Synergistic effects of physical and psychological distress on immunesenescence

by

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Abstract

Ageing is accompanied by impairments in immune responses. In this thesis, an age associated numerical and functional deficit in a novel subset of immunosuppressive $CD19^{+ve}$ $CD24^{hi}$ $CD38^{hi}$ B cells is reported, which might be an additional factor contributing towards increased risk of systemic autoimmunity with advancing age. The immune system is profoundly affected by environmental factors such as stress. This thesis also examined the synergistic effect of the physical stress of a hip fracture and the psychological stress of depressive symptoms on immune function of older adults. It was observed that 37% of our hip fracture patients had developed depressive symptoms 6 weeks post-surgery. A significant decline in neutrophil, monocyte and NK cell functioning was reported in hip fracture patients with depressive symptoms, but not in those with hip fracture alone. Additionally, an increased cortisol: DHEAS ratio was also only found in hip fracture patients with depressive symptoms. An elevated level of pro-inflammatory (IL6, TNFα) and anti-inflammatory (IL10) cytokines were observed in hip fracture patients with depressive symptoms. In the adaptive immune system, a reduction in circulating T cells and an accumulation of senescent and activated T cells was also found in the hip fracture patients who developed depressive symptoms. Finally, the age associated numerical and functional deficit in IL10 production by $CD19^{+ve}$ $CD24^{hi}$ $CD38^{hi}$ B cells was further suppressed on exposure to chronic stress in hip fracture patients with depressive symptoms. In conclusion, depressive symptoms in hip fracture patients have been associated with immune dysregulation.

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Abbreviations

ABC	Age associated B cell
Abs	Antibodies
ACTH	Adreno corticotrophin hormone
ADCC	Antibody dependant cell mediated cytotoxicity
ADL	Activities of daily living
ANA	Anti-nuclear antibody
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASC	Antibody secreting cell
ATCC	American type culture collection
BAFF	B cell activating factor
BBS	Berg balance scale
BCR	B cell receptor
BMI	Body mass index
BSA	Bovine serum albumin
Ca	Calcium
CBG	Corticosteroid binding globulin
CD	Cluster of Differentiation
CD40L	CD40 ligand
CD62L	CD62 ligand
CIA	Collagen induced arthritis
CM	Central memory
CMV	Cytomegalovirus
CNS	Central nervous system
CRF	Corticotrophin Releasing Factor
CRH	Corticotrophic Releasing Hormone
CRP	C-reactive protein

CTL	Cytotoxic lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone – sulphate
DHR	Dihydrorhodamine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DUSP4	Dual specific phosphatase 4
EAE	Experimental autoimmune encephalitis
EDTA	Ethylenediamine tetra acetic acid
E.coli	*Escherchia coli*
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
EMRA	Effector memory RA
ERK	Extracellular signal regulated kinase
FACS	Fluorescent activated cell sorter
Fas L	Fas ligand
Fc	Fragment crystallisable
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FO B cells	Follicular B cells
Foxp3	Forkhead winged helix transcription factor 3
FS	Forward scatter
GC	Glucocorticoids
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GDS	Geriatric depression scale
GPS	Glutamine , penicillin, streptomycin

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HADS	Hospital anxiety and depression scale
HiFCS	Heat inactivated foetal calf serum
HLA	Human leukocyte antigen
HPA	Hypothalamus pituitary adrenal
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cells
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
H_2SO_4	Sulphuric acid
Hsp90	Heat shock protein 90
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
IFNγ	Interferon gamma
IP_3	Inositol triphosphate
IRP	Immune risk phenotype
Jak	Janus kinase
JNK	c-Jun N terminal kinase
KGF	Keratinocyte growth factor
KIR	Killer-cell immunoglobulin like receptor
KLRG1	Killer-cell lectin-like receptor subfamily G member 1
LAMP-1	Lysosomal – associated membrane protein-1
LFA	Lymphocyte functional antigen
LPS	Lipopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal antibody
Mac-1	Macrophage – 1 antigen
MACS	Magnetic activated cell sorting

MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein-1
MMSE	Mini mental state exam
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
MVC	Max voluntary contraction
MZ B cells	Marginal zone B cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NCR	Natural cytotoxicity receptor
NET	Neutrophil extracellular trap
NFkB	Nuclear factor kappa B
NK cell	Natural Killer cell
NKCC	Natural Killer Cell Cytotoxicity
NKT cell	Natural killer T cell
NOD	Non-obese diabetes
OHS	Oxford hip score
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PI_3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear leukocyte
PHA	Phytohemagglutinin
PVN	Para ventricular nucleus of hypothalamus

RA	Rheumatoid arthritis
RAG	Recombinant activating gene
RANTES	Regulated on activation, normal T cell expressed and secreted
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell park memorial institute
SCID	Sever combined immunodeficient
SD	Standard deviation
SDF-1	Stromal derived factor-1
SEM	Standard error of mean
SHP-1	Src homology region 2 domain-containing phosphatase-1
SLE	Systemic lupus erythematous
SS	Side scatter
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGFβ	Transforming growth factor beta
T$_H$ cells	Helper T cells
TLR	Toll like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
T$_2$MZP	Transitional 2 marginal zone precursor B cells
TNFα	Tumor Necrosis Factor alpha
TRAIL	TNF related apoptosis inducing ligand
T$_{reg}$	Regulatory T cells
TREC	T cell receptor excision circles
TREM-1	Triggering receptor expressed on myeloid cells 1
TUG	Timed-up and go
VEGF	Vascular endothelial growth factor

Chapter 1
General Introduction

1.0 Population ageing

We are an ageing population, with a falling birth rate and an increase in the number of people reaching older age. Advances in the field of medicine and improvement in public health, socio-economic developments and scientific advances have led to an increase in human life-span in developed countries. According to the UK national statistics in 2010, 17% percent of the total population were over the age of 65, and if current demographic trends continue it is predicted that by 2035, the population aged 65 and over will account for 23 percent of the total population; resulting in a twenty percent increase in people aged 65 and over [Figure1.1]. The number of people aged 85 and over has been predicted to double over the period1985-2010 (UK National Statistics website).

Figure 1.1 The proportion of individuals aged over 65 is predicted to increase over the time period 2010-2035.

Over the period 1985-2010 a twenty percent increase was observed in the number of individuals aged 65 and above. The number of people aged 85 and above more than doubled. By 2035, the numbers of individuals aged 85 and above are predicted to be almost 2.5 times larger than in 2010. Individuals aged 65 and over are predicted to account for 23 percent of the total population by 2035. Figure taken from the UK National Statistics website (http://www.statistics.gov.uk/hub/population/ageing/older-people)

This increase in life expectancy will have a potential benefit only if the 'added years' are years of relative good health. But, advancing age is accompanied by an increase in susceptibility towards infections (pneumonia, urinary tract infections), cancer and development of chronic illnesses [1, 2] that have a negative impact on an individual's quality of life [3]. Additionally, older adults account for a large proportion of health-care resources [4]. Suggesting, that healthy life expectancy is not keeping pace with the increase in life expectancy [Figure 1.2] , which is a matter of concern for individuals and health care providers. Development of strategies to delay or reverse the negative impact of ageing will have a beneficial effect on health in older adults and are now urgently needed.
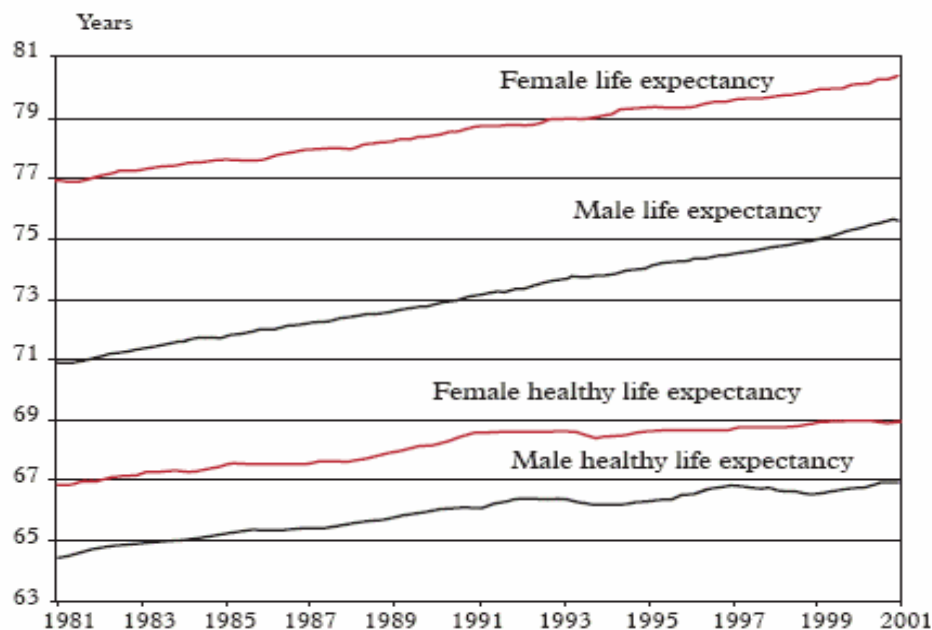
Figure 1.2 The life expectancy and healthy life expectancy proportion of individuals aged over 65 .

For males in the UK , life expectancy has inceased from 70.8 years in 1981 to 75.9 in 2002, for females an increase in life expectancy from 76.8 years in 1981 to 80.5 years in 2002 occurred. At the current rate, an increase in life expectancy of about two years has been reported for each decade. However, healthy life expectancy based on the self-assessed general health questionnaire is not keeping pace with the increase in life expectancy. Figure taken from the House of Lords Science and Technology Committee Report, Ageing: Scientific Aspects, July 2005.

1.1 Immunesenescence

Healthy ageing has been associated with a significant decline in immune competence and ability to mount a robust immune response, termed Immunesenescence [5]. A Swedish longitudinal study reported an association between reduced immune function and individual longevity [6]. The lifelong exposure to pathogenic agents for a longer period than previously encountered during human evolution (chronic antigen load), reduced production of new T lymphocytes from the thymus, increased proliferation of peripheral T and B lymphocytes (homeostatic proliferation) in response to thymic atrophy and an age-related increase in systemic inflammation (inflammaging) are thought to act as major driving forces of immune system ageing [7]. The clinical consequences of this impairment in immune responses imply that immunesenescence might be responsible for an increase in the incidence and severity of infections [8] , impaired vaccine responses [3] and possibly increased risk of cancers and chronic inflammatory disorders with advancing age [9].

All cells of the immune system (myeloid and lymphoid lineage) are formed from the differentiation of the multipotent haematopoietic stem cell (HSC). Hematopoietic stem cells differentiate into multipotent progenitor cells, which can produce cells of either myeloid (neutrophils, monocytes, macrophages, dendritic cells and eosinophils) or lymphoid (T cells, B cells and NK cells) lineage. HSCs in older donors have a reduced ability for self-renewal and a myeloid skewing of their differential potential [10, 11]. Molecular factors contributing to HSC ageing include ; accumulation of DNA damage, altered gene expression patterns and epigenetic dysregulation [12]. Skewing of the HSCs with age is thought to be an additonal factor contributing towards declining immuno-competence [12].

Several innate and adaptive immunological parameters are altered with age, and they can be used as biomarkers of likely mortality in older adults and have been defined as the 'Immune

Risk Phenotype'. The IRP is characterised by an inverted CD4/CD8 ratio, highly differentiated circulating CD8 T cells, CMV seropositivity and high levels of proinflammatory cytokines, specifically IL6 [6, 13, 14].

1.2 Inflammaging

A universal feature of physiological ageing is an increase in circulating levels of pro-inflammatory cytokines (IL-1β, IL-6, IL8, TNFα, IFNγ, and CRP) termed "Inflammaging" [15-17]. A strong association has been reported between elevated pro-inflammatory cytokine levels in older adults and an age-associated increase in mortality [18, 19], frailty [20, 21] prevalence of age related chronic diseases [17, 21] and cognitive impairment [22, 23]. Studies in centenarians [24] and extremely long-lived mice [25] show that long lived individuals maintain the cytokine profile of younger adults.

The various factors driving inflammaging include; increase in adipose tissue (a major source of inflammatory cytokines and adipokines) [26], decreased production of sex steroids many of which are anti-inflammatory [27], sub-clinical infections [28], sedentary lifestyle [29] and a low grade activation of cytokine producing cells such as macrophages [17]. Interestingly, CMV infection was previously proposed as a major driver of inflammaging [30], however this theory has been recently challenged as inflammaging still occurred in a longitudinal study of CMV negative adults [31]. Anti-inflammatory cytokines (such as IL10) that might help to counteract the age associated elevated inflammatory state are known to decline with age [32], resulting in disturbed immune homeostasis. However, this finding has been contradicted with another report of elevated circulating levels of IL10 with age [33]. Interestingly, possession of genetic variations associated with high IL10 production has been associated with successful ageing [32].

