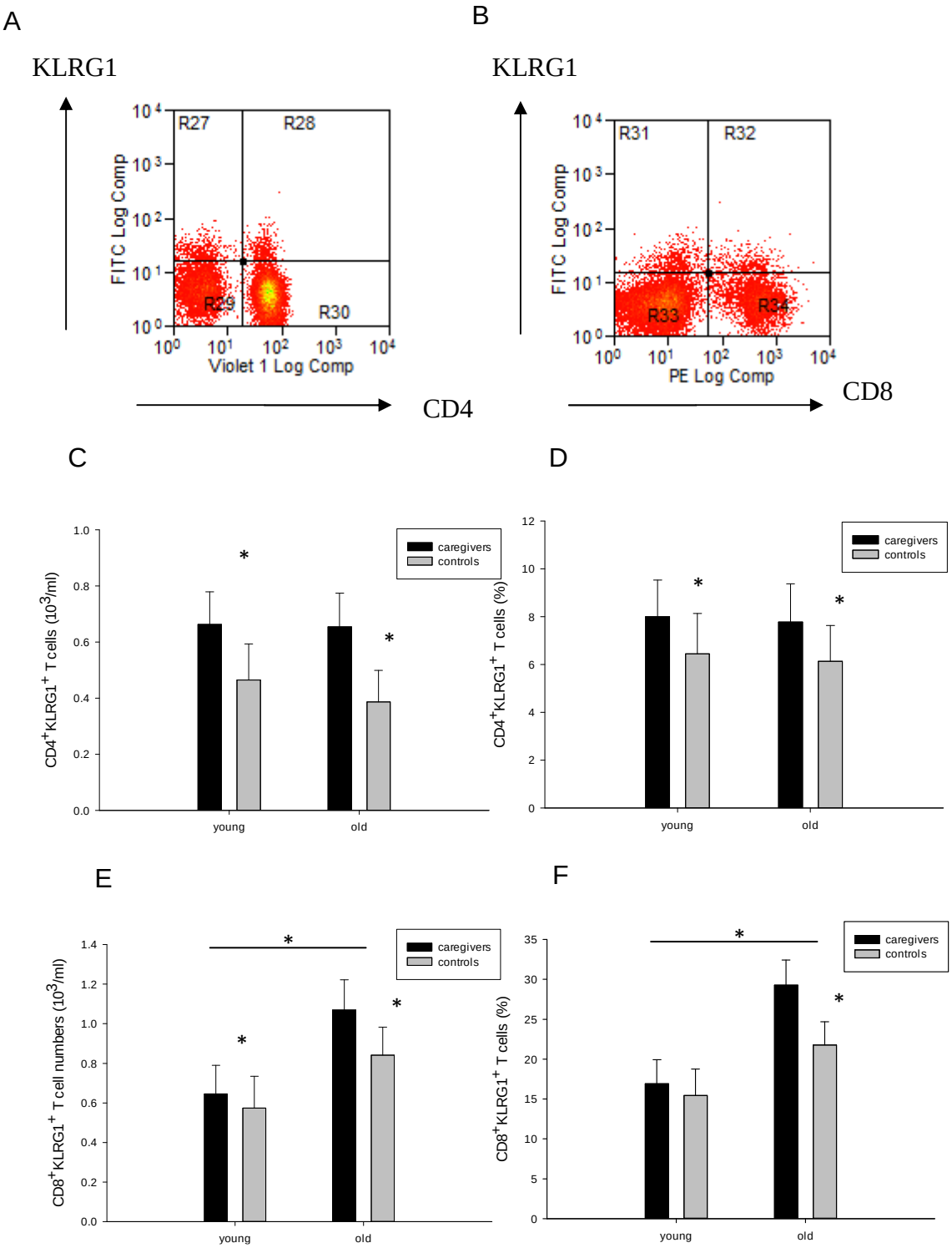

Figure 5. $CD3^+$ cells expressing KLRG1 marker. Representative flow cytometry describing gating method for detecting A) $CD4^+$ and B) $CD8^+KLRG1^+$ within the $CD3^+$ population. $CD4^+$ $KLRG1^+$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls, presented in C) Absolute numbers (10^3/ml) and D) percentages. Under E and F are respective presentations of $CD8^+KLRG1^+$ T cells. Data are mean ± SEM and * indicates $p < .05$.

T cells expressing PD-1 marker of exhaustion

For $CD4^+PD1^+$ T cell count, there was a main effect of age, $F(1,140) = 16.01$, $p < .001$, $\eta^2 = .103$, such that the older group had more of these cells, and a caregiving * age interaction effect, $F(1,140) = 6.30$, $p = .01$, $\eta^2 = .043$, such that older caregivers had the highest number of these cells, but no main effect of caregiving, $F(1,140) = 0.05$, $p = .83$, $\eta^2 = .000$. Subsequent covariate analyses confirmed both main effects of age and caregiving * age interaction effect ($p < .001$ and .04, respectively), see Figure 6C. For the percentage, there was a main effect of age, $F(1,140) = 22.45$, $p < .001$, $\eta^2 = .138$, such that younger participants had lower percentage of T cells expressing this marker of exhaustion, but no main effect of caregiving, $F(1,140) = 1.93$, $p = .17$, $\eta^2 = .014$, nor a caregiving * age interaction effect, $F(1,140) = 0.38$, $p = .54$, $\eta^2 = .003$, as shown in Figure 6D. Repeated analysis after adjusting for covariates revealed the same age effect ($p < .001$).

For the number of $CD8^+PD1^+$ T cells, there was no main effect of age, $F(1,140) = 2.79$, $p = .10$, $\eta^2 = .020$, no main effect of caregiving, $F(1,140) = 0.32$, $p = .57$, $\eta^2 = .002$, nor caregiving * age interaction effect, $F(1,140) = 0.004$, $p = .95$, $\eta^2 = .000$, but after subsequent covariate analyses the main effect of age emerged ($p = .02$). These data are presented in Figure 6E. For the frequency of these cells, there was a main effect of age, $F(1,140) = 17.72$, $p < .001$, $\eta^2 = .112$, such that older adults had higher percentage of T cells expressing this marker of exhaustion, but no main effect of caregiving, $F(1,140) = 0.05$, $p = .83$, $\eta^2 = .000$, nor caregiving * age interaction effect, $F(1,140)= 0.68$, $p= .41$, $\eta^2 = .005$. These effects are shown in Figure 6F. Subsequent covariate analyses revealed the same age effect ($p < .001$).

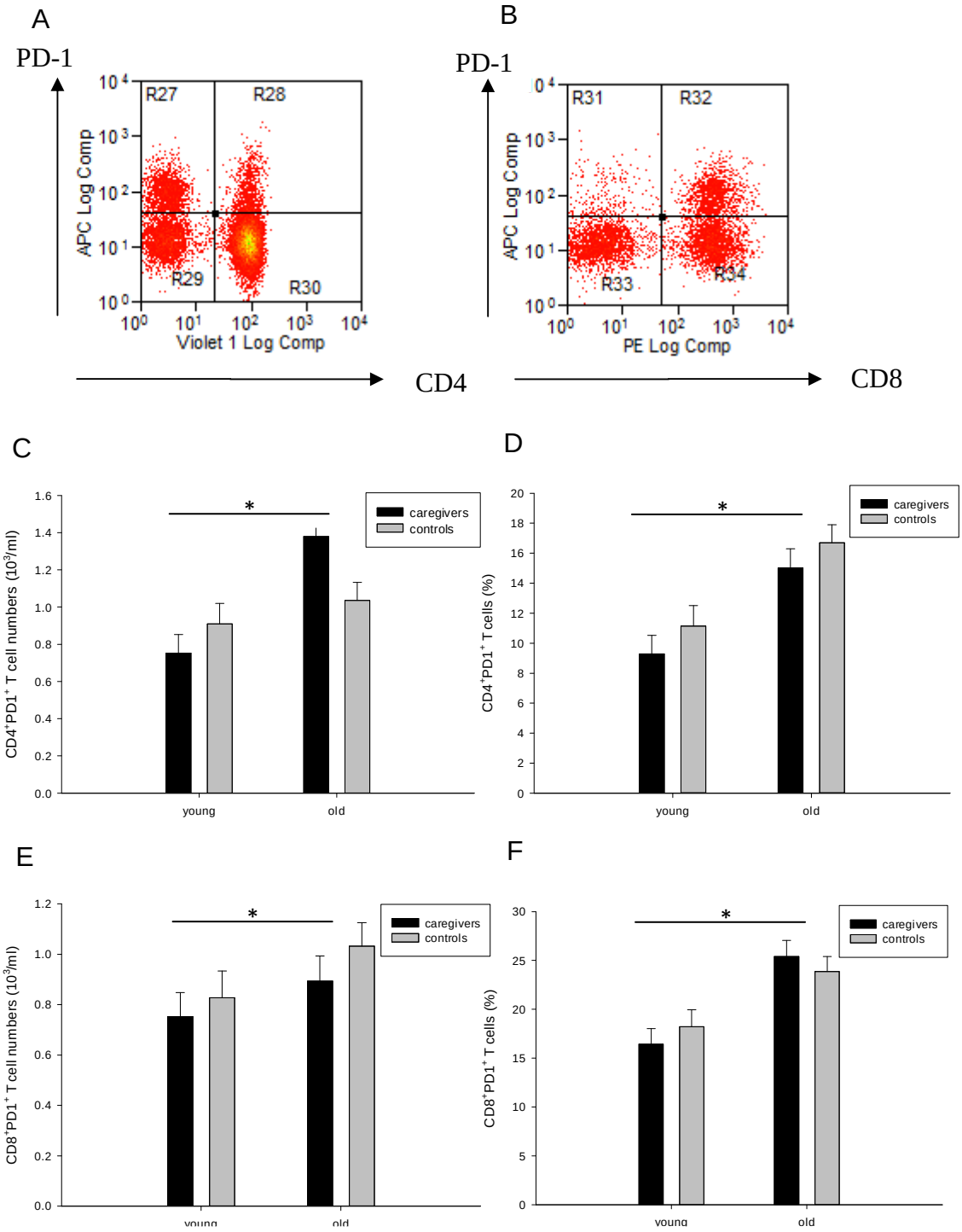

Figure 6. $CD3^+$ cells expressing PD-1 marker of exhaustion. Percentage of T cells showing the expression of PD-1 marker of exhaustion. Representative flow cytometry plot showing gating strategy for detection of A) $CD4^+PD\text{-}1^+$ and B) $CD8^+PD\text{-}1^+$ cells within $CD3^+$ lymphocyte subset. $CD4^+$ $PD1^+$ T cells in old (n = 35) and young (n = 38) caregivers, and old (n = 40) and young (n = 31) controls, presented as C) Absolute numbers (10^3/ml) and D) percentages. E and F present corresponding values for $CD8^+PD\text{-}1^+$ T cells. Data are mean ± SEM, and * indicates $p < .05$.

TREC levels

There was a main effect of age on TREC levels, $F(1,139) = 109.82$, $p < .001$, $\eta^2 = .441$, such that older participants had greater fold decrease of TREC when compared to the calibrator. There was no main effect of caregiving, $F(1,139) = 3.25$, $p = .07$, $\eta^2 = .023$, nor was there caregiving * age interaction effect, $F(1,139) = 1.48$, $p = .23$, $\eta^2 = .011$ (Figure 7). Repeated analyses with covariate adjustment revealed the main effect of age ($p < .001$), but the trend for a caregiving effect disappeared completely ($p = .30$).

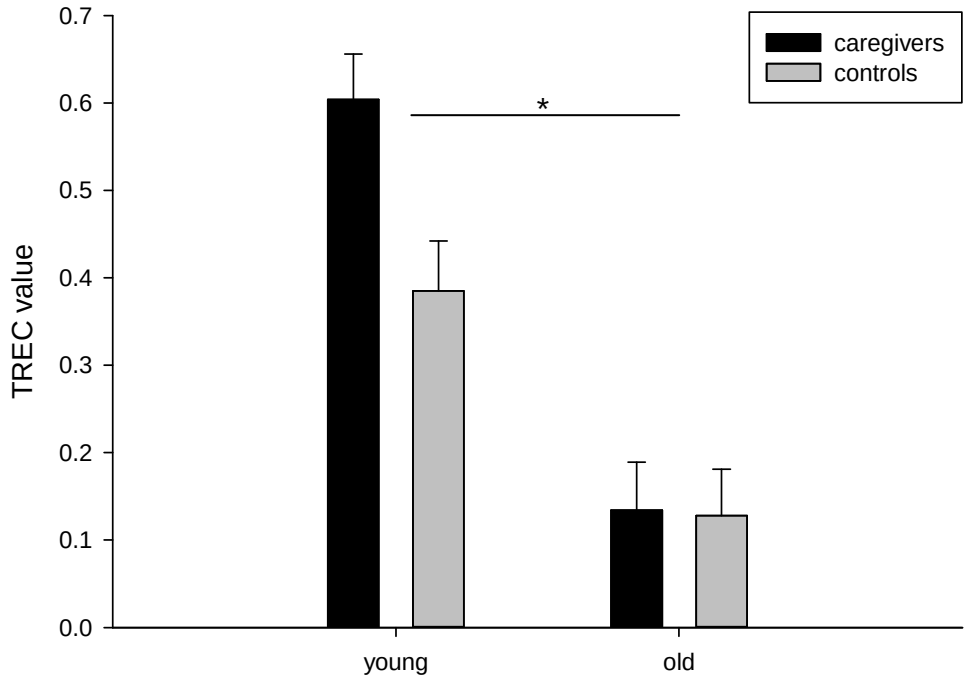

Figure 7. Thymic output presented as TREC expression ratio. Percentage of TREC expression ratio in old (n = 35) and young (n = 39) caregivers, and old (n = 37) and young (n = 32) controls. Data are mean ± SEM and * indicates $p < .05$.

CMV seropositivity and serum antibody titre

Overall, 83 (55%) of subjects were CMV positive, with the number of CMV positive being higher in the younger group, $\chi^2(1) = 4.91$, $p = .03$. In the younger group, 32 (86%) were seropositive overall, with caregivers being less likely to be CMV positive, $\chi^2(1) = 4.43$, $p = .04$. In the older group 51 (64%) participants were CMV positive, and the presence of the CMV infection was similar between older caregivers and controls, $\chi^2(1) = .01$, $p = .92$. Within the CMV positive subset (N = 82), for CMV antibody titre, there was no main effect of age, $F(1,78) = 2.00$, $p = .16$, $\eta^2 = .025$, caregiving, $F(1,78) = 0.66$, $p = .42$, $\eta^2 = .008$, nor caregiving * age interaction, $F(1,78) = 0.21$, $p = .65$, $\eta^2 = .003$. Covariate analysis did not alter these results.