1.3 Ageing of the innate immune system

The innate immune system acts as the first barrier encountered by pathogens and is responsible for immediate and robust immune responses towards micro-organisms. In aged subjects, a breakdown in the integrity of innate physical barriers such as the skin, gastrointestinal tract and lung occurs, resulting in increased susceptibility to invasion by pathogens and an increased burden on the cells of the innate immune system.

1.3.1 Neutrophils

Neutrophils are the most abundant leukocyte and are one of the first cells to migrate into the site of infection. They are responsible for recognising, ingesting and destroying pathogens, most importantly rapidly dividing bacteria, yeast and fungi. The increased incidence of bacterial infections in older adults suggests [2, 34] that neutrophil numbers and/or function might be compromised with age.

Neutrophils have a very short life span (8-12 hr) in peripheral blood and are lost due to spontaneous apoptosis and as a consequence they are produced in large numbers ($1\text{-}2 \times 10^{11}$ per day) [35]. The bone marrow content of precursors for the neutrophil lineage as well as their number in blood remains unchanged in healthy older adults [36, 37]. During infection, an increase in neutrophil production is observed (neutrophilia) in order to combat infection. Studies have shown that older adults are able to mount a normal neutrophilia in response to an infection [38]. Neutrophil life span is controlled by pro-inflammatory cytokines (IFNγ) and chemokines, including granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) [36] that are able to delay apoptosis and extend neutrophil functional efficiency during infections. Neutrophils from older donors have a diminished ability to respond to anti-apoptotic mediators such as GM-CSF [39].

During an infection; neutrophils leave the circulation (extravasation), roll along and then adhere to vascular endothelial cells, followed by migration to the site of infection along a gradient of chemotactic factors. Once neutrophils have reached the site of infection via chemotaxis they engulf the invading microbes by phagocytosis. Pathogen phagocytosis by neutrophils is followed by microbial killing by several mechanisms, including generation of reactive oxygen and nitrogen species [40], release of proteolytic enzymes from intracellular granules [41]. Adherence of neutrophils to endothelium, which is essential for neutrophil extravasation, remains unaltered with age [42]. The expression of adhesion markers CD11a and CD11b, necessary for neutrophil extravasation is stable with age [43], but an increase in the expression of CD15 has been reported in older adults [44, 45].

Chemotactic molecules (IL-8, fMLP, complement C5a) are responsible for attracting neutrophils to the site of infection. There is a significant degree of discordance in the literature regarding chemotaxis (directional movement) of neutrophils from older adults; a few studies have reported that chemotaxis remains unchanged with age [42, 46], whilst others have reported reduced chemotaxis of neutrophils in response to fMLP and GM-CSF in older adults. Neutrophil chemokinesis (non-directional movement) remains unaltered with age [47-49]. A study taking place in our own group has shown a reduction in neutrophil chemotaxis with age [50]. This decrease in neutrophil directional control may compromise the efficiency of recruitment of neutrophils to the infection focus and neutrophils might take a longer route to the infection site, resulting in an increased exposure of tissue to proteases causing damage to healthy tissue.

An age related decline has also been reported in the percentage of phagocytosing neutrophils and the ability of neutrophils to ingest opsonised bacteria and yeast [45, 49]. However,

neutrophil ability to phagocytose unopsonised bacteria remains unaltered with age [51], suggesting that it is the response via opsonin receptors (Fcγ and C3b complement receptors) that is affected by ageing. A number of intracellular defects might contribute to this reduced neutrophil phagocytic ability including; impaired calcium mobilisation, reduced hexose transport, reduced CD16 expression, impaired downstream signalling events and impaired ligand induced actin polymerisation [35, 45, 52]. In addition to these intrinsic factors; extrinsic factors such as altered circulating cytokine levels may also contribute towards reduced neutrophil functioning efficiency with age.

Ingested microbes are destroyed by two different microbicidal mechanisms; production of reactive oxygen species and degranulation, which both lead to the release of cytotoxic proteases. Regarding the first mechanism, studies of neutrophil respiratory burst have reported unaltered superoxide production in response to PMA with age [44, 53, 54]. However, neutrophil superoxide production in response to gram-positive bacteria such as *S.aureus* has been shown to be reduced in older adults [49]. A few studies have reported an unaltered superoxide production in response to *E.coli* with age [35, 55] , but these findings have been contradicted by another study reporting reduced superoxide production [56]. The consensus thus appears to be that superoxide generation is impaired to gram positive bacteria, but may be less affected in response to gram negative infections. Also, a decline in degranulation in response to microbial products occurs with age [57].

In addition to phagocytosing pathogens and destroying them by generation of reactive oxygen species, neutrophils are also capable of engulfing and destroying pathogens via extrusion of neutrophil extracellular traps (NETs) [58]. However, so far, the effect of advancing age on NET generation remains unexplored.

<u>1.3.1.(a) Mechanisms of reduced neutrophil function</u>

Impaired neutrophil functioning detected in older adults might be a result of modifications in signalling pathways and receptor distribution with age. Alterations in physiochemical properties of neutrophil membranes, such as reduced membrane fluidity due to altered cholesterol/phospholipid content and changes in actin cytoskeleton occur with age in rodents, though the situation in humans has not been examined [59]. Altered membrane fluidity could explain the reduced signalling through PI-3 kinase, MAP kinase, protein kinase B, Jak-STAT, and SHP-1 signalling observed in neutrophils with increasing age [60]. Ca^{2+} ion mobilisation plays a central role in intracellular signal transduction, resting neutrophils of older adults have an increased level of Ca^{2+} [49] and are unable to mount a proper Ca^{2+} mobilisation response [61]. TREM-1 is a recently identified receptor responsible for initiating neutrophil responses, and impairment in TREM-1 engagement has also been observed in neutrophils of aged donors [62].

1.3.2. Monocytes / Macrophages

Circulating monocytes play a critical role in defense against invading pathogens by migrating to sites of infection, engulfing and killing microorganisms and cytokine release [63]. The differential expression of CD14 and CD16 has been used to define the three peripheral subsets of monocytes; classical monocytes ($CD14^{+ve}CD16^{-ve}$) represent about 90 % of peripheral monocytes; intermediate monocytes ($CD14^{+ve}CD16^{+ve}$) and non-classical monocytes ($CD14^{+ve}CD16^{++ve}$) comprise the remaining fraction of peripheral monocytes [64]. Classical monocytes are more efficient phagocytes and are major producers of reactive oxygen species, whereas $CD16^{+ve}$ monocytes are major producers of pro-inflammatory cytokines on stimulation [65].

The percentage and absolute number of peripheral monocytes remains unaltered with age [37, 66]. However, monocyte subsets considerably change and an increase in non-classical monocytes occurs with age, resulting in an overall shift from classical to non-classical monocytes [67, 68]. The bone marrow of older adults has lower numbers of CD68[+ve] macrophage precursors [69, 70]. A decline in haematopoietic cell proliferation due to replicative senescence and increased apoptosis could explain the reduction in macrophage precursors. However, the number, size, DNA content and cell surface markers expressed during macrophage maturation (e.g. Mac 1) are similar in macrophages from aged and young mice [71].

Ageing is accompanied by reduced surface expression of adhesion molecules such as CD62L, CX_3CR1 in older adults which might have an effect on monocyte adherence and migration to inflammation site [72, 73]; resulting in impairments in chemotactic responses [74]. Further, a reduction in monocyte phagocytic ability [73] and a decline in production of reactive oxygen species and nitrogen intermediates has also been reported with age; resulting in delayed pathogen clearance [75, 76]. Studies done in mice and humans have also reported a decline in macrophage migration and phagocytic ability with age [77-80]. On the contrary, studies done in aged rats have reported an increase in the phagocytic ability of specialised liver macrophages (Kupffer cells) and alveolar macrophages [81, 82]. Impairments in production of reactive oxygen intermediates in response to IFNγ, PMA or opsonised zymosan stimulation have been reported by macrophages in aged rats [83, 84]. Similarly, reductions in reactive nitrogen intermediate production by macrophages have also been reported in aged rodents [84-86].

Monocytes and macrophages activate other inflammatory cells via secretion of a range of cytokines (TNFα, IL-6), chemokines (IL-8), growth factors (GM-CSF, M-CSF), coagulation

factors and prostaglandin E2. As discussed previously, ageing is accompanied by an increase in levels of serum pro-inflammatory cytokines. Monocytes from aged individuals exhibit increased production of cytokines such as IL6, IL1β in basal conditions [87]. A study of monocytes from 154 young and old individuals found an age-associated reduction in TNF-α and IL-6 secretion after stimulation via TLR1/2 heterodimer [88], these findings have also been confirmed by another study [68]. Similarly, macrophage production of pro-inflammatory cytokines (IL-6, TNFα, IL12, and IL-1β) upon LPS stimulation is also reduced in both aged mice and humans [89-92]. This impairment in cytokine production has been related to decreased cell surface expression of TLR1, TLR2 and TLR4 on aged monocytes [88, 93] and macrophages [89, 92, 94, 95]. Additionally, defects in downstream signal transduction pathways have also been reported in LPS activated aged monocytes including ; impaired protein kinase C (PKC) activation , reduced expression of c-Fos , c-Jun and deficient mitogen activated protein kinase (MAPK) activation [68, 96]. Studies have also reported lower expression of MAP kinases p38 and Jun kinases in macrophages with age [91, 92]. However, a recent study reported an increase in IL8 production by aged monocytes on stimulation via TLR5 pathway, and have correlated this elevated cytokine production with increased levels of expression of TLR5 and increased levels of TLR5 mediated phosphorylation of MAPK p38 and ERK on monocytes with age [93].

Monocytes and macrophages are also involved in T cell mediated immunity. A decline in antigen presentation has been reported with advancing age [97], possibly due to reduced expression of MHC class II molecules on human monocytes [98, 99] and murine macrophages [100]. Co-stimulatory molecules CD80 and CD86 are also involved in mediating T cell response to infectious agents [101, 102]. Ageing is accompanied by diminished monocyte co-stimulatory activity due to reduced CD80 expression [98]. Additionally, an age associated

defect in upregulation of co-stimulatory molecules CD80 and CD86 by monocytes on stimulation via TLR agonists has also been observed, which contributes towards diminished vaccination responses in older adults [101].

1.3.3 Dendritic cells

Dendritic cells (DC) represent a rare population of circulating cells that form about 1% of total peripheral blood cells and play a key role in initiating and regulating pathogen-specific adaptive immune responses [103]. DCs in human peripheral blood have been divided into two subsets - myeloid lineage marker (CD11c) expressing DCs, termed myeloid DC (mDC) and lymphoid marker (CD123) expressing DCs , termed plasmacytoid DCs (pDC) [104]. pDCs are characterised by low phagocytic ability and production of type I interferon that plays an essential role in providing protection against viral infections [105, 106]. On the other hand, mDCs control immune responses by presenting antigens to activate naïve CD4 T cells[107].

A decline in circulating levels of DC precursors has been shown in older adults [108]. Further, a reduction in frequency and absolute numbers of pDCs has also been reported in older adults, which might contribute towards increased susceptibility towards infections [109-112]. However, percentage and absolute numbers of mDCs in peripheral blood of aged donors remain unaltered compared to young donors [111-113] , but one study so far has reported contradictory findings [108]. On examining DC phenotype, a decline in surface expression of MHC class II and unaltered expression of CD80 and CD40 has been reported with advancing age [108, 113, 114]. However, there is a slight contradiction regarding the effect of age on CD86 expression on DC, a few studies reported no effect of age on CD86 expression [113, 114] whilst another study reported an increase in CD86 expression on aged

DC [108]. Further, DCs in older adults show a more mature phenotype (express high levels of maturation marker CD83) [108].

Ageing is accompanied by a decline in DC ability to uptake foreign antigen and phagocytose apoptotic cells [114]. This impaired removal of apoptotic cells by DCs might be a contributing factor towards age associated increase in inflammation and may be responsible for higher risk of autoimmunity [115, 116]. Further impaired DC migratory responses towards chemokines (MIP-3β, SDF-1) also occurs with advancing age, which might have a negative effect on DC ability to migrate to lymph nodes and induce immune responses [114].

Studies examining constitutive cytokine production by DCs with age showed similar expression of IL12 and IL10 in young and aged donors [108]. However, on stimulation of DCs with a pathogen derived stimulus (such as LPS) a decline in IL12, TNFα and IL6 production occurs with age [117]. DCs play a critical role in generating immune responses against viral infections by secretion of interferons. An age associated decline has been reported in DC production and secretion of IFNγ [110, 111, 118], which might be a result of reduction in TLR7 and TLR9 expression [111, 113]. Interestingly, there is recent evidence suggesting that age associated defects in cytokine production by DCs correlates with poor vaccination responses reported in older adults [117].

DCs are involved in stimulation of T cell responses upon encounter of viral pathogens [119]. There is disagreement regarding capacity of aged DCs to stimulate T cells, a few studies have shown an age associated decline in DC capacity to induce proliferation and IFNγ secretion in T cells [120, 121] , but another study has shown that aged DC were effective in stimulation of clonal expansion of antigen-specific T cells [122]. Further, an age associated

decline in p DC capacity to induce perforin and granzyme B expression has also been reported; resulting in impaired ability to prime cytotoxic responses of CD8 T cells [120].

1.3.4 Natural Killer cells

Natural killer (NK) cells are cytotoxic lymphoid cells capable of destroying tumour and virus infected cells [123]. NK cells have been divided into two subsets on the basis of relative expression of CD56 and the Fcγ receptor CD16, namely CD16^{+ve} CD56^{dim} and the CD16$^{-ve/dim}$ CD56^{bright} [124, 125]. Majority of NK cells are CD56^{dim} and these cells have high cytotoxic potential. CD56^{bright} cells have immunomodulatory properties and are capable of initiating adaptive immune responses including; activation of DCs and production of immunoregulatory cytokines (IL-10, IL-13, TNFα, IFNγ) [124, 126].

Granule exocytosis and engagement of death receptors are the two main mechanisms used by NK cells to induce MHC class I-deficient target cell lysis [127, 128]. NK cell killing via granule exocytosis pathways is initiated by target cell recognition, followed by immunological synapse formation between target cell and NK cell[129]. NK cells have lytic granules containing cytotoxic proteins; perforin and granzyme [130]. Post conjugate formation, lytic granules polarise towards the immunological synapse and the cytotoxic content is released into the synapse[131]. Perforin is a 'key shaped' molecule that oligomerises to form pores that disrupt the target cell membrane [132], facilitating delivery of granzymes into target cells and induction of caspase dependent and independent cell apoptosis [130, 133] [Figure 1.3]. The second NK cell killing pathway involves engagement of death receptor with their ligands, for instance TNF –related apoptosis inducing ligand (TRAIL) is capable of inducing apoptosis of TRAIL sensitive target cells [134]. NK cells also express Fas Ligand (Fas L) that can induce caspase- dependent apoptosis by binding to Fas expressed on target tumor cells [127, 135].

An increased in frequency and absolute numbers of circulating NK cells has been observed in older adults [136-138]. This increase in the number of NK cells, results from an expansion of the CD56^{dim} subset, however a decline in the number of CD56^{bright} NK cells [139, 140] occurs with age [139, 141]. Further, the increase in CD56^{dim} NK cell subset suggests a phenotypic shift towards a more mature phenotype in NK cells with age. Additionally, an accumulation of mature CD57[+ve] NK cells has also been associated with ageing of human immune system [142].

A decline in NK cell cytotoxic potential on a per cell basis has been observed in older adults [143-146]. The increase in proportion of CD56^{dim} NK cells with age might be a compensatory mechanism to overcome reduced per cell cytotoxicity. Importantly, this decline in NK cell activity has been associated with a higher incidence of infections in older adults [147]. Preserved NK cell activity in older adults has been associated with better response to influenza vaccines and protection against respiratory tract infections [148]. Interestingly, studies in centenarians have shown preserved NK cell cytotoxicity, suggesting that intact NK cell activity can act as a marker for healthy aging [138, 149].

Studies examining the mechanism responsible for reduced NK cell activity with age have shown that the ability of NK cells to form conjugates with target cells is preserved with age [150]. Further, the ability of cytotoxic granules to fuse with NK cell membrane, examined by expression of CD107a on NK cell surface post stimulation also remains unaltered with age [150, 151]. Another study has suggested that defective NK cell signal transduction following stimulation with target cells due to reduced release of IP_3 with age might be contributing towards impaired NK cell cytotoxicity due to defective granule exocytosis [152]. However, a recent study has proposed that even though perforin levels are preserved with age[153], a

reduction in perforin binding to target cell surface due to reduced release of perforin is the underlying mechanism for reduced NK cell cytotoxicity in older adults [150].

NK cells can also eliminate antibody coated target cells via ligation of the Fc receptor CD16, termed antibody-dependent cellular cytotoxicity (ADCC). CD16 expression on NK cells has been reported to remain unaltered in older adults [150, 154, 155] and CD16 induced cytotoxicity is also preserved with age [156] .

NK cell activation is regulated by a balance of signals from specific activatory and inhibitory receptors [157]. Ageing is accompanied by a decline in the expression of natural cytotoxicity receptors (NCR) ; NKp46 [150] and NKp30 [136]. In young individuals, most of the NK cells express high levels of NKp30 and NKp46 (NCR^{bright} phenotype) and very few NK cells express low levels of these receptors (NCR^{dull} phenotype), but in older adults a higher proportion of NK cells exhibit NCR^{dull} phenotype [141]. Since both these receptors play an essential role in target cell killing by NK cells, reduced expression of these receptors might be a contributing factor towards reduced NK cell cytotoxicity with age [141]. Further, NKG2D expression has been shown to be preserved on NK cells in older adults [136, 150, 154]. There is discrepancy in the data regarding the effect of age on killer immunoglobulin like receptors (KIR), one study has reported an increase in KIR expression [136], whilst another study failed to report an alteration in KIR expression on NK cells of older adults [154]. Further, a decline in KLRG-1 and CD94 expression has been reported on NK cells with age [151].
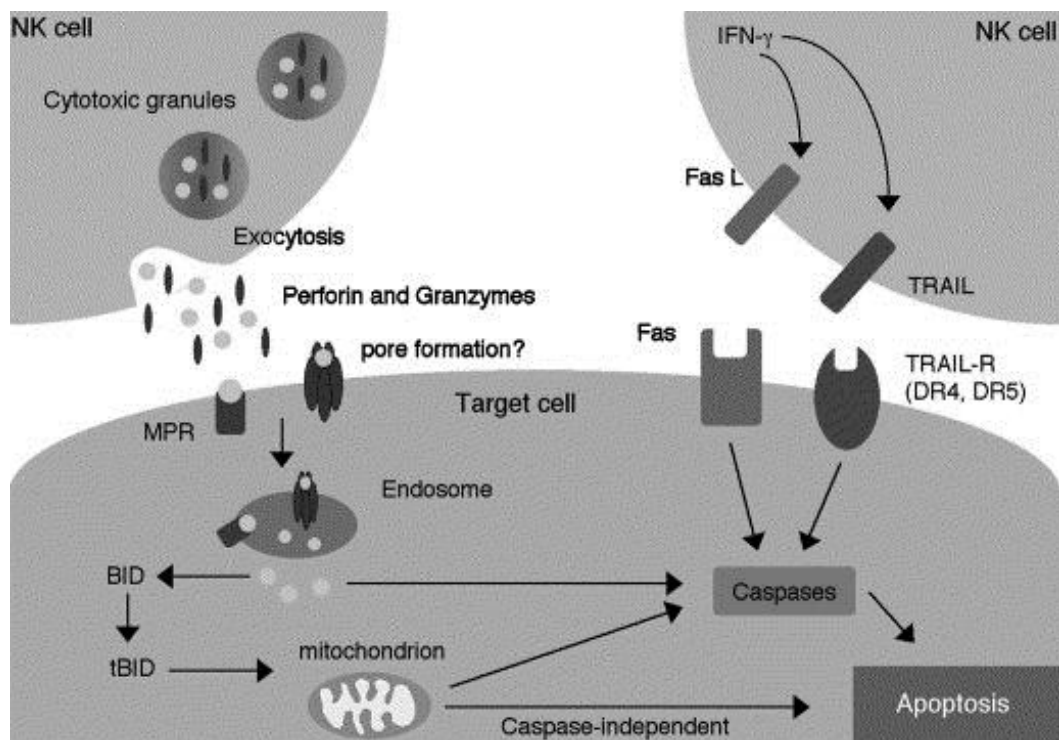
Figure 1.3 The two main mechanisms used by NK cells to induce MHC class I-deficient target cell lysis - Granule exocytosis and engagement of death receptors .

The granule exocytosis pathway begins with immunological synapse formation between target cells and NK cell, followed by polarisation and release of cytotoxic granule contents (Perforin and granzyme B). Perforin form pores in a target cell membrane facilitating Granzyme up take by target cells. Grz trigger apoptosis via activation of caspase pathway or caspase independent pathways that still remain uncharacterised. The second pathway involving ligation of Fas and TRAIL with their ligands induces caspase dependant cell apoptosis. Image taken from Smyth *et al* 2005 [127].

NK cell functional capabilities also include secretion of cytokines and chemokines. According to a study done in older adults, IFNγ production by NK cells remains intact with age [154], but these findings were contradicted by another study showing IFNγ production by CD56^{bright} NK cells increased post stimulation in old donors [151]. Further, an increase in IL-4 and IL-10 production by NK cells and unaltered TNFα production has also been reported with age [158, 159]. A decline in chemokine production (IL8, RANTES, and MIP-1alpha) occurs in aged NK cells on IL2 stimulation [160, 161].

1.3.4 (a) NKT cells

NKT cells are 'innate immune lymphocytes' that in addition to T-cell receptor (TCR) also express certain NK cell receptors [162]. The origin and function of NKT cells are poorly understood, but these cells have been suggested to play an important role in defense against viral infections mainly via cytokine production (especially IFNγ) or via direct killing of target cells [163]. Studies done in rodents have reported an increase in the number of NKT cells with age [164, 165]. Additionally, a decline in NKT cell cytotoxicity and impaired IFNγ production has been reported in aged mice and humans [166]. Studies in centenarians and very old mice have reported increased numbers of NKT cells [167], preserved IFNγ production [167, 168] and cytotoxic potential [168]. Although only limited data on age associated changes in NKT cells are available, so far the age-related changes in NKT cells are similar to those observed in NK cells.

The current literature regarding the effects of ageing on innate immune cells and their function are summarised in Figure 1.4.

Neutrophil + Age

Preserved
Numbers at site of infection
Adherence
Reduced
Response to anti-apoptotic
mediators (GM-CSF)
Chemotaxis
Phagocytosis
Superoxide generation
Signal transduction

Dendritic cells + Age

Preserved
myeloid DC
Reduced
Plasmacytoid DC's
Phagocytosis
Migration
Capacity to stimulate T cells
IFNα production

Monocyte/Macrophage +Age

Preserved
Numbers
Reduced
Chemotaxis
Phagocytosis
Superoxide generation
Production of pro-inflammatory cytokines (stim)
TLR expression
MHC class II expression
Wound healing function
Increased
non-classical CD14$^+$ CD16$^+$ monocytes
Production of pro-inflammatory cytokines (basal)

NK cells + Age

Increased
Numbers
CD56^{dim} cells
Mature CD57^{+ve} NK cells
Reduced
NK cell cytotoxicity
NKp30 and NKp46
expression

Figure 1.4 Summary of age associated changes in innate immune cells; Neutrophils, Monocytes, Dendritic cells and NK cells.

1.4 Ageing of the adaptive immune system

Ageing results in extensive remodelling of the adaptive immune system contributing towards inefficient control of infectious diseases, increased autoimmunity and reduced immune surveillance against cancers and ultimately towards increased mortality.

1.4.1 Thymus and T cells

The thymus is devoted to thymocyte differentiation and maturation and is the first organ to age in the human body. In 1985, Steinman *et al* reported that the thymus reaches its peak size within first year of life after which thymic involution occurs [169]. Further, a decline in recent thymic emigrants also occurs in aged mice [170]. Thymic atrophy and decline in thymopoiesis result from a number of alterations in the thymus including; loss of thymic epithelial cells, replacement of lymphoid with fat tissue, loss of cytokines and hormones essential for thymopoiesis (e.g. IL-7, KGF and Ghrelin) and upregulation of thymosupressive cytokines (e.g. IL-6, TNFα) with age [171-173] . Recently, novel molecular strategies have been developed to measure T cell generation by quantification of T-cell receptor excision circles (TREC) [174, 175], revealing an exponential drop of around 95% in frequency of TRECs between 25 and 60 years of age [176].

Mature T lymphocytes are divided into two distinct subsets on the basis of expression of cell surface markers CD4 and CD8. CD8^{+ve} T cells are cytotoxic cells, whilst CD4^{+ve} T cells are predominantly helper T cells. A few studies have reported a decline in peripheral T cell numbers with age [138, 177-179]. Further, ageing is also accompanied by an increase in CD8^{+ve} T cells, resulting in a slight decline in CD4:CD8 ratio [14, 178] . It has been suggested that the CD4:CD8 ratio can be used as a biomarker of ageing to predict longevity and it is part of the IRP [180].