Further, the analyses regarding T cell subsets expressing markers of senescence and exhaustion were re-run controlling for CMV serostatus as a covariate to determine if any of the effects were driven by CMV infection. All main effects of age reported previously remained the same, while the caregiving effect for $CD8^+CD28^-$ ($p = .20$), $CD8^+CD28^+$ ($p = .17$), as well as $CD8^+CD57^+$ ($p = .20$), disappeared. On the other hand, the main effect of caregiving for T cells expressing the KLRG1 marker remained significant for both $CD4^+$ ($p = .01$) and $CD8^+$ ($p = .02$) T cells.

Finally, median CMV titre was used for splitting CMV seropositive subjects into two groups with high versus low serological responses, and the comparison of T cell immunity parameters between the groups are presented in Table 2. Interestingly, the groups were significantly different on almost all immune parameters with the exclusion of $CD8^+$ T cells expressing the PD-1 marker of exhaustion.

Table 2. T cell immunity parameters between CMV seropositive participants with high and low CMV titre, created using median split of CMV titre.

	High CMV titre	Low CMV titre	
	Mean (SD)		*p*
$CD3^+CD4^+CD28^-$ cells (number x 10^3/ml)	1.3 (1.23)	1.1 (0.73)	.07
$CD3^+CD4^+CD28^-$ cells (%)	13.5 (12.52)	6.1 (7.39)	< .001
$CD3^+CD8^+CD28^-$ cells (number x 10^3/ml)	2.5 (1.56)	1.0 (0.76)	< .001
$CD3^+CD8^+CD28^-$ cells (%)	57.6 (18.39)	35.9 (18.23)	< .001
$CD3^+CD4^+CD28^+$cells (number x 10^3/ml)	6.2 (2.67)	7.7 (2.52)	.001
$CD3^+CD4^+CD28^+$ cells (%)	80.1 (15.56)	88.3 (8.04)	.001
$CD3^+CD8^+CD28^+$ cells (number x 10^3/ml)	1.9 (1.37)	2.1 (1.2)	.12
$CD3^+CD8^+CD28^+$ cells (%)	42.3 (19.07)	61.5 (17.26)	< .001
$CD3^+CD4^+CD57^+$cells (number x 10^3/ml)	1.0 (0.71)	0.5 (0.47)	< .001
$CD3^+CD4^+CD57^+$ cells (%)	15.8 (12.11)	6.5 (6.48)	< .001
$CD3^+CD8^+CD57^+$ cells (number x 10^3/ml)	2.5 (1.56)	1.0 (0.76)	< .001
$CD3^+CD8^+CD57^+$ cells (%)	53.5 (20.76)	29.9 (18.00)	< .001
$CD3^+CD4^+KLRG1^+$cells (number x 10^3/ml)	0.6 (0.59)	0.5 (0.83)	.01
$CD3^+CD4^+KLRG1^+$ cells (%)	8.5 (8.61)	5.7 (9.95)	< .001
$CD3^+CD8^+KLRG1^+$ cells (number x 10^3/ml)	1.0 (1.14)	0.6 (0.56)	.03
$CD3^+CD8^+KLRG1^+$ cells (%)	23.5 (21.59)	18.4 (16.12)	.43
$CD3^+CD4^+PD1^+$cells (number x 10^3/ml)	1.2 (0.81)	0.9 (0.39)	.01
$CD3^+CD4^+PD1^+$ cells (%)	16.1 (9.51)	10.3 (5.17)	< .001

$CD3^+CD8^+PD1^+$ cells (number x 10^3/ml)	1.0 (0.72)	0.8 (0.43)	.25
$CD3^+CD8^+PD1^+$ cells (%)	20.0 (11.27)	21.9 (9.4)	.10
TREC expression ratio	0.3 (0.34)	.4 (.41)	.03

Psychological factors and immune parameters within caregivers in each group

Within the caregiving group overall, analyses showed that caregivers who reported higher anxiety, depression, and stress levels, had lower percentage of $CD57^+$ for both $CD4^+$ and $CD8^+$, and $CD4^+PD\text{-}1^+$ T cells, as well as higher expression ratio of TREC, see Table 3. In addition, those with a higher burden index had a lower percentage of $CD4^+$ T cells expressing PD-1 marker of exhaustion, as well as higher TREC expression ratio, Table 3. Analysis within the caregiving participants in the younger group showed that caregivers who reported higher depression, anxiety, perceived stress, and burden levels, as well as more problematic behaviour in their child(ren), had a higher TREC expression ratio, detailed in Table 4. In the older group, caregivers who reported higher problematic behaviour in their spouses/partners had higher percentage of $CD8^+CD57^+$ T cells, see Table 4.

Table 3. Correlations between the T cell immunity and caregivers' psychosocial characteristics.

	Caregivers overall (N=73)			
	$CD4^+CD57^+$ (%)	$CD8^+CD57^+$ (%)	$CD4^+PD\text{-}1^+$ (%)	TREC
HADS anxiety	r(68) = -.25, *p* = .04	ns	r(68) = -.24, *p* = .05	r(69) = .31, *p* = .01
HADS depression	r(68) = -.26, *p* = .03	r(68) = -.24, *p* = .04	r(68) = -.30, *p* = .01	r(69) = .38, *p* = .001
Perceived stress (PSS)	r(68) = -.30, *p* = .01	r(68) = -.31, *p* = .01	r(68) = -.35, *p* = .003	r(69) = .35, *p* = .003
Caregiver burden score (BI)	ns	ns	r(68) = -.25, *p* = .03	r(69) = .32, *p* = .01

*ns = non-significant correlation

Table 4. Correlations between the T cell immunity and young/old caregivers' psychosocial characteristics.

	Young (N=38)	**Old (N=35)**
	TREC	$CD8^+CD57^+$
HADS anxiety	r(34) = -.34, *p* = .04	ns
HADS depression	r(35) = -.53, *p* = .001	ns
Perceived stress	r(35) = -.52, *p* = .001	ns
Caregiver burden score (BI)	r(35) = .44, *p* = .01	ns
Child behaviour problems (SDQ)	r(35) = .35, *p* = .03	-
Pearlin problematic behaviour	-	r(32) = .42, *p* = .02

*ns = non-significant correlation

4.5. DISCUSSION

Psychosocial measures were significantly different and in the expected direction between the caregivers and controls in both young and old participants. There was an age-related increase in the absolute number and frequency of the $CD8^+$ $CD28^-$ subset but caregivers unexpectedly showed lower numbers of these cells than age- and sex-matched controls, with younger caregivers also having a lower frequency of these cells than controls. $CD8^+CD28^+$ T cells predictably showed age-related changes in the opposite direction than $CD8^+CD28^-$ T cells, and a caregiving effect in favour of caregivers. While age related differences were in agreement with previous reports (Fagnoni et al. 1996, Vallejo et al. 1999, Boucher et al. 1998), the apparent beneficial effect of caregiving effect is unexpected and novel. The negative impact of $CD28^-$ $CD8^+$ T cells on immunity is related to their loss of proliferative capacity due to the loss of the co-stimulatory signal from CD28 (Vallejo 2005) and resulting decreased tolerance (Bour-Jordan et al. 2011). The high percentages of CMV and EBV specific $CD8^+CD28^-$ T cells in older adults (Weng et al. 2009) are also thought to compromise immunity by occupying immunological space leaving limited room for other T cell subsets to expand when new pathogens are encountered (Pawelec et al. 2005). Accumulation of $CD28^-$ T cells with age has been attributed to repeated antigen stimulation, as T cell activation induces decreases in CD28 expression, through lifelong exposure to pathogens and the constant stimulation by chronic viral infections such as CMV, reviewed in (Vallejo 2005). This is further confirmed by the increase in $CD8^+CD28^-$ T cells with poor proliferative capacity in advanced HIV infection (Effros et al. 1996). The present findings confirmed significantly higher numbers of these cells in the CMV positive participants with high CMV antibody titres, but with caregivers having lower incidence of CMV infection.

Therefore, a possible explanation for the lower levels of $CD8^+CD28^-$ T cells in young caregivers is the significantly lower incidence of chronic CMV infection when compared to the young controls, which would reduce the overall infectious burden responsible for expansion of the $CD8^+CD28^-$ T cell subset (Weng et al. 2009, Kern et al. 1996) . Additional confirmation for this was gained in the repeated analyses using CMV status as covariate where the caregiving effect disappeared, while age remained significant.

$CD57^+$ cells in both $CD4^+$ and $CD8^+$ subset of T cells were more abundant in the older sample, as previously reported (Merino et al. 1998, Bandres et al. 2000), and this has been related to limited proliferative capacity and 'clonal exhaustion' (Wood et al. 2009, Brenchley et al. 2003). On the other hand, the preserved T cell profile in case of $CD8^+CD57^+$ cells, and lower percentages of $CD4^+CD57^+$ cells, shown among older caregivers was unexpected. However, increased $CD57^+$ cells in the elderly have previously been related to CMV infection (Pourgheysari et al. 2007). Although there was no difference in serum CMV antibody titre between caregivers and controls amongst older adults in the present study, the percentage of $CD4^+CD57^+$ T cells significantly positively correlated with levels of anti-CMV antibodies (data presented in Appendix, Additional findings from chapter 4), which could explain the unexpected increase in the percentage of these cells amongst older controls. This is further confirmed by the significantly higher numbers of these cells in CMV seropositive participants who had a relatively higher CMV antibody titre. In addition, repeated analyses adjusting for CMV status eliminated this caregiving effect. On the other hand, the increase in $CD57^+$ cells in both $CD4^+$ and $CD8^+$ subsets, although more prominent in the latter, has been shown to occur after acute exercise which mobilised these terminally differentiated highly cytotoxic cells in both young and old (Campbell et al. 2012, Simpson et al. 2007,

Simpson et al. 2008). Thus, perhaps the low percentages of these cells in caregivers in the current sample are a dampening of the numbers of cells with high killing potential due to the chronic stress of caregiving. This decrease in $CD57^+$ cells has been previously reported in some chronic diseases, such as chronic Lyme's disease, where the increase in $CD57^+$ levels relate to improved symptoms (Stricker and Winger 2001). Finally, it has been argued that cells expressing the CD57 marker and/or lacking CD28 represent only terminally differentiated effector T cells with high cytotoxic potential, which then apoptose as a result of their activation, and are therefore not a marker of immune deficiency (Kern et al. 1996).

KLRG1 was increasingly expressed on $CD8^+$ T cells from the present older participants, confirming previous findings (Henson et al. 2009). This is not surprising, as this marker of terminally differentiated T cells is associated with replicative senescence and inhibits cell proliferation (Henson et al. 2009, Henson and Akbar 2009). In addition, KLRG1 is known to down-regulate CD95-mediated cell lysis and, in certain cases, inhibit T cell activation (Rosshart et al. 2008). Interestingly, blockade of this inhibitory marker can restore proliferative function, which indicates the potential involvement of KLRG1 in exhaustion-related pathways (Henson et al. 2009, Akbar and Henson 2011). KLRG1 was also shown to be increasingly expressed on both $CD4^+$ and $CD8^+$ T cells in caregivers when compared to controls, indicating the possibility that chronic caregiving stress can affect the functionality of the T cell immunity. This higher percentage of functionally impaired T cells rich in inhibitory receptors such as KLRG1 is what may explain the poorer vaccination response observed in caregivers (Kiecolt-Glaser et al. 1996, Gallagher et al. 2009a, Vedhara et al. 1999). This is likely as it has been shown that KLRG1 prevents the response of $CD4^+$ T cells to novel antigens (Shi et al. 2014) necessary for

achieving adequate immune protection following vaccination. Further support for this target specific effect of caregiving stress that could explain the higher percentage of T cells expressing this senescence marker in caregivers regardless of their age comes from studies that suggest changes in the methylation profile of DNA, the epigenome, due to stressful life experiences. There is now a growing body of evidence that stress, from prenatal to adult social stress, through epigenetic changes influences not only behaviour and stress responses (Gudsnuk and Champagne 2012), but also, via the HPA axis, has an impact on immune-related genes (Uddin et al. 2010). A recent study compared people with and without posttraumatic stress disorder associated with changes in DNA methylation profiles and gene expression including KLRG1 (Uddin et al. 2010), potentially compromising the individual's immunity (Uddin et al. 2010).

Significant age effects were observed for both $CD4^{+}PD\text{-}1^{+}$ and $CD8^{+}PD\text{-}1^{+}$ T cells, with older adults having a higher percentage of both of these subsets, consistent with some previous research (Lages et al. 2010, Dolfi et al. 2013), reviewed in (Lefebvre and Haynes 2012), but not all (Canaday et al. 2013). Chronic caregiving stress did not appear to affect PD-1 expression as caregivers in the current sample showed a T cell exhaustion profile comparable to that of the controls. Therefore, it seems that although physical stress, such as laparoscopic-assisted surgery, can induce an increase in PD-1 expression on both $CD4^{+}$ and $CD8^{+}$ T lymphocytes (Arai et al. 2012), caregiving stress may not affect this aspect of immunity.

The significant age effect on TREC expression was in agreement with previous research indicating a decrease in thymic output and production of naive T cells with age (Naylor et al. 2005, Betjes et al. 2011, Mitchell et al. 2010). However, there was no negative effect of caregiving stress observed for this immune parameter; if anything, caregivers

showed a trend towards higher thymic output than controls, largely influenced by the younger group.

A higher percentage of CMV positive individuals in the younger group was not expected, and is higher than previously reported for an equivalent age group (Dowd et al. 2009, Staras et al. 2006). However, this could be due to specifically targeting parents, rather than younger adults *per se*, who may have more exposure to CMV through contact with their children whose infection rates can be high due to day-care centre attendance (Adler 1988). Interestingly, comparison between CMV seropositive participants with high and low serological profiles revealed group differences in almost all T cell immunity parameters with the exception of $CD8^+$ T cells expressing the PD-1 marker of exhaustion. This is in agreement with previously reported data that suggest the role of CMV in driving T cells towards senescent or exhaustion profiles through repeated stimulation of T cells in an attempt to control the infection (Almanzar et al. 2005, Chou and Effros 2013).

Higher psychological morbidity was related to lower levels of some immunesenescence markers and higher TREC, which seems counterintuitive. However, it appears that this overall effect results from the higher psychological morbidity scores in the younger caregivers group, that, due to their age, also exhibit the expected lower or absent immunosenescence shown in terms of T cell marker expression and output of naïve T cells. This is further confirmed by the direct relationship between higher problem behaviours in care-recipients and higher percentage $CD8^+CD57^+$ T cells amongst the older caregivers, which suggests that, for older caregivers at least, future research into caregiving should focus on those with higher psychological morbidity.

Overall, the results presented in this chapter provide a support for a mixed picture of the effect of caregiving stress on T cell phenotype in young and old, with the KLRG1 marker of cell senescence being the main responder to caregiving stress. These data also indicate that the contribution of caregiving stress to immunesenescence is not uniform across T cell functional markers. The KLRG1 result may indicate that a prominent negative effect of caregiving stress in both young and old would be seen during active immune stimulation/challenge *in vivo* of the adaptive immune system such as vaccination (Gallagher et al. 2009a, Gallagher et al. 2009b, Glaser et al. 2000, Kiecolt-Glaser et al. 1995, Vedhara et al. 1999). Caregiving stress in these situations may prevent the immune response from a competent reaction and adequate protection, namely a clinically relevant increase in antibody titre. Thus, the higher percentage of $KLRG1^{-}$ T cells in caregivers and controls presents a potential mechanism for the poorer vaccination response previously reported in this stressed group (Shi et al. 2014).

The study has a number of limitations. First, the sample size could be regarded as small, yet it is of the same magnitude as in previously reported caregiving studies (Gallagher et al. 2009a, Gallagher et al. 2009b, Vedhara et al. 1999, Vedhara et al. 2002), and therefore large enough to determine medium effect size reported by the previous studies. Second, one could argue that requirements for the participation in the study biased the sample towards those caregivers with lower burden and stress levels overall and therefore immune integrity. However, attempts were made to avoid this by providing the opportunity of home visits for participants. In addition, scores on psychosocial scales were comparable if not higher to those previously reported by other caregiving research, suggesting that the present caregivers did have high burden and stress levels. Finally, conducting staining on frozen and then thawed PBMCs might give different results to

those that arise from the work on fresh PBMCs, but as all the samples were treated in the same way from the moment of blood sampling until the acquisition of the data on flow cytometer, the comparison between groups would not be affected by this.

In conclusion, caregivers showed poorer KLRG1 T cell profiles compared to controls, but other markers of T cell-related immunesenescence and exhaustion did not differ between the groups. This KLRG1 effect could explain previous caregiving effects on vaccination response and suggest KLRG1 as a target for future intervention. Further, among older caregivers, those who reported higher behavioural problems in care recipients showed a greater senescent profile. These findings underline the importance of considering individual differences in the impact of caregiving stress on immunity, and that future research should focus on those who report higher psychological morbidity.

CHAPTER 5

BEREAVEMENT STRESS AND NEUTROPHIL FUNCTION IN YOUNG AND OLDER ADULTS: ROLE OF THE HPA AXIS AND IMMUNESENESCENCE

5.1. ABSTRACT

The effect of the chronic stress of bereavement on immunity is poorly understood. Previous studies have demonstrated negative effects on immunity in older adults, and those who report higher depressive symptoms. The aim of the present study was to compare the effect of bereavement on neutrophil function in healthy young and old adults, also assessing serum levels of the stress hormones, cortisol and DHEAS. 41 young (mean age 32 years) and 52 older adults (mean age 72 years), bereaved and non-bereaved, took part in the study. They completed questionnaires on socio-demographic and health behaviour characteristics, as well as psychosocial variables, and provided a blood sample for analysis of neutrophil function (phagocytosis and ROS production) and stress hormone analysis. Bereaved participants in both age groups reported more symptoms of depression and anxiety than controls and scored moderately highly on bereavement-specific questionnaires for these symptoms. Despite this, young bereaved participants showed robust neutrophil function when compared to age-matched non-bereaved controls, and comparable stress hormone levels, while reduced neutrophil ROS production and raised stress hormone levels (cortisol:DHEAS ratio) were seen in the older bereaved group compared to their age-matched controls. Reduced neutrophil function among older bereaved participants may be the result of the inability to maintain stress hormone balance, specifically the cortisol:DHEAS ratio.

5.2. INTRODUCTION

Bereavement is a stressful life event often accompanied by grief after the loss of someone close (Stroebe and Stroebe 1987) and, as such, has numerous consequences for physical and mental health (Parkes 1964). In addition to the increase in morbidity and mortality associated with bereavement in older adults (Biondi and Picardi 1996, Kaprio et al. 1987, Stroebe et al. 2007), particularly in the case of the unexpected death (Shah et al. 2013), bereavement has been shown to have a number of adverse effects on immunity (Adrianopoulos and Flaherty 1991). For example, bereavement in the year prior to vaccination related to lower antibody responses to two different influenza strains in older adults (mean age 75 years) (Phillips et al. 2006a), and decreased lymphocyte response to PHA (Bartrop et al. 1977). On the molecular level, the expression of the genes specifically involved in B cell immunity was down-regulated in bereaved older adults (aged 61-83 years) when compared to age- and sex-matched controls (O'Connor et al. 2014). Bereaved parents aged 38-61 years experienced a decrease and increase in the number of regulatory and helper T cells, respectively, compared to matched controls after the sudden and unexpected death of their child (Spratt and Denney 1991). In terms of the innate immune response, bereaved middle aged female spouses aged 57.1 ± 7.9 years (mean ± SD) had a poorer NK cell cytotoxic activity when compared to gender matched controls (Irwin et al. 1987), and neutrophil ROS production was lower in bereaved older adults (mean age 72 years) when compared to the age- and sex-matched non-bereaved participants (Khanfer et al. 2011). In contrast, a group of middle aged widows (mean age 56 years) showed preserved immune response compared to non-bereaved controls (Zisook et al. 1994). However, within the bereaved group, those with depressive symptoms had lower NK cell activity and response to mitogen stimulation than those without (Zisook et al. 1994).

Previous studies of the impact of physical stress (e.g. hip fracture) have shown that impaired immune function, specifically neutrophil ROS production was only seen among older adults with concomitant immunosenescence and did not occur in young patients with a similar level of trauma (Butcher et al. 2003). Importantly in this study HPA axis activity, specifically a raised cortisol:DHEAs ratio was highest in those patients with the lowest neutrophil ROS production and also lower in patients who developed infection. Further, a subsequent study revealed that reduced ROS production and a higher cortisol:DHEAS was observed only in those hip fracture patients with depressive symptoms when compared to both those patients without depression and healthy age-matched controls (Duggal et al. 2013a). These data suggest that the effects of some types of stress on immunity may only be observed among older adults, or among those with poorer psychological status, e.g., high depressive symptoms.

Stress activates the HPA axis and subsequently induces the secretion of cortisol, a hormone with immune suppressive effects (Charmandari et al. 2005). DHEAS, also secreted by the adrenal gland in response to stress, is considered to be immune-enhancing (Phillips et al. 2007). Whilst cortisol has been shown to decrease the adhesion and increase mobility of the neutrophils (Davis et al. 1991, Zak-Nejmark et al. 1998), DHEAS increased neutrophil ROS production *in vitro* (Radford et al. 2010). An imbalance between these two hormones, i.e., a high cortisol:DHEAS ratio can arise in response to stress (Boudarene et al. 2002, McCraty et al. 1998) and have negative implications for immunity including increase risk of bacterial infection (Butcher et al. 2005). Further, our previous research in older adults showed a higher cortisol:DHEAS ratio in bereaved participants when compared to age- and sex-matched controls (Khanfer et al. 2011). Indeed, with ageing, levels of DHEAS decline whereas cortisol continues to be produced, termed adrenopause (Orentreich et al. 1984), thus resulting

in a higher cortisol:DHEAS ratio. Whether the same increased stress hormone ratio and associated reduction in neutrophil function would be observed in younger adults suffering the stress of bereavement is not known.

Consequently, the present study sought to extend our previous research which showed reduced neutrophil function in older bereaved adults (Khanfer et al. 2011). Specifically, it compared neutrophil function and the cortisol:DHEAS ratio in four groups of participants: younger bereaved adults; non-bereaved young participants; older bereaved adults and non-bereaved age-matched controls.

5.3. METHODS

5.3.1. Participants

21 young bereaved adults and 20 age- and sex-matched non-bereaved controls, as well as 26 older bereaved adults and 26 controls participated in the study. Recruitment was conducted mainly via local advertisements and the Bereavement Care Centre, Queen Elizabeth Hospital, Birmingham. The bereaved group comprised participants who suffered bereavement in the past two months. None of the participants suffered from a chronic immune disorder or acute infection, and none were taking immunosuppressive medication.

5.3.2. Study design and procedure

Participants attended a morning testing session where they completed a questionnaire pack and provided a blood sample. Informed written consent was obtained, and the study was approved by the local Ethics Committee.

5.3.3. Questionnaires

Groups were compared on general socio-demographic variables, as well as health behaviours. The latter were assessed using an adaptation of the Whitehall II study questionnaire (Marmot et al. 1991). HADS (Zigmond and Snaith 1983), was used to determine depression and anxiety symptoms in all participants, and the Cronbach's alpha in the present study was .86 for anxiety and .80 for depression. The availability of social support was examined using the MOS Social Support Survey (Sherbourne and Stewart 1991). The Cronbach's alpha in the current sample was .96.

Bereaved participants were asked about their recent bereavement using the Core Bereavement Items questionnaire (CBI, (Burnett et al. 1997)), and the Impact of Event Scale (IES, (Horowitz et al. 1979)). The CBI assesses the feelings of the bereaved on a 4-point scale from 0 - *never*, to 3 - *continuously* happening. An example of a typical item is 'Do reminders of this person such as photos, situations etc. cause you to feel loneliness'. Previously used in bereavement research (Tolstikova et al. 2005, Holland et al. 2013), the scale showed good internal consistency at .91; and .94 in the present study. The IES asks about frequency of feelings about the bereavement (e.g. how frequently 'you had dreams about it'), with higher scores meaning higher negative impact. The scale shows good internal consistency (.79-.92) (Corcoran and Fischer 1994); and .89 in the current sample. They were also asked who the deceased person was in relation to them, and whether the death was expected or not.

5.3.4. Blood sampling and assays

Venous blood was collected in the morning, between 9-11a.m., from the ante-cubital vein into two 6ml tubes (BD Diagnostics, Oxford, UK), one with heparin for neutrophil functional

assessment, and one plain for serum cortisol/DHEAS analyses. The blood samples in the plain tube were left for 30 minutes to coagulate, after which they were centrifuged at 1600xg for 10 minutes and serum from the supernatant layer was stored at -20°C for future analysis. Neutrophil phagocytosis of FITC labelled *E coli* and oxidative burst activity in response to unlabelled *E coli* were assessed using two commercial kits (Phagotest, Orpegen Pharma, Heilderberg, Germany), following the suppliers protocol. Both aspects of neutrophil function were assessed using a three-laser Dako Cyan High Performance flow cytometer (Dako, Carpinteria, California), with Summit v 4.3 software. Phagocytic ability was expressed as phagocytic index which was calculated as: Phagocytic index = % phagocytic neutrophils x MFI, where MFI is mean fluorescence intensity measured by flow cytometry. The difference between MFI in the test sample (which is the sample with *E.coli*) and control sample (sample with wash buffer) was used to measure the oxidative burst activity of neutrophils.

Concentrations of cortisol and DHEAS in the stored serum samples were analysed using corresponding ELISA commercial kits (IBL international, Hamburg, Germany), following the suppliers protocol. The optical density (OD) was measured at 450nm within 15 minutes of stop solution addition. Standard curves were then constructed using GraphPad Prism 6 software by plotting the OD of the standards against their concentrations on semi-logarithmic graph. The unknown concentrations of the samples (in nmol/l for cortisol and μmol/l for DHEAS) were calculated from the standard curve using the cubic spline fit.

5.3.5. Statistical analyses

Comparison between the bereaved and non-bereaved on socio-demographics, and questionnaire scores were conducted by ANOVA and chi-square as appropriate; with effect sizes reported as η^2. Further, 2x2 bereavement group * age group ANOVAs were used to compare immune and hormone measures in the young and old, bereaved and controls. Neutrophil function and hormone levels were skewed and therefore subjected to log transformation. Significantly different demographic or health behaviour variables between groups were controlled for in further ANCOVAs. Correlations were used within younger bereaved group only to examine whether the cortisol:DHEAS ratio or any other questionnaire variables were related to neutrophil function. Further, bereaved participants were divided into two groups (those who lost spouse or parent versus those who lost more distant relative), and differences between them on neutrophil function and hormone status were examined using ANOVAs.

5.4. RESULTS

5.4.1. Demographic, health behaviour and psychosocial characteristics

Table 1 shows that bereaved participants and controls were reasonably well matched on most socio-demographic and health behaviour variables in both young and old groups, with the exception of occupational status (p = .02), and the medication (p=.04) in the young. Young bereaved were more likely to hold manual occupations, and to take medication, mainly anti-hypertensives and non-steroidal asthma treatments. The bereaved in both groups reported more symptoms of depression and anxiety than controls. Social support availability did not differ between groups in either of the age

cohorts. Bereaved participants scored moderately highly on both the CBI and IES, albeit slightly lower than bereaved participants in previous research (Walsh et al. 2002, Burnett et al. 1997, Horowitz et al. 1979, Corcoran and Fischer 1994). In the younger group, two bereaved participants had lost a spouse (9.5%), eight had lost a parent (38.1%), nine a grandparent (42.9%), and two a distant relative, e.g. parent-in-law (9.5%). For the older group, the respective values were 17 (65%), 3 (12%) and 6 (23). The death was expected in 86% of cases in the younger group, and in 84% in the older group.

5.4.2. Neutrophil function

For neutrophil phagocytosis, a 2x2 age group versus bereavement group ANOVA comparing neutrophil phagocytosis in young bereaved and matched controls with older bereaved and controls revealed the significant main effect of age, $F(1,87) = 31.45$, $p < .001$, $\eta^2 = .265$, such that younger participants showed higher phagocytosis overall than older adults, but there was no overall main effect of bereavement, $F(1,87) = 0.26$, $p = .61$, $\eta^2 = .003$, nor bereavement * age interaction effect, $F(1,87) = 1.94$, $p = .17$, $\eta^2 = .022$ (Figure 1A). Repeated analyses with adjustment for occupational status and medication usage revealed the same significant main effect of age, $p < .001$.

Table 1. Socio-demographic, health behaviour and psychosocial characteristics of bereaved and non-bereaved participants.