Antigen exposure results in clonal expansion of naïve T cells and generation of effector cells. On resolution of infection only a few memory T cells survive to provide protection in case of future encounter with the antigen. A decline in the proportion of naïve T cells and an increase in memory T cells have been widely observed with advancing age [177, 178, 181]. This is likely a result of repeated exposure to antigens over the lifetime, homeostatic proliferation of the peripheral pool and reduced thymic output of naïve T cells [182]. Memory T cells have been classified into three distinct subsets on the basis of expression of CCR7 and CD45RA; central memory (CM), effector memory (EM) and effector memory RA (EMRA) [183, 184]. Studies examining the effect of ageing on CD8 memory T cells have reported, that frequency of CM T cells remains unaltered , but an increase in the frequency of EM and EMRA CD8[+ve] T cells is seen in older adults [185, 186] . In the case of memory CD4[+ve] T cells, an increase in CM cells occurs with age [186, 187]. Further , ageing is also accompanied by a skewing of memory CD8[+ve] T cells towards clones of latent viruses, specifically CMV [188, 189], resulting in filling of valuable 'immunological space' and reduced ability of CD8[+ve] T cells in older adults to respond to novel pathogens [190]. T cell ability to provide effective immune response against new antigens depends on naïve T cell receptor diversity [191]. T cell diversity has been reported to be maintained up to the age of 65, after which a drop in T cell diversity occurs ; resulting in contraction of naïve T cell repertoire and poor responses to neoantigens [176, 182].

T cell activation requires two distinct signals. Firstly, an antigen specific signal involving T cell receptor (TCR) followed by a co-stimulatory signal provided by antigen presenting cells via CD28 pathway [192]. Ageing is accompanied by the accumulation of CD28[-ve] T cells [193-195] with impaired proliferation capacity due to shortened telomeres [196] . This loss seems to be more apparent in CD8[+ve] T cells, such that by the age of 80 more than 50% of CD8 T

cells are CD28[-ve] and about 10-15% of CD4[+ve] T cells [197]. Furthermore, accumulation of CD28[-ve] T cells has been associated with CMV seropositivity [198] and correlates with poor vaccine responses in older adults [199]. Expression of NK cell receptors, such as CD57 occurs in these senescent T cells and has been suggested as a marker for T cell senescence [200]. The majority of CD28[-ve] T cells are CD57[+ve] and an increase in CD28[-ve] CD57[+ve] T cells occurs in older adults [200, 201], which has also been associated with CMV seropositivity [201]. An increase in expression of another NK cell receptor, NKG2D has also been reported in CD4[+ve] T cells with advancing age; representing a subset of highly differentiated senescent T cells[202]. Further, on examining the activation status of circulating lymphocytes an increase in frequency of HLADR[+ve] T cells [138, 179, 203] and CD25[+ve] T cells occurs with age [203], but frequency of CD69[+ve] T cells remains unaltered [203, 204].

Ageing is accompanied by a decline in T cell helper activity, resulting in impairment in antigen specific B cell responses [182, 205]. Naive CD4[+ve] T cells from aged mice exhibit reduced expansion and IL2 production upon stimulation [205, 206]. CD40L (CD154) expression by CD4[+ve] T cells also plays an essential role in mediating B cell responses. An age associated decline occurs in CD40L expression on CD4[+ve] T cells [205, 207], which can be overcome by addition of IL2 to in-vitro cultures of T cells [206]. Furthermore, ageing is accompanied by incomplete differentiation of naïve CD4 T cells into Th1 and Th2 cells [208] and a shift of T helper cells from Th1 (IFNγ, IL2) to Th2 (IL10, IL4, IL5) has also been reported [209-211].

An age associated reduction occurs in CD8[+ve] T cell effector function including, cytokine production (IFNγ) and target cell lysis ; contributing towards delayed pathogen clearance[212, 213]. Several factors such as impairments in dendritic cells [214, 215], age-associated alterations in T_regs and CD4 helper T cells contributes towards decreased CD8 T cell responses [216, 217].

Further, intrinsic defects, for instance a reduction in CD8 T cell expansion in response to an infection have also been reported with age [213].

1.4.1 (a) Regulatory T cells

T_{regs} play a central role in immune regulation via cell-contact, secretion of immunosuppressive cytokines (IL10 and TGFβ) or deprivation of cytokines (IL2) essential for expansion and survival of T cells [218, 219]. An age associated increase in the frequency of $CD4^{+ve}CD25^{+ve}Foxp3^{+ve}$ T_{regs} has been reported in aged mice [220-222] and older adults [223, 224], which might contribute towards increased incidence of infections, poor tumor killing and reduced vaccine responses with age [220, 225]. Factors that might be responsible for accumulation of T_{regs} with age include; alterations in expression of trafficking receptors (CD69, CD62L, CCR7) resulting in impaired T cell migration through lymphoid tissue [221] and impaired ability of aged T_{reg} cells to undergo apoptosis[224]. Further, a shift in peripheral T_{regs} from naïve to memory phenotype has also been reported in aged mice [222] and humans [226].

Although, $CD4^{+ve}CD25^{+ve}Foxp3^{+ve}$ T_{reg} numbers increase with age, a decline in the suppressive capacity of T_{regs} has been reported by a few studies [222, 224]. But, these results are not widely confirmed, a few studies have reported intact suppressor function of aged T_{regs} [223, 227]. Interestingly, a recent study reporting an intact ability of aged T_{regs} to suppress effector T cell function has observed defects in T_{reg} capacity to constrain IL17 producing T cells, which might be a contributing factor towards age associated inflammation [228].

1.4.2 B cells

It is well accepted that B cell lymphopoiesis declines with age [229-231]. Multiple mechanisms have been suggested which might contribute towards reduced B cell production including ; decline in the numbers of precursor B cells [232] due to increased apoptosis of precursor B cells in aged mice [233, 234] , reduced expression of transcription factors crucial for B cell generation (e.g. E47) [235] and reduced expression of genes essential for B cell development (e.g. recombination activating gene (RAG)) [229, 236]. In addition to cell intrinsic changes, altered bone marrow microenvironment including ; decline in the sites of naïve B cell production, decline in growth factors and reduced IL7 production by bone marrow stromal cells might also contribute towards impaired B cell lymphopoiesis [232, 237]. Interestingly, B cell depletion results in expansion of B cell precursors in old mice, suggesting a "negative-feedback" from mature B cells in aged mice on B cell lymphopoiesis [238]. It has been recently reported in mice that ageing is accompanied by an accumulation of a CD23^{-ve}/CD21^{-ve} mature B cell subset termed age associated B cells (ABC), that are capable of inducing apoptosis of pro-B cells via TNFα secretion [239]. However a human equivalent of ABC cells is yet to be reported. Taken together it is clear that there is a decline in both frequency and absolute numbers of circulating B cells with age [240-244] .

In addition to a decline in frequency of B cells, an age associated decline in naïve B cells (CD27^{-ve} IgD^{+ve}) [245, 246] and an increase in memory B cells has also been reported [246-248]. Memory B cells have been divided into different subsets; unswitched memory B cells (CD27^{+ve} IgD^{+ve}), IgM memory B cells (CD27^{+ve} IgD^{+ve} IgM^{+ve}) and switched memory B cells (CD27^{+ve} IgD^{-ve}) [249]. Ageing is accompanied by a decline in IgM memory B cells, predisposing older adults to pneumococcal infections [250]. Late memory/exhausted memory B cells (CD27^{-ve} IgD^{-ve}) have also been reported to increase with age [251].

Ageing is accompanied by poor vaccination responses, likely due to reduced antibody response on immunisation and impaired T cell activity [245, 252]. Older adults have been reported to generate tenfold fewer antibody secreting cells relative to young individuals on antigenic stimulation [253], suggesting that the increase in the number of memory B cells is not paralleled by an increase in number of plasma cells [253]. Surprisingly, even though the actual amount of antibody secreted by plasma cells remains unaltered with age [254, 255], the antibodies produced by aged B cells have lower affinity and fewer antibodies are pathogen specific [255, 256]. Antigen encounter induces B cells to form germinal centres which require help of T cells and follicular dendritic cells to provide survival signals and display antigens to B cells. A decline in CD4 T cell and dendritic cell functioning , along with intrinsic changes in B cells might be responsible for the age-associated reduction in number and size of germinal centres [257] and might contribute towards the decline in antibody production by older individuals with age [169].

Additionally, a reduction in B cell diversity has also been reported with advancing age; resulting in impaired ability of B cells to respond to new pathogens [229]. This age associated decline in BCR diversity has an important implication on health status of older individuals and has been linked to frailty [258].

The literature concerning the age-related changes in T and B lymphocytes is summarised in Figure 1.5.
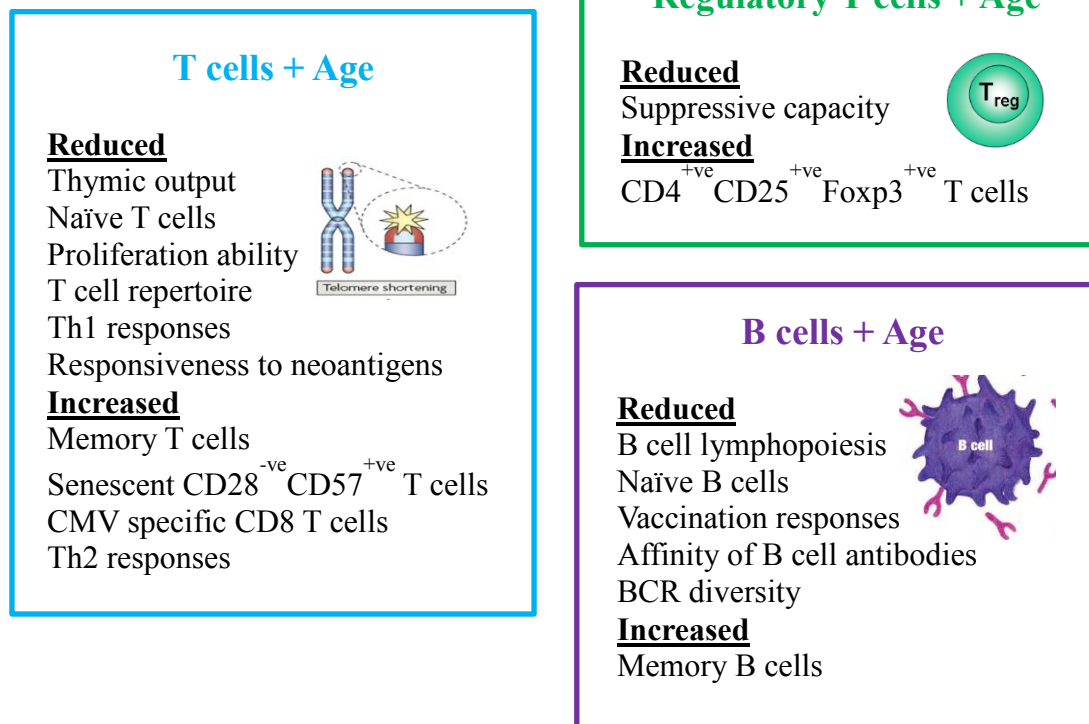
Figure 1.5 Summary of age-associated changes in adaptive immune cells, T cells, Regulatory T cells and B cells.

1.5 Ageing and risk of autoimmunity

As previously discussed, ageing is characterised by remodelling and decline in immune responses; leading to older adults who are more susceptible to chronic diseases and infections. Interestingly, age is also an important risk factor for development of rheumatoid arthritis (RA) [259, 260]. RA is a chronic immune mediated disease involving, infiltration of T cells, B cells and macrophages into the synovium; resulting in synovial inflammation and joint destruction [261]. The synovial joint also accumulates large numbers of neutrophils which contribute to joint destruction. There is evidence suggesting that patients suffering from RA are immunocompromised [262]. Interestingly, immune-ageing emerges at approximately the same age when susceptibility to develop RA peaks [260]; leading to the suggestion that premature immune ageing and reduced immune tolerance might be contributing factors towards development of RA [Figure 1.6] [263].

1.5.1 T cell immunesenescence in rheumatoid arthritis

RA has been associated with premature T cell immunesenescence [264, 265]. As previously discussed, advancing age is accompanied by thymic involution and a subsequent decline in TREC numbers (section 1.4.1). Interestingly, an inverse relationship occurs between thymic capacity and incidence of RA [266]. Also, a reduction in number of TREC expressing T cells has been observed in RA patients [267, 268], such that a 45 year old patient has the TREC levels normally seen in a healthy 70 year old. Further, ageing is accompanied by accumulation of CD4^{+ve} CD28^{-ve} T cells [269], which produce pro-inflammatory cytokines (IFNγ), have cytotoxic abilities and also react with auto antigens and are thus capable of triggering development of RA [266]. An elevated frequency of CD4^{+ve}CD28^{-ve} T cells has also been reported in RA patients [267, 270]. Additionally, an age associated increase in differentiation towards Th17 cells has been reported [271-273]. Th17 cells are involved in

mediating RA [274] and this skewing towards Th17 in older adults might be an additional factor contributing towards autoimmunity associated with age [271].