	Young			**Older**		
	Bereaved (N = 21)	**Non-bereaved (N = 20)**		**Bereaved (N = 26)**	**Non-bereaved (N = 26)**	
	N (%) / Mean (SD)		***p***	**N (%) / Mean (SD)**		***p***
Age (years)	31.8 (9.03)	31.7 (8.41)	.97	71.3 (5.79)	72.6 (5.72)	.42
Gender (Female)	9 (43)	10 (50)	.65	18 (69)	17 (65)	.77
Ethnicity (Caucasian)	21 (100)	17 (85)	.07	26 (100)	25 (96)	.31
Occupational status (non-manual)	16 (76)	20 (100)	.02	19 (76)	21 (81)	.68
Taking medications	4 (19)	0 (0)	.04	15 (60)	15 (58)	.87

Alcohol intake (daily or more)	5 (25)	2 (10)	.21	10 (38)	7 (27)	.38
Smokers	5 (25)	4 (20)	.71	2 (1)	3 (12)	.64
Body Mass Index	24.3 (4.20)	23.0 (2.70)	.27	26.2 (3.93)	25.5 (3.36)	.52
Exercise score	7.8 (5.95)	9.6 (4.83)	.30	5.5 (1.35)	8.0 (1.35)	.21
Fruit and vegetable consumption score	9.8 (2.96)	8.9 (2.27)	.28	9.3 (2.03)	9.9 (2.04)	.27
Fat consumption score	10.6 (3.53)	11.3 (2.65)	.49	10.6 (3.66)	10.0 (3.21)	.56
HADS anxiety score[a]	8.0 (4.63)	5.2 (3.08)	.03	8.6 (4.90)	4.2 (2.95)	<.001
HADS depression score[a]	4.7 (3.15)	2.5 (2.50)	.02	6.1 (5.54)	2.42 (2.32)	.003
Social support score (MOS)[a]	73.8 (17.68)	80.4 (12.65)	.18	70.4 (19.61)	80.2 (16.26)	.06
Core bereavement items (CBI)	23.2 (10.93)	-	-	29.8 (13.28)	-	-

The Impact Event Scale (IES)	33.1 (15.52)	-	-	33.2 (15.6)	-	-
Death expected - yes	18 (86)	-	-	21 (84)	-	-
Bereavement type						
Spousal	2(10)	-	-	17 (65)	-	-
Close relative (parent)	8 (38)	-	-	3 (12)	-	-
Distant relative/friend	11 (52)	-	-	6 (23)	-	-

For neutrophil ROS generation 2x2 ANOVA comparing neutrophil ROS production between young and old, bereaved and control revealed a significant main effect of age, $F(1,87) = 34.4$, $p < .001$, $\eta^2 = .284$, such that older participants had higher neutrophil superoxide burst than younger subjects. There was, however, no main effect of bereavement overall, $F(1,87) = 1.02$, $p = .31$, $\eta^2 = .012$, nor bereavement * age interaction effect, $F(1,87) = 2.63$, $p = .11$, $\eta^2 = .029$. Pairwise comparison revealed that the lack of the effect between bereaved subjects and controls was driven by the comparable ROS production in the younger group ($p = .69$), while there was a significant effect of bereavement in the older group ($p = .05$), such that older bereaved subjects had lower ROS production than older controls (Figure 1B). Repeated analysis with covariate adjustment also revealed a main effect of age ($p < .001$).

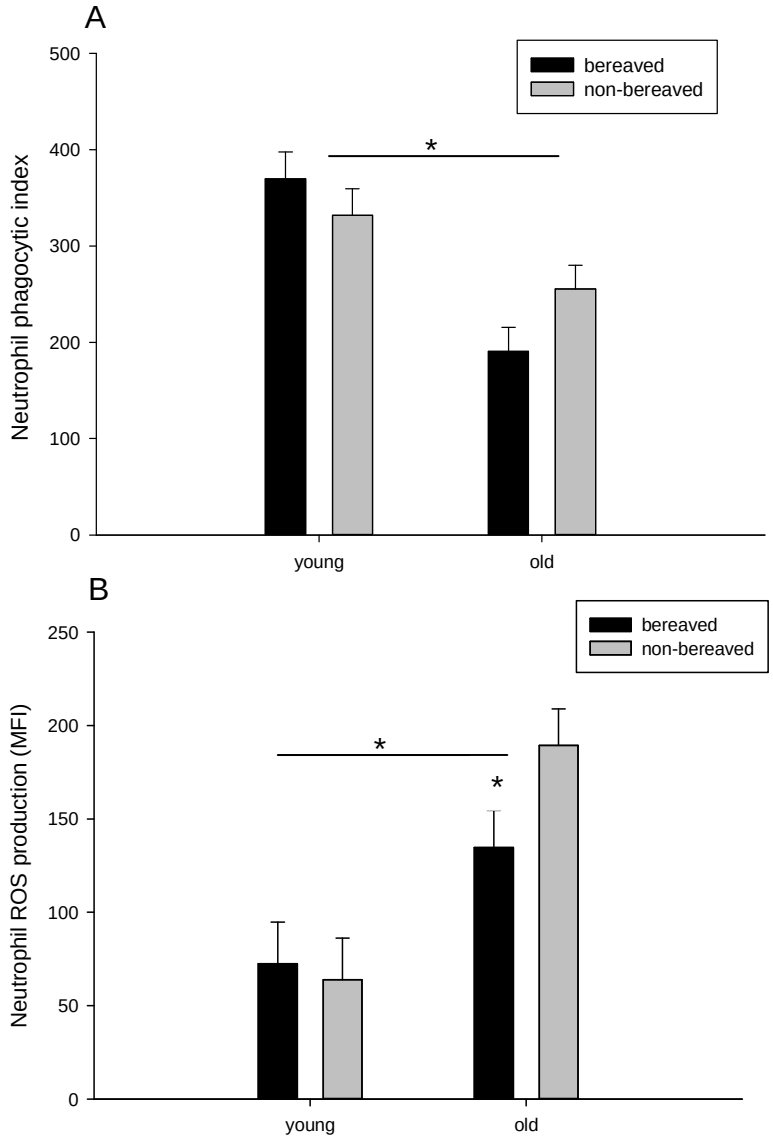

Figure 1. Neutrophil function in response to bacteria *E.coli* in young and old, bereaved and non-bereaved. A: Neutrophil phagocytosis of FITC labelled *E.coli* presented as phagocytic index (bacteria ingested (MFI) x % neutrophils uptaking bacteria) between young and old, bereaved and non-bereaved subjects. B: Neutrophil superoxide production in response to *E.coli* presented as MFI, between young and old, bereaved and non-bereaved. Error bars are SEM and * indicates $p < .05$.

5.4.3. Stress hormone concentrations

For cortisol, 2x2 ANOVA between young and old bereaved and controls showed a significant main effect of age, $F(1,84) = 8.80$, $p = .004$, $\eta^2 = .095$, such that younger participants had higher serum cortisol levels, but no main effect of bereavement, $F(1,84) = 3.28$, $p = .07$, $\eta^2 = .038$, nor bereavement * age interaction effect, , $F(1,84) = 1.42$, $p = .24$, $\eta^2 = .017$. Pairwise comparison revealed a significant effect of bereavement in the older group ($p = .03$), such that older bereaved subjects had higher cortisol levels than controls, while there was no difference in the young ($p = .68$) (Figure 2A). Repeated analyses with covariates adjustment showed a similar main effect of age ($p = .03$).

For DHEAS, 2x2 ANOVA using young and old, bereaved and non-bereaved showed a significant main effect of age, $F(1,84) = 62.08$, $p < .001$, $\eta^2 = .425$, such that younger subjects had higher serum DHEAS, but no main effect of bereavement, $F(1,84) = 1.95$, $p = .17$, $\eta^2 = .023$, nor bereavement * age interaction effect, $F(1,84) = 1.77$, $p = .19$, $\eta^2 = .021$, was seen (Figure 2B). Pairwise comparison revealed a significant bereavement effect in the older group ($p = .04$), such that older bereaved had lower DHEAS than non-bereaved older controls, while the levels of this hormone were comparable between the groups in the young ($p = .97$). Repeated subsequent analyses including the covariates showed a similar main effect of age, $p < .001$.

For the cortisol:DHEAS ratio, 2x2 ANOVA revealed a significant main effect of age, $F(1,84) = 14.35$, $p < .001$, $\eta^2 = .146$, and the trend towards an effect of bereavement, $F(1,84) = 3.59$, $p = .06$, $\eta^2 = .041$, such that younger participants and control participants had a lower cortisol:DHEAS ratio, respectively. There was, however, no bereavement * age interaction effect, $F(1,84) = 2.33$, $p = .13$, $\eta^2 = .027$. Pairwise comparison revealed that the trend towards a bereavement effect was driven by the differences in older group

(p = .01), while the ratio was comparable between the young (p = .80) (Figure 2C). Covariate analyses confirmed a main effect of age, p =.002.

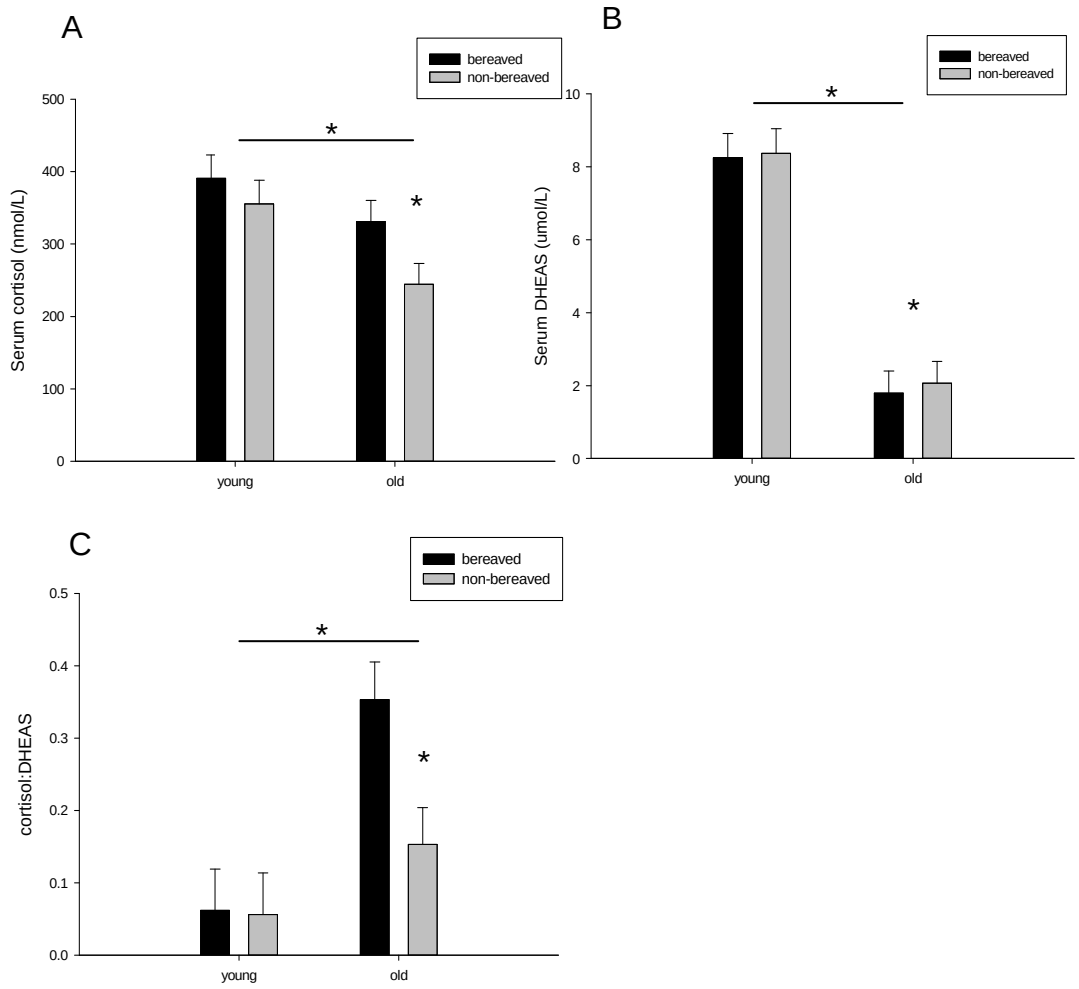

Figure 2. Serum stress hormone levels in young and old, bereaved and non-bereaved. Serum A. cortisol, B. DHEAS or C. cortisol:DHEAS ratio for young and old, bereaved and non-bereaved subjects. Error bars are SEM and * indicates $p < .05$.

5.4.4. Psychological factors and immune and hormone measures

Correlations within the bereaved groups revealed no association between neutrophil function and any of the psychosocial and socio-demographic variables. There was also no significant difference in neutrophil function between those bereaved participants who had lost a spouse or parent and those who had lost a more distant relative. Correlation analysis within the bereaved group revealed that those with higher CBI scores, indicative of greater grief had a higher cortisol:DHEAS ratio, $r(42) = .34$, $p = .03$, and those who reported higher social support had lower cortisol:DHEAS ratio, $r(42) = -.31$, $p = .04$. When bereaved participants were separated into two groups based on who they had lost, there was a difference between the groups in their cortisol:DHEAS ratio, $F(1,42) = 9.04$, $p = .004$, $\eta^2 = .177$, such that those who had lost someone more distant had a lower cortisol:DHEAS ratio.

5.5. DISCUSSION

The results presented in this chapter showed that there was no difference in neutrophil function and serum hormone levels between bereaved and controls overall, with the main differences emerging between the two age groups. This was despite the differences in psychosocial variables that showed higher depressive and anxiety symptoms in the bereaved. Closer analyses revealed the younger group as responsible for these null findings, since neutrophil function and stress hormone levels were comparable between the groups in the young. On the other hand, older bereaved subjects had poorer ROS production, and higher cortisol:DHEAS ratios when compared to the matched non-bereaved older adults, consistent with the previous studies of bereavement and immune function in older adults (Khanfer et al. 2011, Phillips et al. 2006a). The absence of an

effect of bereavement on neutrophil function in the younger sample is perhaps surprising given the high levels of depression and anxiety symptoms among the bereaved, similar to those recorded for the older bereaved sample (Table 1). In addition, responses to questionnaires measuring grief, and impact of the bereavement indicated significant feelings of loss in the present study in both groups. However, only a limited number of studies have examined the effect of bereavement on immune function in younger adults. Lower numbers of regulatory T cell and helper T cells (Spratt and Denney 1991), and lower NK cell cytotoxicity (Gerra et al. 2003) were reported for individuals who had experienced sudden/unexpected death of a close friend or family member. Further, no group differences in NK cell activity were observed between middle aged widows and married controls (Zisook et al. 1994), although NK cell activity and the response to mitogens was poorer in a small sample of widows with symptoms of major depression. In the present study, although depressive symptomatology was higher among the bereaved, only one bereaved participant met the criteria for severe depression or higher (HADS $\geq$ 11). There are several potential explanations for the present null findings for neutrophil function in the young bereaved. It is possible that the intact neutrophil function was attributable to losses in the present study being of less close relationships than those of older adults, only 10% of the bereavements were spousal in the younger sample, the comparable figure for the older participants was 65% (Table 1). However, there was no difference in neutrophil function in the present study between those who have lost a close relation (spouse, parent) and those who have lost a more distant relative (grandparent, parent-in-law). Further, social support is an unlikely explanation for preserved immunity in the present study as the support scores of the young bereaved

were virtually identical to those found in older bereaved participants, who showed reduced neutrophil ROS production.

A plausible explanation for the preservation of neutrophil function in young but not older bereaved subjects is the difference in the HPA axis response between the two groups, superimposed upon the aged neutrophil. Previous research indicates that stress may affect immune function more readily in the context of concomitant immune ageing. For example, lower sIgA, (Gallagher et al. 2008), and higher antibody titres against CMV (Pariante et al. 1997) were specifically characteristic of older caregivers. In general, there is consistent evidence of compromised immune function in older spousal caregivers for partners with dementia (Glaser et al. 2000, Vedhara et al. 1999), whereas the results from the studies of younger caregivers are more variable (Gallagher et al. 2009a, Vedhara et al. 2002). Here the cortisol:DHEAS ratio was only raised in the older bereaved subjects compared to their controls and not in the younger bereaved group. With the well documented and opposing effects of cortisol (Bekesi et al. 2000) and DHEAS (Butcher et al. 2005, Radford et al. 2010) on neutrophil ROS production, this proposal had biological validity.