Breakdown in immunological self-tolerance also contributes towards the development of autoimmunity. Functional deficiencies in T_{regs} occur in autoimmune disorders, including RA [275, 276]. Studies of animal models of autoimmunity have shown that defects in T_{regs} cell numbers or immune suppressive functions can contribute towards disease development [277]. Even though older adults are characterised by higher numbers of T_{regs}, a decline in per cell immune suppression activity of these cells has been reported [278, 279]. Thus, increased incidence of autoimmune disorders, such as RA might also be a result of failing ability of T_{regs} to suppress autoimmunity [225, 280].

1.5.2 B cell immunosenescence and autoimmunity

B cells are known to play a pathogenic role in autoimmune disorders [281]. Profound changes have been reported in B cells with advancing age , such as a shift in antibody specificity from non-self to self-antigens [282]. Ageing is accompanied by an increase in B cells with autoreactive properties and higher levels of autoantibody levels such as rheumatoid factor (RF) and anti-nuclear antibodies [283, 284]. It has been hypothesised that accumulation of self-reactive memory B cells with age that may become reactivated later in life due to deficiency in immune tolerance in aged individuals might be responsible for elevated autoantibody levels in older adults [285].

Additionally, as detailed above (section 1.4.2) novel peripheral subset of B cells known as ABCs have been reported to accumulate in aged mice [286]. ABCs contribute towards age associated tendencies towards autoimmunity by producing autoantibodies, presenting antigens to T cells and enhancing polarisation towards Th17 phenotype [286, 287].

1.5.3 Innate immunosenescence and autoimmunity

In addition to the adaptive arm of the immune system, innate immune cells are also involved in development of autoimmunity. Dendritic cells are responsible for presenting antigens to autoreactive T cells and promote B cell activation and differentiation into plasma cells and increase autoantibody production [288]. Aged DCs react more strongly than DCs from young individuals to self-antigen [289]. Moreover, presentation of arthritogenic self-antigens by DCs to auto reactive CD4^{+ve} CD28^{-ve} T cells might be an additional contributing factor towards the development of RA in older adults [263].

1.5.4 Inflammaging and autoimmunity

TNFα and IL-6 have pleiotropic functions and are known to promote processes involved in pathogenesis of RA including ; release of leukocytes driving tissue destruction and promoting infiltration of inflammatory cells into the synovium [290]. Ageing has been associated with chronic inflammation and elevated serum levels of proinflammatory cytokines [291] (discussed in detail in section 1.2) and such elevated levels of pro-inflammatory cytokines in older adults might promote processes involved in pathogenesis of RA.

1.6 B cells with immunosuppressive properties

B cells are known to play a pathogenic role in human autoimmune diseases due to their ability to secrete autoantibodies, serve as antigen presenting cells to T cells and induce T cell expansion and cytokine secretion [281]. Janeway and colleagues provided the first evidence that B cells could be involved in suppression of inflammation, reporting that the absence of B cells exacerbated T cell mediated autoimmune reactions [292], which has also been confirmed by other groups [293]. Over the last decade, evidence has emerged for an
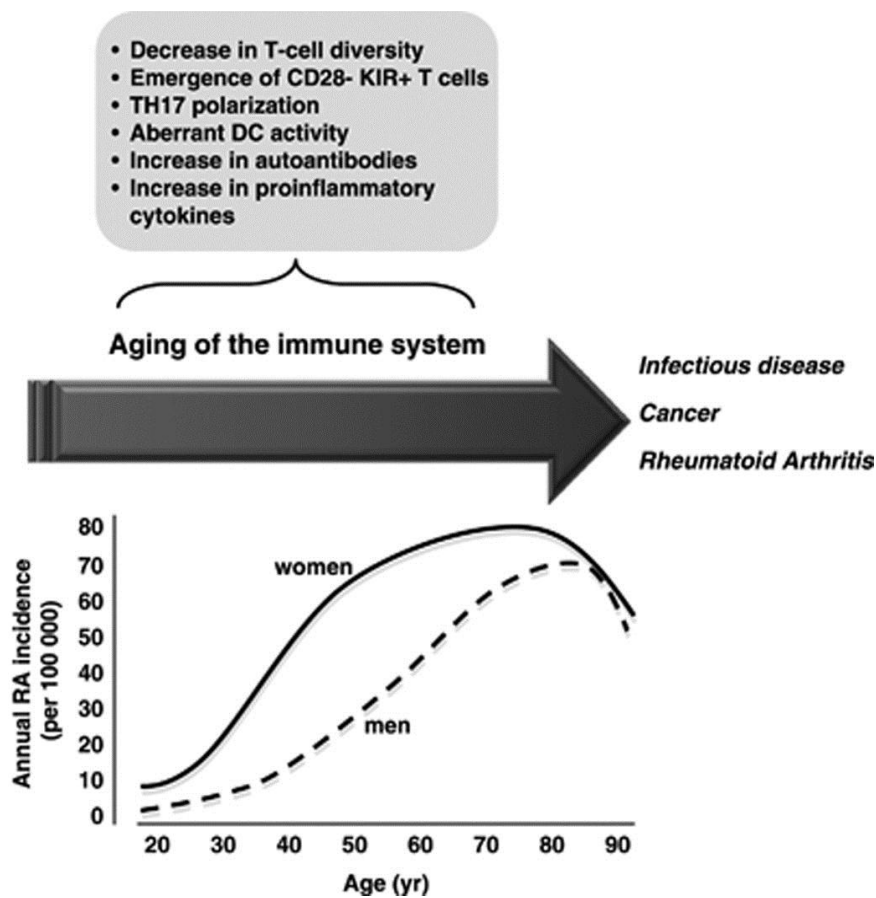
Figure 1.6 Ageing of immune system increases susceptibility to autoimmune disorders especially rheumatoid arthritis.

The arrow indicates a progressive decline in integrity of the immune system, involving a decline in T cell diversity, accumulation of CD28[-ve] senescent T cells and increase in levels of pro-inflammatory cytokines and autoantibodies. Immunesenescence is accompanied by an increase in incidence of several diseases, including infectious disease, cancer and rheumatoid arthritis. The line graph of RA incidence is a schematic approximation of the general trend in incidence with age. Image taken from Lindstrom *et al* 2010 [263].

immunoregulatory role of B cells, analogous to the suppressive activity of regulatory T cells. B cells may therefore play both protective and pathogenic roles in the same pathological setting. Since then a subset of B cells with immunosuppressive properties, have been shown to suppress inflammation in a number of murine models of chronic inflammation, including collagen induced arthritis (CIA), experimental autoimmune encephalitis (EAE) , inflammatory bowel disease (IBD) as well as skin inflammation [294-297].

Mauri *et al* were the first to assess the role of B cells at different stages of development in the pathogenesis of autoimmunity. They isolated splenocytes from mice during the remission phase of CIA and sorted B cells into different subsets; transitional 2 marginal zone precursor B cells (T2MZP), follicular B cells (FO) and marginal zone B cells (MZ). These B cells were transferred into syngeneic mice on the same day as experimental induction of arthritis, after which the disease incidence and severity was monitored.

The mice in which T2-MZP B cells were transferred were the only mice that were protected from arthritis, by rendering T cells hyporesponsive to collagen re-stimulation. This data lead to the hypothesis that transitional B cells (recent emigrants from the bone marrow) have immunosuppressive properties [298].

Three major populations of circulating B cells have been identified in human peripheral blood discriminated on the basis of relative distribution of developmentally regulated markers CD24 and CD38; memory B cells ($CD24^{hi}$ $CD38^{-ve}$) form about 20-40 % of peripheral B cells, 60-70 % of B cells are mature B cells ($CD24^{int}$ $CD38^{int}$) and transitional B cells ($CD24^{hi}$ $CD38^{hi}$) form about 2-7 % of the peripheral B cell population [299, 300]. Interestingly, $CD19^{+ve}$ $CD24^{hi}$ $CD38^{hi}$ immature transitional B cells have been shown to

exhibit immunosuppressive properties and were the main IL10 producers amongst B cells [301, 302].

Tedder and colleagues have suggested that CD19[+ve]CD5[+ve]CD1d^{hi} B cells referred to as 'B10 cells' possess immunoregulatory properties and are capable of producing IL10 in mice [303, 304]. Adoptive transfer of CD5[+ve]CD1d^{hi} B cells into CD19^{-/-} mice inhibits inflammatory responses during EAE [297] and contact hypersensitivity [303]. However, this IL10 producing B10 subset still remains largely unexplored in humans. Phenotypic identification has confirmed that the majority of CD19[+ve] CD1d^{hi} CD5[+ve] B cells are contained within the CD24^{hi} CD38^{hi} B cell subset [305].Similarly, Tedder and colleagues have also reported a high CD24 expression in CD5[+ve]CD1d^{hi} B cell subset [303] and the same group have reported CD24^{hi}CD27[+ve] B cells , with 60% of these cells CD38[+ve] are main IL10 producers in humans and parallel mouse B10 cells [306]. Since, a number of cell surface markers are shared by both subsets of B$_{regs}$ (transitional B cells and B10 cells), it might be possible that both these subsets are derived from a common B$_{reg}$ cell precursor and the phenotypic differences between these subsets is a result of activation of a common B$_{reg}$ precursor in different microenvironments [283, 305].

1.6.1 Mechanism of immune regulation by B$_{reg}$ cells

Immunosuppressive B cells are known to exert their effect via two main modes of action: cytokine production (IL10 and TGFβ) and direct cell-cell contact. B cells are capable of producing IL10 was reported over twenty years ago [307], but over the past decade literature highlighting the protective role of IL10 producing transitional B cells and B10 cells has accumulated in both mice and humans [294, 301, 308]. IL10 plays an essential role in maintaining immune homeostasis [309] via down regulation of antigen presentation by macrophages and DCs, suppressing production of pro-inflammatory cytokines by CD4 T

cells, monocytes and macrophages and inhibition of antigen specific T cell proliferation [310-312]. Exacerbated inflammatory responses and an increased incidence and severity of autoimmune disorders have been reported in IL10 deficient mice [313-315] and IL10 administration can have a beneficial effect on autoimmune responses [316, 317]. Interestingly, the mRNA expression of IL10 in central nervous system (CNS) correlates with remission in EAE [318]. Studies using a bone marrow chimeric system in which IL10 deficiency was restricted to B cells have highlighted the importance of B cell IL10 production in recovery from autoimmune disorders [293]. Further, adoptive transfer studies in mice have shown that IL10 producing B cells are capable of ameliorating established inflammatory responses [304, 319]. It has been recently reported that IL10 producing CD19[+ve]CD24^{hi} CD38^{hi} B cells are capable of suppressing T cell proliferation , cytokine production by CD4 T cells (IFNγ, TNFα) and impairing differentiation of naïve T cells to Th17 cells [301, 302] [Figure 1.7]. Interestingly, B$_{regs}$ are also capable of regulating innate immune responses. For instance, reductions in TNFα production has been reported in monocytes when cultured with activated human B10 cells [306].

Even though IL10 production is the main mechanism of immune suppression by B$_{reg}$ cells, not all of these cells produce IL10 post-stimulation, suggesting the presence of alternative immune suppressive mechanisms. For instance, production of transforming growth factor (TGF-β1) by B cells upon LPS stimulation plays an essential role in preventing inflammation and autoimmune disorders [320]. Studies have shown that transfer of in-vitro generated TGF-β1 producing B cells into non-obese diabetic (NOD) mice, can suppress the development of diabetes in recipient mice and down regulate pathogenic responses by inducing apoptosis of effector T cells and/or inhibition of APC activity [321].

The B7 family of co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) are capable of regulating T cell growth, survival and differentiation via ligation to CD28 and cytotoxic T lymphocyte protein 4 (CTLA4) receptor on CD4^{+ve}T cells [322, 323]. In addition to cytokine secretion, contact between CD4^{+ve} T cells and B$_{regs}$ cells via CD80 and CD86 ligation to CTLA4/CD28 plays an important role in suppression of activated CD4^{+ve} T cells [298]. Interestingly, mice with B7- deficient B cells were unable to recover from EAE , a result of delayed upregulation of IL10 expression and mobilisation of T$_{reg}$ cells into site of inflammation [324]. In humans, blocking antibodies against CD80 and CD86, inhibit CD19^{+ve} CD24^{hi} CD38^{hi} cells from suppressing TNFα and IFNγ production by CD4 T cells [301, 325].