The present study is not without limitations. First, the sample size can be regarded as small; however, bereaved participants are notoriously difficult to recruit and the sample size is comparable to that recruited to previous studies of immunity and bereavement (Gerra et al. 2003, Khanfer et al. 2011). Second, it could be argued that the preserved immune function in the present sample was due to bias such that those who are less stressed by or coping better with bereavement might be more likely to take part. However, the scores on CBI and IES suggested that the bereavements were significantly stressful.

In conclusion, unlike older bereaved adults, younger bereaved participants showed no detrimental effect of bereavement on neutrophil function and stress hormone concentrations when compared to the matched non-bereaved controls. This is most likely attributable to the absence of immunosenescence and adrenopause in the younger aged bereaved group.

CHAPTER 6

OVERALL DISCUSSION

6.1. SUMMARY OF THE MAIN THESIS FINDINGS

The research in this thesis was concerned with the relationship between chronic stress (caregiving and bereavement), ageing and different aspects of immune and endocrine function, in particular neutrophil function, serum anti-CMV antibody titre, the predominance of T cell exhausted and senescent phenotypes and stress hormone (cortisol, cortisone and DHEAS) levels.

Chapters two, three and four focused on the chronic stress of caregiving, examining its effect on aspects of both innate and adaptive immunity, as well as stress hormone levels; while chapter five compared the effect of the chronic stress of bereavement on neutrophil function and the HPA axis in younger adults, and compared it to the results obtained from older participants.

Chapter two examined the effect of caregiving stress in parents of children with developmental disabilities on CMV seropositivity and serum CMV antibody titre by comparing them to age- and sex-matched control parents of typically developing children. The aim was to determine whether caring for a child with a developmental disability negatively influenced caregiving parents' immunity, responsible for keeping latent virus infection under control. This would be evidenced through an increased titre to the corresponding virus in younger caregivers' serum when compared to the controls. The study showed that caregiving parents were no more likely to be CMV seropositive than parents of typically developing children. It also did not find any differences between the groups in serum antibody titres against CMV. However, in focusing on the caregiving group alone, the study also aimed to elucidate whether CMV titre in seropositive caregiving parents was

associated with any health behaviours and/or psychological/caregiving variables. An association emerged between CMV antibody titre and negative health behaviour variables in CMV seropositive caregivers, such that those with a higher BMI, lower exercise level, smokers and those eating less well overall had higher CMV antibody titres. Overall, this study confirmed a lack of negative effect of caregiving stress on latent virus control in a younger population, but suggested a possible negative influence of detrimental health behaviours practiced by some stressed caregivers on this aspect of immunity.

Chapter three focused on the effect of caregiving stress and/or age on neutrophil function (phagocytosis and ROS production), outcomes previously not investigated in the caregiving research, as well as stress hormone (cortisol, cortisone and DHEAS) levels using the previously described four groups of participants. Despite the differences in psychosocial/caregiving variables, such that caregivers in both age groups reported higher psychological morbidity than controls, no difference was found in neutrophil function between caregivers and controls, and cortisol, DHEAS and their ratio was comparable between the groups. Younger caregivers exhibited, however, an increased cortisol:cortisone ratio, which is an accepted indicator of systemic generation of cortisol via the enzyme 11β-HSD1, a novel finding in caregiving research. Finally, focusing on caregivers alone, those with higher anxiety symptoms and caregiving burden had lower neutrophil phagocytosis and poorer ROS production was seen in those reporting poorer sleep quality. This suggests that the focus of future caregiving research should perhaps be less on caregiving status in general and more on the individuals who demonstrate higher psychological morbidity.

Chapter 4 focused primarily on the parameters of T cell immunity, examining, in particular, the effect of caregiving stress with and without ageing on T cell senescent and exhaustion profile and thymic output, immune parameters not previously examined in this group of chronically stressed individuals, as well as serum CMV antibody titre. Caregivers overall demonstrated a generally robust T cell profile, with the main differences emerging between young and old, with older adults showing greater cell senescence as might be expected. The exception was an increased percentage of T cells expressing the KLRG1 senescent profile in caregivers overall when compared to controls, an effect that supports previous findings of a poorer vaccination response in old and young caregivers, and that could be used in future interventions. In addition, amongst older caregivers, those who reported higher problematic behaviour in their spouses with dementia showed poorer $CD57^+$ T cell senescent profile. This suggests, once again, that the focus of the future caregiving research should be more on caregiving individuals with a higher caregiving burden.

Chapter five examined the effect of a different type of chronic stress, suffering bereavement, on neutrophil function, and serum stress hormone (cortisol and DHEAS) levels in the younger and old adults. Due to the difficult nature of recruiting bereaved participants, the effect of the stress of bereavement is largely under-researched and frequently suffers from small research sample sizes. This is the first study to examine the effect of bereavement on neutrophil function in young adults and compare it to an older sample, in order to examine possible reasons for the different patterns of findings in the different age groups. Despite scoring higher on depression and anxiety questionnaires than controls, as well as reporting high levels of grief and feelings of loss, younger bereaved participants showed neutrophil function comparable to that of the young controls. In addition, serum stress hormone (cortisol and DHEAS) levels were similar between the groups. Comparison with results of the effect of

bereavement on older adults, who showed poorer ROS production and higher cortisol:DHEAS, revealed a significant age effect for all the variables measured. This suggests that the presence of immunosenescence is necessary for a negative effect of bereavement on neutrophil function and stress hormone levels to be observed, and that younger adults are protected against the negative immune impact of bereavement due to their younger immune systems.

6.2. CAREGIVING STRESS, IMMUNE FUNCTION, CORTISOL, CORTISONE AND DHEAS

The model of stress of caregiving in young used was parents of children with developmental disabilities. This way an attempt was made to replicate the caregiver burden due to behavioural problems seen in dementia patients, and find the equivalent model in a younger group to compare the effect of caregiving stress in younger adults to the frequently examined older spousal dementia caregivers. The findings presented imply robust immune and endocrine responses in young caregiving parents, comparable to that of the age- and sex-matched control parents of typically developing children. CMV positivity, anti-CMV antibody titre, neutrophil function, and cortisol and DHEAS levels did not significantly differ from those measured in the control group. These findings are consistent with some previous studies examining caregiving stress in a younger population. For example, spousal caregivers of multiple sclerosis patients had similar levels of IgG and hemagglutination-inhibition antibodies, and cytokines IFN-γ and IL-4, as matched controls (Vedhara et al. 2002). On the other hand, the concentration of salivary stress hormones (cortisol and DHEAS) was higher in non-caregivers than in caregivers, while their ratio remained the same (Vedhara et al. 2002),

which is comparable to the present findings. Another study showed no difference in salivary sIgA levels between caregivers and matched controls in younger aged cohorts from the West of Scotland Twenty-07 study (Gallagher et al. 2008). No difference was also observed for serum anti-CMV antibody titres between younger female caregivers of handicapped people and non-caregiving controls (Pariante et al. 1997).

However, chapter 3 demonstrated a significant difference in the serum cortisol:cortisone ratio between younger parental caregivers and age- and sex-matched controls, a marker of systemic generation of cortisol. A raised cortisol:cortisone ration has been previously reported in the surgical patients, during the acute-phase response and after Synacthen stimulation and, as such, is considered an indicator of systemic 11β-HSD1 activity (Vogeser et al. 2001, Vogeser et al. 2002, Vogeser et al. 2003). The 11β-HSD1 enzyme is responsible for converting inactive glucocorticoids, such as cortisone, into their active forms, cortisol in humans (Chapman et al. 2013). An increase in the cortisol:cortisone ratio in younger caregivers could, therefore, indicate an increase in 11β-HSD1 activity caused, for example by pro-inflammatory factors, such as cytokines TNF-α and IL-1 (Escher et al. 1997, Cai et al. 2001). It is important to note here, however, that serum cytokine concentrations of caregiving parents were similar to those of the controls (see Appendix with the Additional findings from chapter 3). On the other hand, this does not eliminate the possibility of 11β-HSD1 being influenced by pro-inflammatory cytokines from other sources, such as autocrine signals (Cooper et al. 2001, Ignatova et al. 2009, Yang et al. 2009). However, it was beyond the scope of the present study to measure this, or any impact of this increased inflammatory marker in our sample. A longitudinal study with more inflammatory markers included, such as CRP, would be necessary to examine the potential implications of the increased cortisol:cortisone ratio in younger caregivers. In summary, an increased cortisol:cortisone ratio in younger parental

caregivers could be seen as further support for a negative effect of caregiving stress on certain parameters in younger adults, such as those previously reported for vaccination response (Gallagher et al. 2009a, Gallagher et al. 2009b).

Chapter 4 reported comparable T cell immunity between caregivers and controls in both older and younger adults, with the exception of the $CD28^-$ T cell subset, where paradoxically caregivers had lower numbers of senescent $CD28^-$ T cells. In addition, the percentage of CMV positive individuals among young caregivers was lower than those in controls. Therefore, one possible explanation for the unexpectedly robust immune response in younger caregivers could be the lower incidence of chronic CMV infection which is known to be an important predictor of $CD8^+$ $CD28^-$ T cell subset expansion (Kern et al. 1996, Weng et al. 2009). This is further confirmed by the positive association between the $CD8^+CD28^-$ T cell subset and CMV antibody titre in the younger group ($r(41) = .48$, $p = .001$, see Appendix, Additional findings from chapter 4), and subsequent ANCOVAs using CMV status as a covariate that eliminate this difference between T cells expressing CD28 marker in caregivers and controls.

Overall, the results from chapters 2, 3 and 4 that refer to the differences in immune and hormone status between younger caregivers and controls add to the mixed findings reported by a number of studies that aimed to elucidate the effect of caregiving stress on health in the young, suggesting that this effect might not be straightforward, but instead has differential effects on different immune and endocrine parameters.

6.3. AGEING, IMMUNE FUNCTION, CORTISOL, CORTISONE AND DHEAS

Chapters 3 and 4 also attempted to examine the differences in immune and hormone parameters that are a consequence of the different ages of our mixed caregiver and controls sample. The results from chapter 3 showed consistent differences between young and older adults on neutrophil function and most of the hormone measures, with the exception of the cortisol:cortisone ratio. Older adults unexpectedly had higher neutrophil function (both phagocytosis and ROS production), but also an expected higher cortisol, lower DHEAS and a higher cortisol:DHEAS ratio. Increased neutrophil function in the older group is surprising considering the overall consensus about decreasing immune function with age (Franceschi et al. 2007, Panda et al. 2009, Shaw et al. 2010). However, increased superoxide production has been previously reported (Cannizzo et al. 2011), and it was further supported by higher ROS production seen in older age cohort in our bereavement study. In addition, this is not the first study to indicate the possible perseverance and potential dominance of innate immunity in older age, suggesting that perhaps the negative effect of immunosenescence is more clearly marked in the adaptive branch of immune response (Franceschi et al. 2000). Age-related differences in the stress hormone status, in particular increased serum cortisol and lower serum DHEAS, and subsequently higher cortisol:DHEAS ratio among older adults, confirms previously reported and well established findings (Buford and Willoughby 2008). The imbalance between these hormones with age is considered to be a contributing factor to immunosenescence, and its negative effects on different immune parameters.

For T cell immunity in chapter 4, there was a main effect of age for most of the parameters measured, with the exception of $CD4^+$ both $CD28^-$ and $KLRG1^+$ T cells, indicating an overall greater senescent and exhausted T cell profile in older group. This is consistent with the previously reported data (Fagnoni et al. 1996, Henson and Akbar 2009, Weng 2006, Wherry

2011), and is related to the increase in the replicative senescence of T cells and their inhibition of the proliferation as well as the loss of functionality (Akbar and Henson 2011). One of the main drivers of this increase in senescence and exhaustion in T cells is thought to be repeated antigen stimulation as a consequence of chronic latent viral infection present over the years (Boucher et al. 1998, Chidrawar et al. 2009, Kern et al. 1996). A higher TREC expression ratio in the younger compared to the older adults is again in concordance with previously reported research on thymic atrophy with age (Mitchell et al. 2010, Naylor et al. 2005). TREC represent extra-chromosomal DNA segments that are transported from thymus in so called recent thymic emigrants, mature $CD4^+$ and $CD8^+$ thymocytes that emigrate from thymus (Jamieson et al. 1999). As such, TREC is directly associated with thymic output for $CD4^+$ T cells, and with thymic output when peripheral T cell division and death are taken into account, for $CD8^+$ T cells (Ye and Kirschner 2002). Therefore, a decrease in TREC expression ratio in older adults indicates a decreasing ability to produce novel naive T cells in later life, leaving the organism potentially less able to fight off the infections that the body is exposed to for the first time. This is likely to underlie, at least in part, the reduced vaccination response to new antigens exhibited by older adults (Goodwin et al. 2006).

6.4. CAREGIVING STRESS AND AGEING, IMMUNE FUNCTION, CORTISOL, CORTISONE AND DHEAS

Chapters 3 and 4 also aimed to elucidate the potential additive effects of caregiving stress and ageing on immune function and serum stress hormone levels. They presented the findings of the studies that used four groups of participants as models of caregiving stress with and without ageing, as well as age-matched controls. To our knowledge, these are the first studies

to simultaneously examine the effect of caregiving stress in the cross-sectional experimental set up, using two distinct age groups (23-50 and >60 years). This enabled direct examination of individual and synergistic effect of chronic stress and ageing on person's health. However, the hypothesised additive impact of caregiving and ageing on immunity was, on the whole, not observed. With the exception of serum cortisol:cortisone ratio, there was no caregiving * age interaction effect for any of the measured variables, suggesting that, at least in the present sample, caregiving stress and ageing did not act synergistically to result in reduced immunity.

In terms of the effect of caregiving stress on immune function between older spousal caregivers of dementia patients and age- and sex-matched controls, chapter 3 reported no difference in neutrophil function, cortisol, DHEAS, cortisol:DHEAS and cortisol:cortisone ratio between the groups. This was opposite to expectations based on the previous extensive research that showed a detrimental effect of caregiving stress on spousal dementia caregivers (Esterling et al. 1994, Glaser et al. 2000, Kiecolt-Glaser et al. 1991a, Vedhara et al. 1999). The most parsimonious explanation for this discrepancy between the present study findings and the previously reported research would be lower psychological morbidity in the current sample and selection bias towards those caregivers who are less stressed and are therefore much more likely to take part in the study. However, from the scores on the range of psychological and caregiving-specific questionnaires used in the study it was clear that on the basis of the perceived stress, caregiving burden and problematic behaviour of the care-recipient, the present group of caregivers was comparable in terms of stress levels to those used previously in caregiving research (Bauer et al. 2000, Bedard et al. 2001, Li et al. 2007, Roepke et al. 2008). Another possible explanation emerged after closer analysis of the nature of the immunological assays used in the previous research when compared to those in the

present studies. For example, caregivers of dementia patients showed poorer NK cell response only after stimulation with recombinant cytokines IFN-γ and IL-2 (Esterling et al. 1994, Esterling et al. 1996). In contrast, measure of the innate immunity in the study presented in chapter 3 was neutrophil function after *in vitro* stimulation with bacteria *E.coli* only, and therefore more resembling the studies that looked at the NK cytotoxicity without cytokine induction (Esterling et al. 1996, Irwin et al. 1991), which, on the contrary, did not observe any significant differences between older dementia caregivers and matched controls. Further, in previous studies, older caregivers showed a poorer immune response to influenza vaccination (Glaser et al. 1998, Kiecolt-Glaser et al. 1996, Vedhara et al. 1999), lower antibody titre against pneumococcal pneumonia antigen after 6 months follow up (Glaser et al. 2000), and slower wound healing (Kiecolt-Glaser et al. 1995) than age-matched controls. This further confirms the inability of the immune response to respond adequately in the presence of caregiving stress when actively challenged. In other words, perhaps the strongest negative effect of caregiving stress is exerted once the organism is confronted with the pathogenic challenge or injury, such as in the case of administering a vaccination antigen, or causing a small biopsy wound, while the effect of stress on immunity in the resting state, such was the case in the present studies, was not clearly visible. This is perhaps further confirmed by a study that reported no difference in the incidence of the upper respiratory infection between older caregivers and controls, but instead showed that caregivers who succumbed to the infection, took significantly more days to heal then the controls in which the same illness has occurred (Kiecolt-Glaser et al. 1991a). This however, cannot be the case for all components of the immune response, since a different study found shorter telomere length in PBMCs of caregivers of Alzheimer's dementia patients than in controls at the basal (unstimulated) level (Damjanovic et al. 2007).

There was no difference in serum hormone levels (cortisol, cortisone and DHEAS) between the older caregivers and controls in the present study, despite the high perceived stress reported by older caregivers comparable to the levels of stress reported in previous studies (Bauer et al. 2000). This contrasts with the higher cortisol levels reported in previous research in dementia caregivers when compared to controls (Bauer et al. 2000, Vedhara et al. 1999), but is in concordance with others (Jeckel et al. 2010). It is important to note, however, that the 2010 study that showed comparable salivary cortisol levels between the groups, still found lower salivary DHEAS levels and higher cortisol:DHEAS ratio in caregivers (Jeckel et al. 2010), contrary to the present findings.

The results from chapter 4 showed a comparable T cell exhaustion profile in caregivers overall when compared to the controls. In terms of the T cell senescence, there was no difference between caregivers and controls in T cells expressing CD57 marker, and controls showed higher expression of CD28 than caregivers only in young, while the expression of this marker was comparable between older caregivers and controls. The present findings are the first to examine exhaustion and senescent profiles of T cells in detail between caregivers and controls, both young and old, and to demonstrate a difference in $KLRG1^+$ T cell senescent profile in both caregivers and older participants. The only other study that examined the percentage of $CD28^-$ T cells found no difference between older dementia caregivers and matched controls (Damjanovic et al. 2007), consistent with the results presented in chapter 4.

Caregivers in both age groups demonstrated a higher percentage of $CD4^+$ and $CD8^+$ $KLRG1^+$ T cells than controls. This marker is associated with replicative senescence and inhibition of T cell proliferation (Henson and Akbar 2009), but it is also involved in the exhaustion-specific pathways (Henson and Akbar 2009). KLRG1 is an inhibitory marker increasingly expressed on highly differentiated T cells with ageing. It has been shown that blockage of

KLRG1 can induce proliferative activity of these T cells (Henson et al. 2009), which suggests the potential for targeting pathogen-specific, highly differentiated T cells by blocking KLRG1 receptor in order to increase the poor vaccination response seen in older adults (Henson et al. 2009, Henson and Akbar 2009). This is further confirmed by a study that reported the inhibitory role of KLRG1 in $CD4^+$ T cells response to novel antigens (Shi et al. 2014). In addition, the increase in KLRG1 expression in caregivers presented in chapter 4 could explain consistently poorer vaccination response in caregivers when compared to the matched controls (Gallagher et al. 2009a, Kiecolt-Glaser et al. 1996, Vedhara et al. 1999). Thus, this chapter gives further support to the notion that some but not all aspects of immunity are influenced by caregiving stress, but also contributes a potential mechanism by which some aspects of immunity are impaired in both young and older caregivers.

6.5. PSYCHOSOCIAL AND CAREGIVING FACTORS, IMMUNE RESPONSE AND HORMONES

Various studies have previously examined the relationship between self-reported psychological distress and impaired immune response in caregivers. For instance, spouses and children of Alzheimer's dementia patients who reported higher depression symptomatology and perceived stress on the day of the vaccination against tetanus exhibited a lower antibody response (Li et al. 2007). Also, those caregivers who reported more negative thoughts such as worry and rumination, had poorer antibody response than those whose thoughts were more positive (Segerstrom et al. 2008). Further, higher stress levels and anxiety symptoms were correlated with PHA proliferation, but not *in vitro* IL-2 production (Bauer et al. 2000). In younger caregivers, those parental caregivers who reported more child

behavioural problems had also poorer antibody response to vaccination against pneumonia (Gallagher et al. 2009b). The findings in chapter 3 build on this previous literature by comparing neutrophil function (phagocytosis and ROS production) with all psychosocial and caregiving variables measured. The results showed that caregivers with higher depression and anxiety symptomatology, perceived stress and caregiving burden had poorer neutrophil phagocytosis. In addition, those with poorer sleep quality had lower neutrophil ROS production. Subsequent sensitivity analysis further confirmed this for anxiety, caregiving burden and poorer sleep quality.

The results in chapter 4 also relate higher psychological morbidity to lower levels of some aspects of immunity, specifically immunosenescence markers and higher TREC expression ratio, which at first appears counterintuitive. However, the explanation for this discrepancy lies in the overall better senescent and exhausted profile in the younger caregivers, who, in addition, reported higher psychological morbidity than older caregivers (see Table 1, chapter 3). This is further confirmed with the findings that more problematic behaviour by care-recipients is reported by older caregivers whose T cells express more CD57 senescent markers. Overall, the results of chapters 3 and 4 add to the mixed picture of a differential effect of caregiving stress on various immune parameters. In addition, they emphasise the importance of future research to focus not on caregiving status *per se,* but in particular on those individuals who show higher psychological morbidity, and highlight this sub-sample of caregivers as those most in need of intervention to address both psychological distress and its associated immune impairment.

6.6. BEREAVEMENT STRESS, NEUTROPHIL FUNCTION, CORTISOL AND DHEAS IN YOUNG AND OLDER ADULTS

Chapter 5 examined the effect of chronic stress after suffering bereavement on neutrophil function and serum cortisol and DHEAS levels in younger and older adults. Bereaved participants reported higher depression and anxiety symptomatology than age- and sex matched controls, and significant levels of feelings of loss and grief. Despite this, there was no difference in neutrophil function (both phagocytosis and ROS production), nor serum stress hormone levels between the younger caregiver and control groups.

To our knowledge, this is the first study to simultaneously examine the effect on neutrophil function and stress hormone levels after recent bereavement in young and older adults. The present findings of no difference in immune and endocrine parameters are perhaps surprising considering previously reported study by our laboratory that found significantly lower oxidative burst activity in older bereaved adults when compared to the matched controls (Khanfer et al. 2011). However, closer analyses revealed the younger group as the main driver of this null finding, and that the older bereaved adults did show impaired immunity. The levels of depression and anxiety symptoms, as well as feelings of loss and grief reported by the young bereaved in the present study were high, and similar to those recorded for older bereaved adults (see Table 1, chapter 5) but did not extend to an impact upon immunity in the older group.

Previous findings from bereavement research showed poorer NK cell function (Irwin et al. 1987), vaccination response (Phillips et al. 2006a) and neutrophil ROS production (Khanfer et al. 2011) in older bereaved adults when compared to the matched controls. For younger adults, however, poorer immune function was reported only if accompanied by symptoms of

major depression (Zisook et al. 1994), or in the case of the sudden and unexpected death of the loved one (Gerra et al. 2003, Spratt and Denney 1991). In the study presented in chapter 5, only one bereaved participant met the criteria for severe depression or higher (HADS $\geq$ 11), and only 14% of losses were unexpected. Therefore, in the absence of severe psychological morbidity, one could conclude that presence of immunosenesence, the deterioration of the immune system that occurs with age, is necessary for the bereavement to exert its full negative effect on the immune function.

In terms of serum hormone levels, cortisol and DHEAS, as well as their ratio were similar in young bereaved to those of the control group. This is again in contrast with the data from older group where, consistent with the previously reported data (Khanfer et al. 2011), bereaved had higher cortisol:DHEAS ratio, further confirming seeming ability of the young to resist the negative immune effect of chronic bereavement stress. Interestingly, however, DHEAS was lower and ratio between the two hormones was higher in those young bereaved who lost closer relative (parent/spouse) when compared to those who lost more distant relative (grandparent, parent-in-law), and this ratio was higher in those who reported higher grief on CBI scale, and lower in those with higher social support, suggesting that those worst affected by bereavement did show some physiological decrements. This was, however, not the case with the neutrophil function which was comparable between the groups, suggesting that perhaps specific nature of the loss might affect hormonal status, but not extend to an impact on immune parameters such as neutrophil phagocytosis and ROS production. It is not known what the longer term effects of the raised cortisol:DHEAS ratio in the most distressed younger caregivers might be, which would need longitudinal study. These findings also do not minimise the need to address the high levels of psychological distress in younger bereaved adults, despite their seeming protection against immune system down-regulation.

6.7. CHRONIC STRESS, IMMUNE RESPONSE AND THE HPA AXIS

Based on the findings presented in this thesis as well as previously reported results it appears that the effect of chronic stress on immune function and stress hormones is dependent upon the type of chronic stress, specific parameters of immunity, and the circumstances in which the effect in question is measured. With this in mind, it could be argued that caregiving stress, in both young and old, is most likely to exhibit its negative effect on an already stimulated immune response, such as during vaccination and wound healing (Esterling et al. 1994, Gallagher et al. 2009b, Gallagher et al. 2009a, Vedhara et al. 1999), or in case of NK cell function when stimulated by cytokines (Esterling et al. 1994). Similarly, a higher number of T cells expressing senescent KLRG1 marker in caregivers regardless of their age links to, and offers a possible explanation for the poorer vaccination response in those affected by caregiving stress, suggesting a negative effect of stress even in the absence of immunosenescence, at least for some parts of immune response. On the other hand, measuring immune system function at rest, so in the absence of the prior immune challenge, may not show the expected negative effect, as seen for neutrophil function and some aspects of T cell immunity, presented in chapters 3 and 4, respectively, as well as in case of NK cell function without prior cytokine stimulation (Irwin et al. 1991). In addition, the mechanisms by which caregiving stress demonstrates its effect might be more complex than HPA axis and stress hormone levels alone. Another type of chronic stress, that of suffering with bereavement, appears to differently affect young and old individuals, with the HPA axis appearing to have an important role in exerting the negative effect of bereavement on the immune response in the old. A proposed model of the relationship between chronic stress, immune and hormone parameters based on the data in the present thesis is presented in Figure 1.

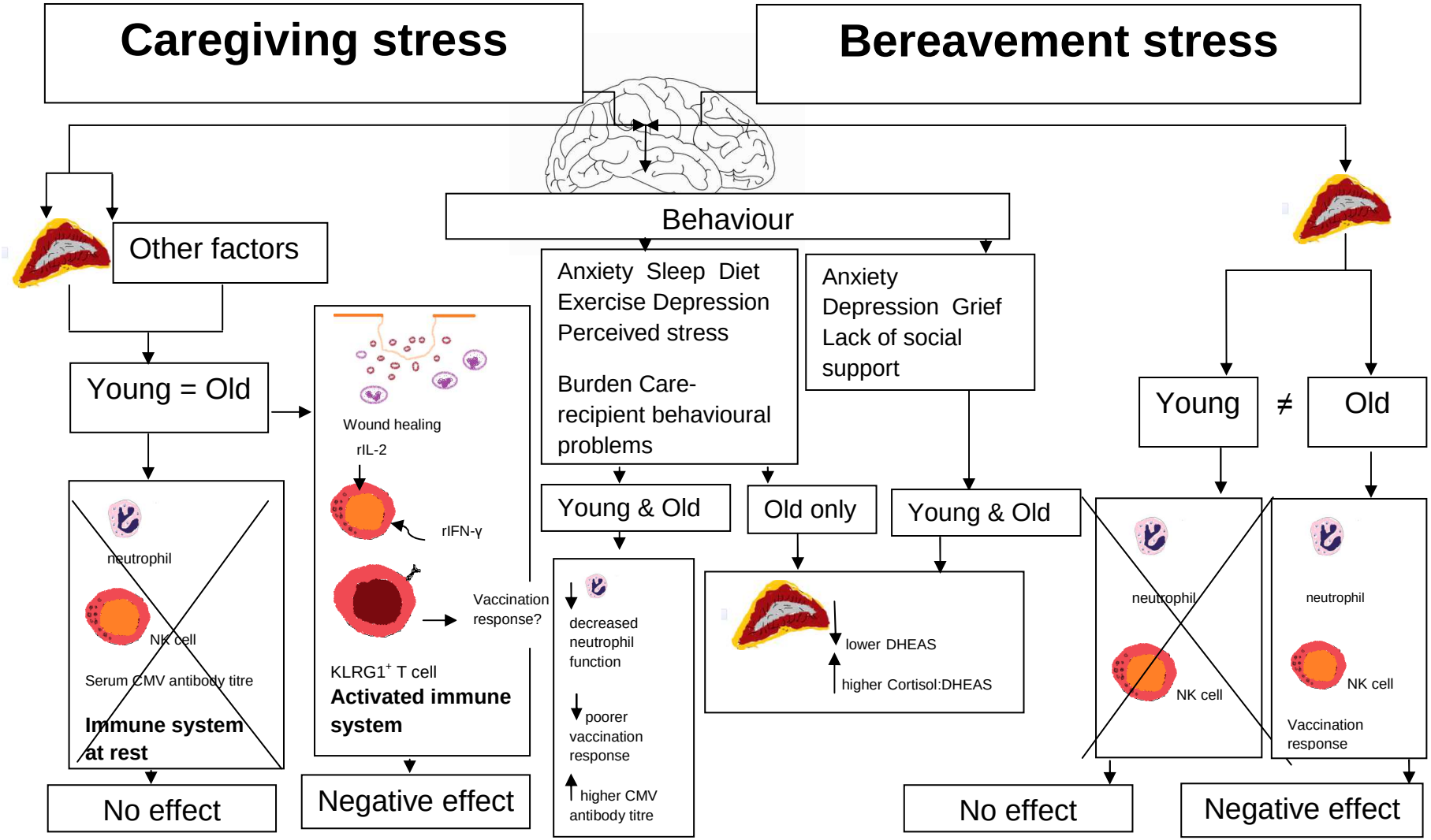

Figure 1. Proposed model of the relationship between chronic stress, immune response and stress hormones.