B$_{regs}$ are also capable of mediating immunosuppressive effects via induction of Foxp3 expression in CD4^{+ve}CD25^{+ve} Tcells. Interestingly, CD40 activated B cells have a higher capacity to induce T$_{regs}$ generation than DC's [326]. Additionally, B cells have also been reported to play a role in recruitment of T$_{regs}$ to the site of inflammation [Figure 1.7] and B cell deficiency results in delayed emergence of T$_{regs}$, which might prevent recovery from inflammation [324]. However, depletion of T$_{regs}$ does not alter the capacity of B$_{regs}$ to regulate immune responses, suggesting that T$_{regs}$ are unlikely to play a role in B cell mediated immune regulation [327].

1.6.2 Induction of transitional B cells and B10 cells

Development, maturation and expansion of immunosuppressive B cell subsets require specific external signals that inform B cells of their extracellular environment. According to current data BCR, CD40, LPS and CpG generated signals are involved in B$_{regs}$ development [308, 328] [Figure 1.7].

CD40 signalling mediates B cell survival, stimulates B cell proliferation and up regulation of cytokines (IL10, TNFα, IL6) and co-stimulatory molecules (CD80 and CD86) [329]. Interestingly, T cell mediated B cell activation is capable of inducing immunosuppressive function in B cells [325, 330]. CD40 engagement halts B cell differentiation into antibody producing plasma cells and maintains the undifferentiated state of B cells to facilitate differentiation into cells with immunosuppressive properties [331]. The importance of CD40 stimulation in immune regulation was highlighted in a murine model of autoimmunity, in which transfer of splenocytes stimulated with agonistic anti-CD40 results in amelioration of arthritis due to an increase in IL10 producing B cells [294]. Similarly, chimeric mice reconstituted with CD40 deficient B cells were unable to recover from EAE compared with wild type mice due to impairment in IL10 production by B cells [293]. BCR ligation combined with CD40 signalling results in the production of pro-inflammatory cytokines (TNFα, Lymphotoxin) and promotes B cell development and plasma cell differentiation [332].

A range of studies have used CD40 stimulation to induce IL10 production in B$_{regs}$ in both mice [293, 294] and humans [301] [Figure 1.7]. CD40 signalling results in the activation of multiple signalling cascades, including nuclear factor kappa B (NFkB), mitogen activated protein kinase (MAPK) and signal transducer and activator of transcription-3 (STAT3) [333]. CD40 receptor binding with its ligand results in auto phosphorylation of Janus kinase (Jak) which in turn induces phosphorylation of tyrosine residue of STAT3, resulting in its dimerisation and nuclear translocation, where it binds to promoter region of target genes and regulates their expression [334].

Toll-like receptors (TLRs) are pattern-recognition receptors that can activate immune responses in the presence of pathogen associated molecules such as lipopolysaccharides (LPS), viral ribonucleic acid, viral and bacterial DNA, lipoteichoic acids and flagellin [335].

Recently expression of TLR1, TLR2, TLR4, TLR7 and TLR9 transcripts has been reported in B cells, suggesting that B cells are capable of recognising a range of pathogenic antigens through TLRs [336, 337]. Stimulation via agonists of TLR2, TLR4 and TLR9 has been shown to stimulate IL10 production in murine B cells [337, 338]. Further, LPS and CpG stimulations also induce clonal expansion and IL10 production by B10 cells in mice [308, 339] and humans [306] [Figure 1.7]. TLR agonists can also induce IL10 production in transitional B cells in mice [319].

TLR-induced IL10 producing B cells suppress differentiation of Th1 and Th17 cells, limiting T cell mediated inflammation from driving autoimmunity [338]. TLR agonists can also suppress autoimmunity in mice models [340, 341]. Defective IL10 production by B cells on TLR stimulation has been reported during autoimmunity [342] and mice with B cell restricted deficiencies in TLR4 develop an aggravated EAE [338]. TLR9 gene polymorphisms resulting in reduced expression have been associated with an increased predisposition towards systemic lupus erythematosus (SLE) in a Japanese population [343]. Interestingly, early life exposure to LPS in mice results in delayed onset and diminished severity of EAE, an increased spinal cord transcript level of IL10 has been reported in LPS exposed mice [344].

Thus, B cell activation for IL10 production can occur via two different modes; T-cell dependant (via CD40 pathway) or T-cell independent (via TLR), which has led to development of a two-step model for B cell mediated regulation of immune responses by Lampropoulou *et al*. According to which, TLRs initiate IL10 mediated suppression by B cells, but it is possible that TLR stimulation induces IL10 production in only a few B cells. Suggesting that a second phase involving signalling via CD40 pathway might amplify the IL10 producing B cell population and re-stimulate B cells activated via TLRs to maintain the suppressive program resulting in effective immune suppression [338, 345].

In addition to CD40 and TLR stimulation, IL10 production by B cells can be triggered by a range of other stimuli. For instance, B cell activating factor (BAFF), a member of TNF family cytokines that acts as a key regulator of B cell maturation and survival has been identified as an additional stimulus for induction of IL10 production in $CD5^{+ve}$ $CD1d^{hi}$ B cells[346]. Stimulation of B cells with GM-CSF and IL15 fusion protein known as GIFT15, has also been shown to convert naive B cells into B cells with immunosuppressive properties and may serve as a novel treatment for autoimmunity [347]. Interestingly, apoptotic cells have also been shown to exert a protective effect on CIA development, via induction of IL10 secretion by B cells [348].

1.7 Ageing, stress and immunity

Stress has been defined as a state of reduced homeostasis, following exposure to extrinsic or intrinsic adverse forces (stressors), resulting in precipitation of a spectrum of adaptive physiological and behavioural reactions to re-establish homeostasis [349]. Healthy older individuals have been reported to experience greater levels of stress, anxiousness and depression than young adults [350, 351]. Meta-analysis of literature over the past few decades has led to the development of the hypothesis according to which chronic stressful life events are suppressors of immune function [352, 353]. Immune dysregulation associated with chronic distress has clinical implications, for example individuals who report relationship conflict lasting a month or more are at increased risk of developing illness when exposed to an infectious agent [354] and relationship stress has also been associated with slower wound healing [355].
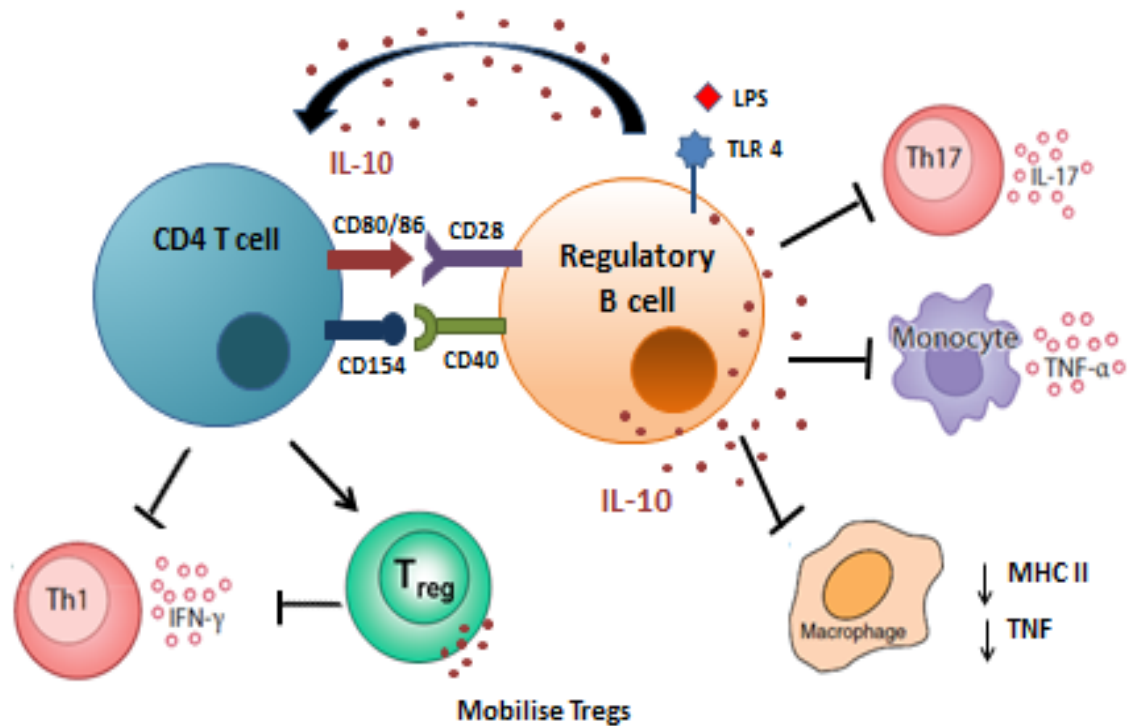
Figure 1.7 Generation and mechanism of action of regulatory B cells.

The primary mechanism of immune tolerance mediated by transitional B cells and B10 cells involves IL10 production which supress unwanted immune responses. Activation of B cells by CD154^{+ve} CD4^{+ve} T cells via CD40 pathway or via T cell independent TLR stimulation (LPS) induces IL10 production in B$_{regs}$. IL10 produced by these B$_{regs}$ has immunosuppressive effects; including limiting differentiation of Th1 and TH17 cells, modulating differentiation and migration of T$_{regs}$ and inhibiting pro-inflammatory cytokine production by monocytes and macrophages.

Immunesenescence has already been discussed (section 1.1) and there is now accumulating evidence suggesting that the effects of stress and age are interactive; chronic stress exacerbating the effects of ageing on immune function [4, 356]. For example, a study examining the effect of the chronic stress of caregiving on vaccine responses reported that even though there was a deficit in the vaccine response of young caregivers when compared with young controls, this defect was magnified in older adults who were caregivers [357]. Additionally, a negative association between exposure to stressful life events such as bereavement and marital dissatisfaction and a significant positive effect of marital satisfaction on vaccination responses has been reported in older adults [358]. A previous study done in our own group has also shown that innate immunity is susceptible to the effects of physical stress, specifically a reduction in neutrophil superoxide production is seen on exposure to the trauma of a hip fracture [359] or the psychological distress of bereavement in older adults [360]. These interactive effects of stress and ageing on the immune system in older adults are particularly troubling, as older adults are already at a higher risk of health complications due to immunesenescence. In addition, older adults have an increased risk of exposure to life events such as hip fracture, care-giving, bereavement and social isolation.

1.8 The Hypothalamus-Pituitary-Adrenal axis

The nervous, endocrine and immune systems are closely integrated and play an essential role in enabling organisms to adapt to stressful situations [349]. The hypothalamus-pituitary-adrenal (HPA) axis acts as a pivotal regulator of the stress responses by mobilising energy reserves and modulating immune responses, enabling individual to adapt to environmental changes [361]. The central control point of this system is in the hypothalamus. Corticotrophin releasing hormone (CRH) secreted by the hypophysiotropic neurons localised in the para

ventricular nucleus (PVN) of the hypothalamus is the principal regulator of the HPA axis [362]. CRH binds to its receptor on pituitary corticotropes stimulating the secretion of adrenocorticotrophic hormone (ACTH) [363]. The adrenal cortex is the principal target for circulating ACTH, where it stimulates secretion of glucocorticoids (GC) from zona fasciculata [Figure 1.8]. GCs are the final effectors of the HPA axis and act as principal hormone mediators of the stress system. Additionally, ACTH also stimulates secretion of another steroid, Dehydroepiandrosterone (DHEA) by the zona reticularis of the adrenal gland [364].

GCs play a prominent role in regulating the magnitude and duration of the HPA axis activation [362] and are responsible for terminating stress responses through negative feedback. They exert a negative effect on secretion of ACTH, limiting the total exposure of organism to glucocorticoids, thus minimising the catabolic and immune suppressive effects of these hormones [361, 365]. A dual glucocorticoid receptor system exists in the CNS, glucocorticoid receptor type I responds to low concentrations of GCs, glucocorticoid receptor II responds to both basal and stress concentrations of GCs and is responsible for mediating negative feedback control [363].

The HPA axis exhibits a diurnal rhythm of secretion. In non-stressful events, CRH is secreted in portal system in a circadian fashion, the increased amplitude of CRH in the early morning results in ACTH and cortisol secretory bursts [361, 366]. Cortisol secretion rises during sleep and reaches its peak 30-45 minutes after awakening, cortisol levels steadily decline throughout the day [367]. Although DHEA levels are elevated post awakening, the diurnal rhythm of DHEAS (the sulphated and major form of DHEA in the circulation) does not display an awakening secretory activity. Post awakening DHEAS levels displays a flat secretion pattern and a decline in DHEAS levels has been reported 3 hr post-awakening [368].