6.8. LIMITATIONS

The studies presented in chapters 2 to 5 of this thesis suffer from several limitations. One problem present throughout is the small sample sizes used. This is a common problem for caregiving and bereavement research, mainly due to the challenges regarding the difficult recruitment of these groups of participants. Therefore, one could assume that the limited power of these studies is the reason for the lack of the significant effects. However, the studies used G-power analysis programme to determine the required number of participants for a medium effect size. For example, the studies described in chapters 3 and 4, for the medium effect size, with power at .80, and alpha at .05 with 4 groups, required a sample size on the basis of power calculation of 134. The aim was to recruit at least 35 participants in each group to allow for some small amount of missing data. Further, the sample size in the present studies is of the similar magnitude and in some cases even greater than in previous research that reported significant effects on immunity (Gallagher et al. 2009a, Glaser et al. 2000, Vedhara et al. 1999).

Another limitation that applies to studies in chapters 2 through to 5 is the method of recruitment. Since the target of all the studies were hard-to-reach groups of participants, parents of children with developmental disabilities, spouses of dementia patients or bereaved younger adults, it could be argued that there is the bias in these studies towards those that cope better and are therefore more likely to take part in the studies, but may not be representative of the caregiving/bereaved population. However, in all cases attempt was made to minimise this bias by giving the option of the home visits to participants and overall organising the testing session so that they best suit individual's needs, and the participants reported similar high levels of psychological morbidity i.e., stress and burden to previously reported samples. Further, most of the younger caregivers from chapters 2, 3 and 4 were

recruited via syndrome groups, and from our experience, those were the parents that indicated these support days as one of the very few qualitative support mechanisms available to them, where they could gain the relief from their very stressful daily life.

A third limitation relevant to all the studies was that they were all cross-sectional, and therefore unable to determine changes in certain aspects of immune function in the stressed groups over time. This was particularly relevant for the CMV studies presented in chapter 2 and a part of chapter 4 where comparing the antibody titre against CMV with the control group could not determine if comparable antibody levels were indeed the consequence of the adequate immune control over the virus or there were other factors involved such as time since initial infection. In that respect, future longitudinal studies that measure immune function over the several time points using the same sample subset will be able to shed light on this question, although it is envisaged that this would be particularly challenging in these difficult to reach groups.

A potential limitation which applies to the studies presented in chapters 3 and 5 is related to the diurnal variations of stress hormone levels such as cortisol and cortisone (Nomura et al. 1997). Therefore, measuring the serum levels of these hormones in a single time point could be interpreted as inaccurate. However, attempts were made to overcome this limitation by, where possible, conducting the blood sampling in the narrow time window, between 9-11a.m. In addition, our initial aim for the study presented in chapter 3 was to collect and analyse 24-hour urine samples, as a more accurate measure of the steroid hormone levels. However, the analysis of these samples was not possible due to the costs of the assay required.

Finally, studies in chapters 2 and 3 would benefit from introducing another measure of immune response. In chapter 2, measuring CMV-specific T cell response would provide a

better understanding of adequate control of CMV infection. In chapter 3, in addition to neutrophil function, measuring another component of innate immunity, such as NK cell function, would increase the power of analyses. However, in both cases, additional analyses would require a large amount of the whole blood, unavailable in the present studies. In addition, for the study in chapter 3, analyses of NK cell function have already been conducted and measured in the older spousal dementia caregivers (Esterling et al. 1996, Esterling et al. 1994).

6.9. FUTURE DIRECTIONS

The findings from this thesis can be used as a basis for several directions of future research. A common path for all the studies would be replicating it using the larger sample in order to either confirm or refute the present findings. In addition to this, the research in each of the chapters can be taken forward in different ways.

The lack of differences in serum anti-CMV antibody titre between younger parental caregivers and controls should be further examined by conducting a longitudinal study that will monitor serum CMV seropositivity and corresponding titre over several time points using the same group of participants. Only then the conclusion can be made if the comparable levels between these two groups are indeed the consequence of preserved latent virus control in younger caregivers or present results are the artefact of, for example, different infection times. Further, other parameters of the immune system directed against CMV should be measured. This includes CMV-specific T cell immunity, as the main effector system responsible of controlling CMV infection, as well as IgM antibody titre, that, in addition to

IgG would provide a clearer picture about the time of the infection and if re-infection of the virus has occurred.

The findings of the study presented in chapter 3 suggested that those caregivers with higher psychological morbidity show poorer neutrophil functioning. Therefore, they emphasise the importance of extending the future research focus from caregivers in general, towards those with greater stress, depression, anxiety and burden. In other words, rather than generalising the effect of caregiving stress across the population it is perhaps more important to focus on detecting individual feelings, needs and ability to function under particular circumstances. This will not only enable early detection of those individuals that need greater psychosocial support, but will also provide the insight to the method of the intervention that might be the most helpful in order to preserve effective immune function. For example, this particular group of caregivers showed poorer neutrophil phagocytosis associated with higher anxiety and burden and poorer neutrophil ROS production with poorer sleep, suggesting therefore, that those individuals would most benefit from the interventions targeting lowering anxiety and providing more respite time. The need for individualisation of treatment is further supported by the findings in chapter 4 that identified problematic behaviour in care-recipient as the driver of the higher senescent profile amongst older caregivers, but no association between other psychosocial variables and immune functioning, again suggesting a need for respite care and possibly interventions to help cope with dementia patients' challenging behaviours.

Higher expression of the KLRG1 marker in caregivers despite comparable senescent and exhausted profiles assessed through CD28, CD57 and PD-1 marker presented in chapter 4 is perhaps the most relevant for the future studies focussing on immunity. First, it complements the previously reported studies that found a poorer vaccination response in both younger and

older caregivers when compared to the age- and sex-matched controls. Second, KLRG1 is starting to emerge as the safest target for the reversion of the early senescence and exhaustion (Akbar and Henson 2011), and therefore potentially the best pathway for the future interventions. One direction would be to further explore KLRG1 as a potential mechanism for increasing the function of specific T cells. This could then be used in future medical interventions to boost vaccination response in older and stressed adults.

The final study in chapter 5 showed no difference in neutrophil function and stress hormone levels between bereaved and controls, which was driven by the lack of the effect in younger group. There was also no indication of an association between psychological morbidity in bereaved and immune or endocrine parameters, with the exclusion of those losing closer relative and reporting higher grief having higher cortisol:DHEAS ratio, while those with higher social support having lower ratio. This suggests that in the case of bereavement, immunosenescence is perhaps necessary for exerting negative effect on neutrophil and endocrine function. However, these findings are only preliminary and need to be further confirmed in larger studies. Further, additional measures of immune function would help in understanding if the preservation of neutrophil function in young bereaved adults is in fact representative of the immune response in general. Interventions that address grief in younger caregivers could also be a useful model for the study of the potential to improve immune function in those at risk due to high psychological morbidity. Finally, additional decrease in DHEAS in those caregivers and bereaved from the older cohort who report higher anxiety symptomatology, perceived stress, and for bereaved participants, higher feelings of grief point to, and further support the idea of an randomised controlled trial of DHEA replacement therapy to the stressed older adults.

6.10. CONCLUSIONS

In conclusion, the present studies demonstrated that the effect of chronic stress on immune and endocrine function, be it caregiving or bereavement, is not straightforward, but depends on many factors. These factors include the specific immune parameters or hormones measured; the age of stressed person; and, at least in the case of caregiving stress, the psychological resilience or burden of the individual. There was no evidence that immune control over CMV virus is compromised in younger parental caregivers, and similarly for neutrophil function and serum cortisol and DHEAS in young bereaved, suggesting that previously reported decrements in these immune parameters for older populations were most likely a consequence of the synergistic effect of chronic stress and immunosenescence. On the other hand, neutrophil function, as well as some of the markers of T cell-related exhaustion and senescence, were preserved in both young and older caregivers when compared to the age-matched controls. Similarly, serum cortisol and DHEAS as well as their ratio were comparable between the groups, with the main differences emerging between young and old. However, the differences between caregivers and controls emerged for the KLRG1 T cell profile, confirming previously the reported decrease in the vaccination response in both young and old caregivers when compared to controls, and emphasising the potential for manipulating KLRG1 related pathways in future immunotherapeutic measures for boosting the immunity in the aged and stressed. In addition, caregivers had a higher serum cortisol:cortisone ratio, which could indicate a raised 11β-HSD1 activity in this stressed group. Finally, the caregiving studies indicated the differential effects of caregiving stress-influenced behaviours or emotions on different immune parameters. In the case of CMV antibody titre, it was unhealthy behaviours such as poorer diet and exercise levels and higher BMI that was associated with the higher CMV antibody titre. Further, caregivers with

higher anxiety and burden levels as well as poorer sleep showed poorer neutrophil function, while older caregivers who reported more problem behaviours in their spouses with dementia had higher senescent profile. Finally, although there was no association between psychological morbidity and neutrophil function in the young bereaved adults, those who lost closer relative had lower serum DHEAS, and higher cortisol:DHEAS ratio. These findings implicate the importance of measuring individual differences in chronically stressed groups more closely rather than simply focusing on stress exposure *per se*, as a better way of examining mechanisms linking stress and health, and targets for potential intervention.

APPENDIX

ADDITIONAL FINDINGS FROM CHAPTER 3

The Table below presents the raw serum cytokine values (in pg/ml) collected from the older and young, caregiving and control participants presented in the chapter 3. Symbol [a] presents significant age effect; symbol [b] significant difference between caregivers and controls; and symbol [c] significant age * caregiving interaction effect. Serum levels of the cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-15, IFN-γ, TNF-α, and GM_CSF were simultaneously detected using a Human Cytokine 10-Plex Panel for 96-well plate (Invitrogen). The assay was performed following suppliers instructions (Invitrogen, Cat. no.LHC0001). Briefly, the polystyrene beads internally dyed with red and infrared fluorophores of different intensities are conjugated to cytokine-specific capture antibodies. The wells were coated with 50µl of mixed beads, aspirated and washed twice, after which 100µl of and 100µl of previously diluted standards were added to the each well and incubated on the shaker for 2 hours at 250rpm. After washing, cytokine-specific biotinylated detector antibodies were added and incubated for 1 hour allowing binding to the corresponding immobilized cytokines. Washing step was preformed to remove the excess of biotinylated antibody, after which PE-streptavidin was added and incubated for 30 minutes to allow the formation of a four-member solid phase sandwich. The excess PE-streptavidin was washed and the beads were analysed using a Luminex detection system (Luminex 100). The specific cytokines and their concentrations were determined by monitoring the bead colour (analyte) and the signal strength of the assay (PE fluorescence).

	Mean (SD)			
	Young		Older	
	Caregivers (N = 52)	**Controls (N = 32)**	**Caregivers (N =37)**	**Controls (N =40)**
IL-1β [a]	13.4 (24.63)	9.7 (9.16)	12.2 (28.24)	8.7 (37.56)
IL-6	6.8 (11.07)	4.0 (4.87)	14.0 (27.02)	5.1 (8.17)
GM-CSF [c]	20.1 (28.0)	27.6 (36.92)	35.7 (52.99)	24.1 (41.41)
IL-5	32.6 (101.19)	5.3 (9.09)	13.3 (40.12)	13.1 (36.20)
IFN-γ	6.6 (21.75)	2.4 (1.95)	6.8 (13.01)	2.7 (2.68)
TNF-α	44.0 (100.46)	10.7 (14.78)	43.4 (100.47)	22.9 (47.84)
IL-2 [a]	6.5 (10.11)	6.5 (7.74)	6.8 (15.36)	5.8 (13.67)
IL-4 [b]	24.93 (55.44)	8.6 (7.68)	29.8 (65.24)	16.8 (39.52)
IL-8 [a, b, c]	21.7 (33.42)	16.8 (24.50)	58.9 (156.38)	0.2 (1.37)
IL-10 [c]	66.5 (98.49)	81.2 (126.28)	103.4 (146.27)	70.2 (122.39)

ADDITIONAL FINDINGS FROM CHAPTER 4

The Table below presents correlations between CMV titres and the T cell senescent ($CD28^-$ and $CD57^+$) profiles split between young and older adults.

	r	df	p
Young			
$CD4^+CD28^-$ T cells	.32	41	.04
$CD8^+CD28^-$ T cells	.48	41	.001
$CD4^+CD57^+$ T cells	.43	41	.001
$CD8^+CD57^+$ T cells	.32	41	.04
Older			
$CD4^+CD28^-$ T cells	.45	53	.001
$CD8^+CD28^-$ T cells	.43	53	.001
$CD4^+CD57^+$ T cells	.39	53	.004
$CD8^+CD57^+$ T cells	.22	54	.11

HEALTH BEHAVIOUR QUESTIONNAIRE

Please circle the appropriate answer

1.	Over the last year, how many cigarettes, on average, did you smoke per day?	None	1-5	6-10	11-20	21+	40+
2.	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

3.	Over the last year, on average, how many units did you drink per week?	None	1-5	6-10	11-20	20-40	41+
4.	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+

5.	Over the last year, how often have you taken vitamin/mineral supplements?	Never	Special Occasions only	1-2 per month	1-2 per week	Almost daily	2 per day or more

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

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

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

If other, please state…….

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

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

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

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)							
Beef/lamb/pork/ham/ bacon							
Soft drinks (non-caffeinated)							
Pure fruit juice							

PITTSBURGH'S SLEEP QUALITY INDEX (PSQI)

1. During the past year, what time have you usually laid down to go to sleep?
   Usual Bed time: _____

2. During the past year, how long (in minutes) has it taken you to fall asleep at night?
   Number of minutes: _____

3. During the past year what time have you usually gotten up in the morning?

   Getting up time: _____

4. During the past year how many hours of actual sleep did you get a night? (This may be different than the number of hours you spent in bed)
   Hours of sleep per night:_____

For each of the remaining questions, mark the one best response. **Please answer all questions**.

5. During the **past year**, how often have you had **trouble sleeping** because you……..

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
Could not get to sleep within 30 min…..				
Woke up in the middle of the night or early in the morning…..				
Had to get up to use the bathroom…				
Could not breathe comfortably…				

Felt too hot…				
Felt too cold …..				
Had bad dreams….				
Had pain ……				

6. During the past year, how would you rate your sleeping quality overall?

Very bad **Fairly bad** **Fairly good** **Very good**

7. During the past year, how often have you taken medicine (prescribed or 'over the counter') to help you sleep?

Not during the past month **Less than once a week** **Once or twice a week** **Three or more times a week**

8. During the past year, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

Never **Less than once a week** **Once or twice a week** **Three or more times a week**

9. During the past year, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

Not problem at all **Only a very slight problem** **Somewhat of a problem** **A very big problem**

HOSPITAL ANXIETY AND DEPRESSION SCALE (HADS)

This questionnaire is about how you feel in general. Read each item and circle the reply which comes closest to how you have been feeling in the past week. Don't take too long over your replies: your immediate reaction to each item will probably be more accurate than a long thought-out response.