Thus DHEAS levels do not display significant diurnal fluctuations and are relatively stable[369].

1.8.1 Glucocorticoids in humans

Cortisol is a major glucocorticoid, synthesised from cholesterol by adrenocortical cells in response to ACTH in humans [370]. Upon secretion, only 5-10% of cortisol circulates unbound and is biologically active, the remaining cortisol is bound to corticosteroid binding globulin (CBG)[371]. Cortisol is a pleiotropic hormone that influences gene expression of up to 20% of human genes [349]. GCs are responsible for controlling whole body homeostasis by mediating a range of metabolic processes including; mobilisation of energy, glucose utilisation, enhancing cardiovascular output and respiration, redistributing blood flow, increasing energy and substrate delivery to brain and muscle , increasing cerebral perfusion rates and modulating immune functioning [372, 373].

GCs exert their effects via interaction with glucocorticoid receptors (GR). In the absence of GCs, the non-activated glucocorticoid receptor resides in the cytoplasm as a multiprotein complex consisting of two molecules of hsp90 and several other proteins [374]. Upon ligand binding, GR undergoes a conformational change and dissociates from the multiprotein complex and translocates to the nucleus, where it binds to glucocorticoid response elements (GREs) located in promoter region of target genes and regulates expression of glucocorticoid responsive genes [363].
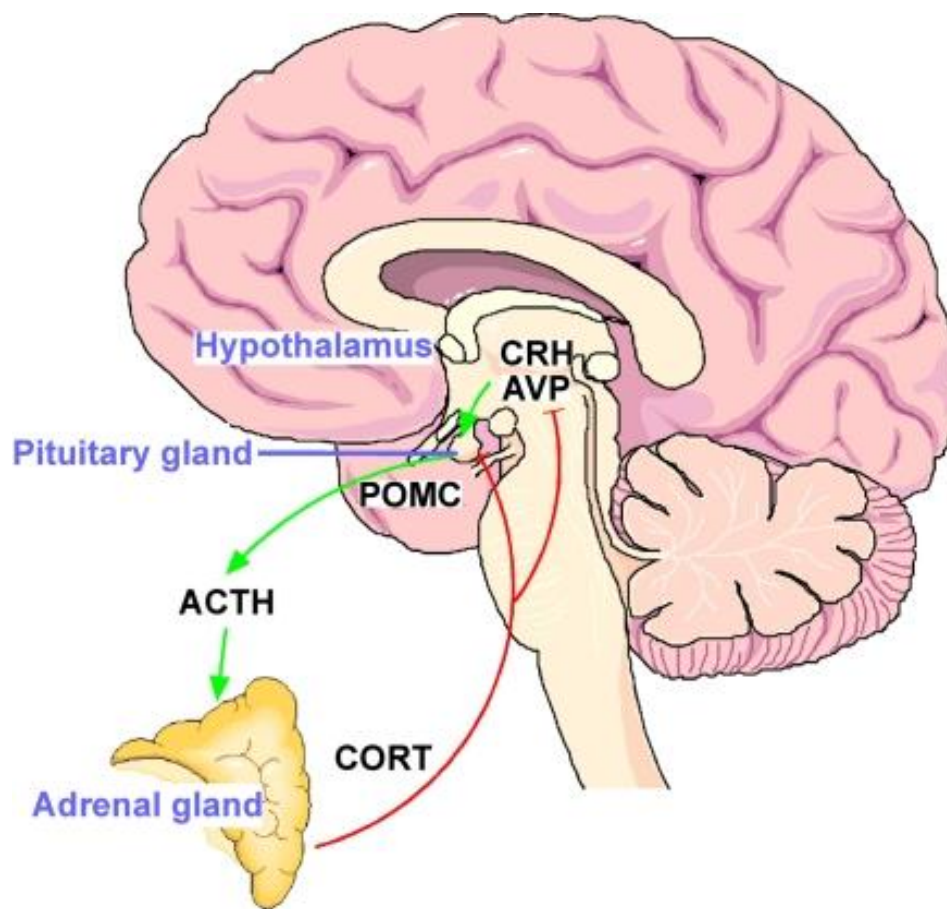
Figure 1.8 The Hypothalamus-Adrenal-Pituitary Axis.

During stressful situations, the parvocellular neurons of the hypothalamus release neuropeptide CRH into portal blood vessels that stimulate ACTH secretion by anterior pituitary which in turn stimulates secretion of glucocorticoids by adrenal glands. Glucocorticoids secreted regulate HPA axis activation by negative feedback mechanism. Image taken from Murgatroyd *et al* 2011[375].

1.8.2 DHEAS/DHEAS

Dehydroepiandrosterone (DHEA) together with its sulphated precursor DHEAS are the most abundant steroids in the human circulation [364]. A small fraction (< 1%) circulates in unsulphated form, as DHEA. Large quantities of DHEAS (3.5 to 20 mg) are produced on a daily basis in both sexes [376]. DHEA is predominantly synthesised in the human adrenal cortex from cholesterol, and serves as a precursor to sex hormones estradiol and testosterone. The human adrenal cortex is divided into three histologically distinct zones, which produce different classes of steroid hormones. The inner zone (zona reticularis) produces DHEA and its sulphate ester DHEAS [364, 377]. Even though the physiological role of DHEAS is poorly understood, it has been reported to have memory-enhancing, cardio protective, anti-diabetic, anti-obesity, anti-depressive, anti-glucocorticoids and immune-enhancing properties [364, 378].

1.8.3 Alterations in HPA axis in older adults

Healthy ageing is accompanied by relative activation of the HPA axis, due to a decline in production of DHEA/S and maintained or slightly increased production of cortisol [379]. Studies examining the effects of ageing on diurnal cortisol secretion have yielded conflicting results. A few studies have reported flattening of the diurnal pattern of secretion in older adults [379-381], which might be due to hippocampal atrophy [381]. Whilst other studies have reported either normal diurnal rhythm of cortisol [382, 383] or decreased diurnal rhythm [384, 385] with age . An overall elevated basal cortisol levels has been reported with age [386, 387]. However, a high variability has been reported in plasma cortisol levels with age, according to a longitudinal study about 40% of older adults exhibit an increase in cortisol levels and a similar proportion showed only moderate increase in cortisol levels whilst the remaining showed a decline in basal cortisol levels [387].Genetic factors are likely to be responsible for this high degree of variability in cortisol levels with age [388]. Further, there have also been a

few reports of unchanged cortisol levels with age [389, 390], with a trend towards elevated cortisol levels during evening in older subjects [391].

A major regulatory switch controlling tissue-specific activation of glucocorticoids is the enzyme 11β-hydrosteroid dehydrogenase type I (11 β- HSD1), which converts inactive cortisol to active cortisol [392]. Inflammatory cytokines can upregulate expression of 11β-HSD1 [393]. As ageing results in increased levels of pro-inflammatory cytokines (discussed in section 1.2), this might result in increased activity of 11 β-HSD1, leading to increased tissue exposure to the damaging effects of glucocorticoid [394, 395].

Sapolsky *et al* proposed the 'glucocorticoid cascade hypothesis' in 1986, according to which the ability to switch off the HPA axis declines with age [396] . During stressful events, impairments in feedback regulation of the HPA axis occur in older adults resulting in hyper secretion of glucocorticoids. Further, an age-related decline in levels of glucocorticoid receptors and their binding capacity, resulting in impaired feedback inhibition, might be the underlying cause for HPA axis hyperactivation [397].

DHEAS secretion follows a specific age associated secretion pattern [Figure 1.9]. High circulating DHEAS levels have been reported on birth, which rapidly drop and only rise again between the ages of 6-10 years, termed adrenarche [398, 399]. DHEAS levels reach peak concentrations during third decade of life. Once peak levels have been reached a steady decline occurs with age (1-2 % per year), such that by the age of 80 DHEA/S levels are only 10-20% of that seen in young adulthood [400-402]. This fall in DHEA/S levels, termed adrenopause, occurs in both sexes [403, 404]. Reduction in the size of the zona reticularis and reduced sensitivity towards ACTH are thought to contribute to diminished DHEA production with age [378, 405].

DHEA and DHEAS have been shown to act as immune enhancers, thus it can be hypothesised that age associated decline in DHEAS levels might contribute towards deterioration in immune functioning in older adults [406]. For instance, ageing is accompanied by dysregulated IL6 production, resulting in elevated serum IL6 levels with age, DHEAS supplementation in old mice has been shown to restore regulation of IL6 production [407]. Also, studies in animal models have reported success in using DHEA as a vaccine adjuvant to improve vaccine responses with age [408, 409]. *In-vitro* studies have reported that pre-treatment of human T cells with DHEA increased IL2 production and enhanced cytotoxic potential of T cells [410] and in vitro treatment of human neutrophils with DHEAS increased superoxide generation and was able to overcome inhibition of neutrophil function by cortisol [411].

Importantly, the elevated cortisol/DHEA(S) ratio that occurs with age might have a negative impact on the health of older adults [412].

1.9 Hip Fracture in older adults

Hip fracture is defined as any fracture of the femur between the articular cartilage of the hip joint to 5 cm below the distal point of the lesser trochanter [413] . Age is an important risk factor for hip fracture [414] and the majority of hip fractures are reported in older adults [413]. 370,000 older adults fall every year, 67000 of these resulted in a hip fracture in 2010 and this is predicted to rise to 117,000 by 2016 [415, 416]. Additionally, an estimated cost of about £12,000 was reported per hip fracture to the National Health Services in 1996 [417] which continue to increase, highlighting the burden of hip fractures on health care costs.
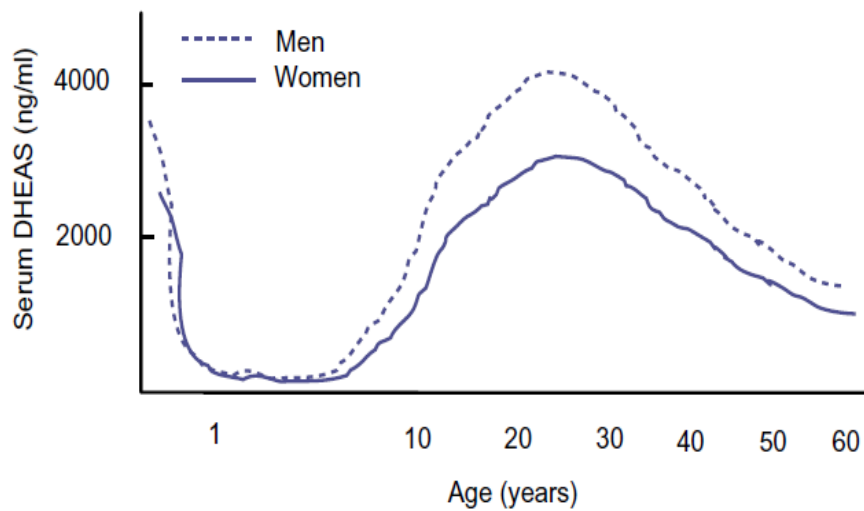
Figure 1.9 Schematic representations of serum DHEAS levels in men and women lifespan.

Immediately after birth, high circulating levels of DHEAS were reported, after which serum DHEAS levels rapidly decline and only begin to rise around the age of 10 years, termed 'adrenarche'. DHEAS levels reach maximum levels during the third decade of lifespan, after which a drop has been reported. DHEAS levels decline to 10-20% of its maximum levels by the age of 70. Image taken from Arlt *et al*, 2004[402].

Increased incidence of hip fracture with age might be a result of increased risk of falling in older adults due to neuromuscular deterioration and osteoporotic changes [418]. Even though hip fracture is treatable, it has a devastating effect on the life of older individuals and has been associated with poor outcomes including: increased mortality [419], reduced life expectancy [420, 421] and physical disability [422, 423] . Magaziner *et al* reported that only one-third of patients return to their pre-fracture level of physical functioning one year after a hip fracture [424] .

In a previous study by our group we have reported that about 37% of the older hip fracture patients that were in good health prior to the fracture succumbed to serious infections (the majority were bacterial infections) within 5 weeks of sustaining the fracture and were readmitted to hospital [359]. Diminished neutrophil superoxide production was reported in older hip fracture patients compared with younger individuals suffering from a similar injury, which might contribute towards the increased risk of infection in these individuals [359]. Further, older hip fracture patients had an increased serum cortisol: DHEAS ratio compared with young trauma patients and healthy older adults, which could mediate the suppressed neutrophil bactericidal function [411].