1. I feel tense or wound up	Most of the time	A lot of the time	Time to time	Not at all
2. I still enjoy the things I used to enjoy	Definitely as much	Not quite so much	Only a little	Hardly at all
3. I get a sort of frightened feeling as if something awful is about to happen	Very definitely and quite badly	Yes, but not too badly	A little, but it doesn't worry me	Not at all
4. I can laugh and see the funny side of things	As much as I always could	Not quite as much now	Definitely not so much now	Not at all
5. Worrying thoughts go through my mind	A great deal of the time	A lot of the time	From time to time but not too often	Only occasionally
6. I feel cheerful	Not at all	Not often	Sometimes	Most of the time
7. I can sit at ease and feel relaxed	Definitely	Usually	Not often	Not at all
8. I feel as if I am slowed down	Nearly all the time	Very often	Sometimes	Not at all
9. I get a sort of frightened feeling like butterflies in the stomach	Not at all	Occasionally	Quite often	Very often
10. I have lost interest in my appearance	Definitely	I don't take so much care as I should	I may not take quite as much care	I take as much care as ever
11. I feel restless as if I have to be on the move	Very much indeed	Quite a lot	Not very much	Not at all
12. I look forward with enjoyment to things	As much as ever I did	Rather less than used to	Definitely less than I used to	Hardly at all
13. I get sudden feelings of panic	Very often indeed	Quite often	Not very often	Not at all
14. I can enjoy a good book or radio or TV programme	Often	Sometimes	Not often	Very seldom

CAREGIVING ACTIVITY SURVEY (CAS)

These questions ask about your caregiving time.

On average, for any given day or night, how many hours do you spend doing the following for your children.....?

1. Taking them to various places (other than shopping) by car or public transport (please include preparation time).
   _____ Hours _____Min

2. Helping them to dress (undress, dress, reminding them what to wear etc)
   _____ Hours _____Min

3. Helping them with their eating (cutting food, feeding, cleaning them after eating)
   _____ Hours _____Min

4. Helping them to wash, brush their teeth, maintain their appearance over the day
   _____ Hours _____Min

5. Watching and supervising them in case they get into difficulty
   _____ Hours _____Min

6. On average, for any given week, how many hours do you feel you have lost either for work or leisure because of your caregiving role.

   _____ Hours _____Min

7. On average, for any given month, how many hours respite (social services or friends and family) from your caregiving duties do you receive?
   _____ Hours _____Min

CAREGIVING BURDEN INDEX (BI)

This questionnaire assesses your stress experience whilst caring for your child/children. There are no right or wrong answers. It is just your own personal feeling that count. For each item, please indicate how often **you** have felt that way.

1. Do you feel that because of the time you spend with your child that you don't have enough time for yourself?	Never	Rarely	Sometimes	Quite frequently	Nearly always
2. Do you feel stressed between caring for your child and trying to meet other responsibilities for your family or work?	Never	Rarely	Sometimes	Quite frequently	Nearly always
3. Do you feel angry when you are around your child?	Never	Rarely	Sometimes	Quite Frequently	Nearly always
4. Do you feel that your child currently affects your relationship with other family members or friends in a negative way?	Never	Rarely	Sometimes	Quite frequently	Nearly always
5. Do you feel that your health has suffered by your involvement with your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
6. Do you feel strained when you are around your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
7. Do you feel that you don't have much privacy as you would like, because of your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
8. Do you feel that your social life has suffered because you are caring for your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
9. Do you feel you have lost control of your life because of your child's disability?	Never	Rarely	Sometimes	Quite frequently	Nearly always
10. Do you feel uncertain about what to do about your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
11. Do you feel you should be doing more for your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always
12. Do you feel you could be doing a better job of caring for your child?	Never	Rarely	Sometimes	Quite frequently	Nearly always

STRENGHTS AND DIFFICULTIES QUESTIONNAIRE (SDQ)

For each item, please mark the box on the basis of the child's behaviour over the last six months. It would help us if you answered all items as best you can, even if they seem daft! If you have more than one child with developmental disabilities, please complete the questionnaire regarding the child with what you consider to be the most difficult behaviour.

	Not True	**Somewhat True**	**Certainly True**
1. Considerate of other people's feelings			
2. Restless, overactive, cannot stay still for long			
3. Often complains of headaches, stomach-aches or sickness			
4. Shares readily with other children (treats, toys, pencils etc)			
5. Often has temper tantrums or hot tempers			
6. Rather solitary, tends to play alone			
7. Generally obedient, usually does what adults request			
8. Many worries, often seems worried			
9. Helpful if someone is hurt, upset or feeling ill			
10. Constantly fidgeting or squirming			
11. Has at least one good friend			
12. Often fights with other children or bullies them			
13. Often unhappy, down-hearted or tearful			
14. Generally liked by other children			
15. Easily distracted, concentration wanders			
16. Nervous or clingy in new situations, easily loses confidence			
17. Kind to younger children			

18. Often argumentative with adults			
19. Picked on or bullied by other children			
20. Often volunteers to help others (parents, teachers, etc)			
21. Can stop and think things out before acting			
22. Can be spiteful to others			
23. Gets on better with adults than with other children			
24. Many fears, easily scared			
25. Sees tasks through to the end, good attention span			
• Takes a long time to settle to sleep at night			
• Wakes in the night often for long periods			
• Is awake very early before anyone else in the family			

During the past year how many times did you awake per night on average? (This may be due to young child(ren) waking and needing care) **Times awake per night**:_____

During the past year, how often have you had trouble sleeping because your child(ren) is/are wakeful and need care	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week

PERCEIVED STRESS (PSS)

The questions in this scale ask you about your feelings and thoughts during the **LAST MONTH**. In each case, indicate by ticking in the appropriate space how often you felt or thought a certain way. Although some of the questions are similar, there are differences between them and you should treat each one as a separate question.

	Never	Almost never	Sometimes	Fairly often	Very often
1. In the past month, how often have you been upset because of something that happened unexpectedly?					
2. In the past month, how often have you felt that you were unable to control the important things in your life?					
3. In the past month, how often have you felt nervous or stressed?					
4. In the past month, how often have you dealt with irritating life hassles?					
5. In the past month, how often have you felt that you were effectively coping with important changes that were occurring in your life?					
6. In the past month, how often have you felt confident about your ability to handle personal problems?					

7. In the past month, how often have you felt that things were going your way?					
8. In the past month, how often have you felt that you could not cope with all the things you had to do?					
9. In the past month, how often have you been able to control irritations in your life?					
10. In the past month, how often have you felt that you were on top of things?					
11. In the past month, how often have you been angered because of things that happened that were outside of your control?					
12. In the past month, how often have you found yourself thinking about things that you have to accomplish?					
13. In the past month, how often have you been able to control the way you spend your time?					
14. In the past month, how often have you felt difficulties were piling up so high that you could not overcome them?					

SUPPORT FUNCTION SCALE - SHORT FORM (SFS-SF)

Listed below are 12 different types of assistance which people sometimes find helpful. This questionnaire asks you to indicate how ***much*** help you need in these areas. Please ***circle*** the response that best describes your needs. Please answer all the questions.

How often is each of the following kinds of support available to you if you need it?

1. Someone to talk about things that worry you	Never	Once in a while	Sometimes	Often	Quite Often
2. Someone to help take care of your child	Never	Once in a while	Sometimes	Often	Quite Often
3. Someone to talk to when you have questions about raising your child	Never	Once in a while	Sometimes	Often	Quite Often
4. Someone who loans you money when you need it	Never	Once in a while	Sometimes	Often	Quite Often
5. Someone to encourage or keep you going when things seems hard	Never	Once in a while	Sometimes	Often	Quite Often
6. Someone who accepts your child regardless of how (s)he acts	Never	Once in a while	Sometimes	Often	Quite Often
7. Someone to help with household chores	Never	Once in a while	Sometimes	Often	Quite Often
8. Someone to relax or joke with	Never	Once in a while	Sometimes	Often	Quite Often
9. Someone to do things with your child	Never	Once in a while	Sometimes	Often	Quite Often
10. Someone to provide you or your child with transportation	Never	Once in a while	Sometimes	Often	Quite Often
11. Someone to hassle with agencies or individuals when you can’t	Never	Once in a while	Sometimes	Often	Quite Often
12. Someone who tells you about services for your child or family	Never	Once in a while	Sometimes	Often	Quite Often

MEDICAL OUTCOMES STUDY: SOCIAL SUPPORT SURVEY (MOS-SSS)

About how many close friends and close relatives do you have (people you feel at ease with and can talk to about what is on your [ ] mind)?

People sometimes look to others for companionship, assistance, or other types of support.

How often is each of the following kinds of support available to you if you need it?

	None of the time	A little of the time	Some of the time	Most of the time	All of the time
1. Someone to help you if you were confined to bed					
2. Someone you can count on to listen to you when you need to talk					
3. Someone to give you good advice about a crisis					
4. Someone to take you to the doctor if you needed it					
5. Someone who shows you love and affection					
6. Someone to have a good time with					
7. Someone to give you information to help you understand a situation					
8. Someone to confide in or talk to about yourself or your problems					

9. Someone who hugs you					
10. Someone to get together with for relaxation					
11. Someone to prepare your meals if you were unable to do it yourself					
12. Someone whose advice you really want					
13. Someone to do things with to help you get your mind off things					
14. Someone to help with daily chores if you were sick					
15. Someone to share your most private worries and fears with					
16. Someone to turn to for suggestions about how to deal with a personal problem					
17. Someone to do something enjoyable with					
18. Someone who understands your problems					
19. Someone to love and make you feel wanted					

(PEARLIN) PROBLEMATIC BEHAVIOUR

In the **past week**, how many days did you personally have to deal with the following behaviour of your spouse/partner? On how many days did she/he:

1. Keep you up at night	No days	1-2 days	3-4 days	5/more days
2. Repeat questions/stories	No days	1-2 days	3-4 days	5/more days
3. Try to dress the wrong way	No days	1-2 days	3-4 days	5/more days
4. Have a bowel or bladder "accident"	No days	1-2 days	3-4 days	5/more days
5. Hide belongings and forget about them	No days	1-2 days	3-4 days	5/more days
6. Cry easily	No days	1-2 days	3-4 days	5/more days
7. Act depressed or downhearted	No days	1-2 days	3-4 days	5/more days
8. Cling to you or follow you around	No days	1-2 days	3-4 days	5/more days
9. Become restless or agitated	No days	1-2 days	3-4 days	5/more days
10. Become irritable or angry	No days	1-2 days	3-4 days	5/more days
11. Swear or use foul language	No days	1-2 days	3-4 days	5/more days
12. Becomes suspicious, or believe someone is going to harm him/her	No days	1-2 days	3-4 days	5/more days
13. Threaten people	No days	1-2 days	3-4 days	5/more days
14. Show sexual behaviour or interests at the wrong time/place	No days	1-2 days	3-4 days	5/more days

THE QUESTIONS ABOUT THE BEREAVEMENT

These questions are about your recent bereavement.

1. How long is it since you were bereaved? (days/months – please delete one)

2. How was the person who has died related to you (spouse/relative/friend)?

3. How old was this person when they died?

4. Was the death of this person expected/unexpected? (please delete one)

Please tick below if this event has happened to you.

For each event that happened to you, please score how serious (stressful, worrying, disruptive) a problem was on a scale of 1 to 10, where 1 is something really small and unimportant and 10 is the worst thing that could ever happen to you.

Spouse / partner died	Happened to me		⇨	1	2	3	4	5	6	7	8	9	10
	Didn't happen												

Other household member died	Happened to me		⇨	1	2	3	4	5	6	7	8	9	10
	Didn't happen												

Other close family (parent, child, sibling)	Happened to me		⇨	1	2	3	4	5	6	7	8	9	10
	Didn't happen												

Other more distant family died	Happened to me		⇨	1	2	3	4	5	6	7	8	9	10
	Didn't happen												

Friend died	Happened to me		⇨	1	2	3	4	5	6	7	8	9	10
	Didn't happen												

CORE BEREAVEMENT ITEMS (CBI)

These questions ask you about your feelings since your recent bereavement. Please circle the appropriate answer.

1. Do you experience images of the events surrounding this person's death?	Continuously	Quite a bit of the time	A little bit of the time	Never
2. Do thoughts of this person come into your mind whether you wish it or not?	Continuously	Quite a bit of the time	A little bit of the time	Never
3. Do thoughts of this person make you feel distressed?	Always	Quite a bit of the time	A little bit of the time	Never
4. Do you think about this person?	Continuously	Quite a bit of the time	A little bit of the time	Never
5. Do images of this person make you feel distressed?	Always	Quite a bit of the time	A little bit of the time	Never
6. Do you find yourself preoccupied with images or memories of this person?	Continuously	Quite a bit of the time	A little bit of the time	Never
7. Do you find yourself thinking of reunion with this person?	Always	Quite a bit of the time	A little bit of the time	Never
8. Do you find yourself missing this person?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
9. Are you reminded by familiar objects (photos, possessions, rooms etc.) of this person?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
10. Do you find yourself pining for/yearning for this person?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
11. Do you find yourself looking for X in familiar places?	A lot of the time	Quite a bit of the time	A little bit of the time	Never

12. Do you feel distress/pain if for any reason you are confronted with the reality that this person is not present/not coming back?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
13. Do reminders of this person such as photos, situations, music, places etc. cause you to feel longing for this person?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
14. Do reminders of this person such as photos, situations, music, places etc. cause you to feel loneliness?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
15. Do reminders of this person such as photos, situations, music places etc. cause you to cry about this person?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
16. Do reminders of this person such as photos, situations, music places etc. cause you to feel sadness?	A lot of the time	Quite a bit of the time	A little bit of the time	Never
17. Do reminders of this person such as photos, situations, music places etc. cause you to feel loss of enjoyment?	A lot of the time	Quite a bit of the time	A little bit of the time	Never

THE IMPACT OF EVENT SCALE (IES)

Below is a list of comments made by people after stressful life events like bereavement. Using the following scale, please indicate (with a tick) how frequently each of these comments were true for you DURING THE PAST SEVEN DAYS.

With regard to your bereavement…	Not at all	Rarely	Sometimes	Often
1. I thought about it when I didn't mean to				
2. I avoided letting myself get upset when I thought about it or was reminded of it				
3. I tried to remove it from memory				
4. I had trouble falling asleep or staying asleep because of pictures or thoughts about it that came into my mind				
5. I had waves of strong feelings about it				
6. I had dreams about it				
7. I stayed away from reminders of it				
8. I felt as if it hadn't happened or wasn't real				
9. I tried not to talk about it				
10. Pictures about it popped into my mind				
11. Other things kept making me think about it				
12. I was aware that I still had a lot of feelings about it, but I didn't deal with them				
13. I tried not to think about it				
14. Any reminder brought back feelings about it				
15. My feelings about it were kind of numb				

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