1.10 Depressive symptoms in hip fracture patients

Depression is the most common mood disorder reported in older adults [425], characterised by cognitive, behavioural, somatic and affective impairments that have a negative effect on functional activity, quality of life and social participation of an individual [426]. Stressful life events such as bereavement or a disabling medical event (e.g. heart attack, rheumatoid arthritis, cancer, lung disease) are frequent precipitants of depressive symptoms [427, 428]. Depressive symptoms have been associated with poor outcomes and an increased mortality in medical inpatients [429, 430].The development of depressive symptoms in medical

conditions rather than being a physiological reaction to the incapacitation and pain caused by the physical disease might also be in part a result of immune activation and cytokine secretion induced by the illness as many pro-inflammatory cytokines can lower mood [431, 432].

A high incidence of depression (9 - 47%) has been reported in UK and USA based studies of older adults with hip fracture [433-435] compared with the general population. Hip fracture is thus a risk factor for depression [426]. Depression is a highly disabling condition and has been identified as a predictor for health outcomes and recovery after hip fracture [436, 437]. Mossey *et al* reported that hip fracture patients with few depressive symptoms were nine times more likely to return to their pre-fracture level of physical functioning than those hip fracture patients with more depressive symptoms [438]. Interestingly, a two year follow up study has shown that diagnosis of depressive symptoms in older adults soon after hip fracture substantially elevates the risk of mortality [435].

1.11 Aims of this thesis

A novel subset of B cells with immunosuppressive properties has been recently identified in humans. Although most aspects of immunity have been studied with respect to ageing, the effect of ageing on this subset of CD19^{+ve}CD24^{hi}CD38^{hi} B cells is unknown. Thus the first aim of this thesis was to examine the effect of age on peripheral count and IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} B cells.

Ageing, physical stress and psychological stress are key interacting factors that have a detrimental effect on immune function. A previous study done in our group had reported suppressed neutrophil superoxide production [359] and an elevated serum cortisol:DHEAS ratio in older individuals post hip fracture [411]. Additionally, a high incidence of

development of depressive symptoms has been previously reported in hip fracture patients, even in those with no previously known psychiatric history [439]. However the potential combined effect of hip fracture and depressive symptoms on immune and endocrine variables in older adults has not been examined. The second aim of this thesis was to examine the effects of hip fracture and depressive symptoms on innate immune function (neutrophils, monocytes, and NK cells), adaptive immune function (T cells, B cells and regulatory cells), serum stress hormone levels (cortisol, DHEAS) and serum cytokine levels in older adults.

Chapter 2

The effect of ageing on immunosuppressive properties of Transitional B cells

2.1 Introduction

2.1.1 Background

It is well accepted that ageing is accompanied by immune dysregulation and an increase in the incidence of autoimmunity, for example RA, has been reported with advancing age [260]. Age associated accumulation of senescent T cells, skewing towards Th17 phenotype, reduced immunosuppressive properties of T_{regs}, increased B cell autoreactive properties, an elevated circulating level of pro-inflammatory cytokines have all been previously discussed as factors that might be contributing towards increased risk of RA in older adults [263, 265].

B cells are known to play a pathogenic role and exacerbate autoimmune diseases [281]. In recent years, evidence has emerged for the presence of a subset of B cells with immunoregulatory properties in a number of murine models of inflammation [294, 304]. Two phenotypically distinct subsets of B cells: transitional $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells [301] and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ "B10" cells [306] have been reported to possess immunosuppressive properties, mainly via IL10 production. CD40 pathway stimulation induces expansion and IL10 production in both transitional B cells and B10 cells [301, 308, 319]. Interestingly, stimulation via TLRs has also been shown to induce IL10 production in B10 cells [306, 308], whereas transitional B cells are capable of producing IL10 on stimulation via the CD40 pathway [301]. In addition to IL10 production, murine B_{regs} have been shown to secrete TGFβ, but the inhibitory effect of human $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells is TGFβ independent [440]. Additionally, the suppressive effect of $CD19^{+}CD24^{hi}CD38^{hi}$ B cells is also mediated via engagement of CD80 and CD86 [301, 328].

In this chapter, the effect of age on peripheral numbers of B_{reg} cells and their IL10 production capacity was investigated to examine if defects in B_{reg} cells might be an

additional factor contributing towards the age-associated increase in incidence of autoimmunity, especially RA.

2.1.2 Aims

The aims of this chapter were:

(a) To confirm that it was possible to stimulate human B10 cells and transitional B cells via CD40 and TLR stimulations;

(b) To determine if ageing is accompanied by numerical alterations in transitional B cells and B10 cells;

(c) To examine if advancing age has an effect on IL10 production by both subsets of B_{regs} on stimulation via TLR and CD40 pathway and if so to determine the mechanisms involved;

(d) To determine if there is an association between IL10 production by $CD19^{+ve}CD24^{hi}CD38^{hi}$ and levels of serum autoantibodies (Rheumatoid Factor) with age.

2.2 Materials and Methods

2.2.1. Media, solutions and antibodies

Complete RPMI medium consisted of RPMI 1640 (Sigma Aldrich, UK) containing 1% GPS (2 mM glutamine, 100U/ml penicillin, and 100µg/ml Streptomycin; Sigma Aldrich, UK) and 10% Heat inactivated fetal calf serum (HiFCS). Fetal calf serum (FCS; Sera Laboratories, UK) was incubated for 30 minutes in water bath at 56°C to heat-inactivate it and prepare HiFCS. Phosphate buffer saline (PBS) was NaCl (137 mM), KCl (2.7 mM), NA_2HPO_4 (10 mM) and KH_2PO_4 (2 mM) in double distilled H_2O. PBS was prepared by dissolving one PBS tablet (Sigma Aldrich, UK) in 200 ml of distilled H_2O. The PBS solution prepared was autoclaved prior to use. MACS buffer was PBS containing 2mM ethylenediamine tetra-acetric acid (EDTA; Sigma Aldrich, UK) and 0.5% bovine serum albumin (BSA; Sigma Aldrich, UK). The antibodies used in the thesis, their working dilution and source are shown in Table 2.1.

2.2.2. Subjects and blood sample collection

56 young (mean age 26.81, range 20 - 36 years, 29 females) and 65 older (mean age 70.12, range 60 – 84 years, 32 females) subjects participated in this study. A venous sample was taken from each subject between the hours of 0900-1100 am after obtaining informed consent. At the time of blood sampling, none of the subjects had infections, and fulfilled a health criterion that was implemented to exclude subjects on steroid medication and those suffering from co-morbidities including; autoimmune diseases, infectious diseases, malignancy, diabetes and asthma. The study was approved by the North Staffordshire Research Ethics Committee.

2.2.3 Peripheral Blood Mononuclear (PBMC) Isolation

Peripheral blood was collected in vacutainers and transferred into a 50 ml falcon tube (Becton Dickinson, UK). The blood was diluted 1:1 with RPMI medium (Sigma Aldrich, UK). The diluted blood was gently layered on top of 6mls of Ficoll-Paque^{TM} PLUS (GE Healthcare, Sweden) in a 25 ml universal tube (Starsted, UK). The tube was centrifuged at 420 x g for 30 minutes with no break using a centrifuge (Jouan CR3i, DJB Labcare Ltd, UK). Post centrifugation, the mononuclear cell layer containing PBMC, which resided at the interface between plasma and Ficoll-Plaque was removed using a plastic pasteur pipette (Starsted, UK) and was added to a new universal tube and the tube was topped up with PBS. The cells were centrifuged at 300 x g for 10 minutes. This wash step was repeated and the cells were resuspended in PBS and were counted using a haemocytometer (Sigma Aldrich, UK).

2.2.4 Freezing and thawing PBMCs

PBMCs were frozen down by re-suspending cells in freezing medium consisting of 10% DMSO (Sigma Aldrich, UK) in heat inactived fetal calf serum (Biosera, UK) and transferring them in small aliquots into cryovials. The cryovials were transferred into a freezing container (Mr Frosty, Sigma Aldrich, UK) containing isopropanol (VWR International, UK). This allowed the samples to freeze gradually at the rate of 1°C per minute. Cells were then stored at -80°C. When thawing out frozen samples, they were put in a water bath at 37°C. Once the cells had been thawed out they were washed in 10 ml of RPMI 1640 (Sigma-Aldrich, UK) to remove DMSO and cells were pelleted by centrifugation at 300 x g for 10 minutes.

2.2.5 Surface immunostaining for phenotypic analysis of B cells and T cells

Isolated PBMCs resuspended in PBS (1×10^6/ml) were transferred into polypropylene tubes (Becton Dickinson, UK) for immunostaining with a combination of fluorochrome conjugated antibodies including; anti-human CD19-PE (eBiosciences, UK), anti-human CD24-FITC (eBiosciences, UK), anti-human CD38-PEcy7(eBiosciences, UK), anti-human CD19-FITC (eBiosciences, UK), anti-human CD5-PEcy7 (eBiosciences, UK), anti-human CD1d-PE (eBiosciences, UK), anti-human CD40-APC (eBiosciences, UK), anti-human TLR4 (CD284)-APC (eBiosciences, UK), anti-human CD80-APC (Biolegend, UK), anti-human CD86- APC (Biolegend, UK), anti-human CD3-FITC (Dako, UK), anti-human CD4-PE (eBiosciences, UK), anti-human CD154-APC (eBiosciences, UK), anti-human CTLA4-APC (Biolegend, UK) and anti-human CD28-APC (BD Biosciences, UK) antibodies at the dilutions shown in Table 2.1. Staining for cell surface markers took place for 20 minutes in dark at 4°C. Following incubation, cells were washed twice with 300 µl of PBS and centrifuged at 250 x g for 5 minutes. Post washing, cells were resuspended in 300 µl of PBS for flow cytometric analysis on a Cyan ^{TM} ADP (Dako Ltd, UK) [441].

2.2.6 B cell functional assays – CD3 stimulation of PBMCs

Human cell cultures were performed in complete RPMI1640 medium (described in section 2.2.1) which was pre-warmed to 37°C. For CD3 stimulation, a 2µg/ml solution of anti-CD3 mAb (BD biosciences, UK) was prepared, 50µl of this solution was added into each well of a 96 well round bottom microtitre plates (Becton Dickinson, UK). The plate was incubated at 37°C for 1 hr prior to addition of 150µl of PBMCs in culture medium (at 1×10^6/ml) to each well. The cells were cultured for 72 hr at 37°C in a humidified atmosphere of 5% CO_2 [441]. Brefeldin A (10µg/ml; Sigma Aldrich, UK) was added for the last 6 hr of the

stimulation along with PMA (50ng/ml; Sigma Aldrich, UK) and Ionomycin (500ng/ml; Sigma Aldrich, UK).

Antibody	Dilution	Company
Anti-human CD19 PE (clone : H1B19)	1/50	eBiosciences
Anti-human CD19 FITC (clone : H1B19)	1/50	eBiosciences
Anti-human CD24 FITC (clone : eBioSN3)	1/20	eBiosciences
Anti-human CD 38 PEcy7 (clone : HIT2)	1/100	eBiosciences
Anti humanCD5 PE cy7 (clone :UCHT2)	1/50	eBiosciences
Anti-human CD 1d PE (clone 51.1)	1/50	eBiosciences
Anti-human CD40 APC (clone : 5C3)	1/20	eBiosciences
Anti-human CD3 FITC (clone : UCHTI)	1/100	Dako
Anti-human CD4 PE (clone : RPA-T4)	1/100	eBiosciences
Anti-human CD154 APC (clone : 24-31)	1/100	eBiosciences
Anti-human TLR4 APC (clone : HTA125)	1/20	eBiosciences
Anti-human CD80- APC (clone :2D10)	1/20	Biolegend
Anti -human CD86 - APC (clone : IT2.2)	1/20	Biolegend
Anti-human CTLA4-APC (clone : L3D10)	1/20	Biolegend
Anti-human CD28-APC (clone : CD28.2)	1/1000	BD biosciences
Anti-human Stat3 (p Y705) Alexa fluor 647	1/10	BD biosciences
Anti-human IL10 Alexa fluor 647(clone: JES3-9D7)	1/40	eBiosciences
Isotype controls		
APC mouse IgG2b (clone: MPC-11)	1/100	Biolegend
FITC mouse IgG1 (clone : DAK-GO1)	1/100	Dako
RPE mouse IgG2b (clone : DAK-GO9)	1/100	Dako
PEcy7 mouse IgG2a (clone : P 3.6.2.81)	1/100	eBiosciences
Alexafluor 647 mouse IgG2b (clone : MOPC-173)	1/100	BD biosciences

Table 2.1.Primary and isotype control antibodies used for flow cytometry.

2.2.7 Measuring CD154 expression on CD4 T cells post CD3 stimulation

PBMCs were stimulated with plate bound CD3 for 72 hr (described in 2.2.6), but Brefeldin A was not added during the last 6 hr of stimulation. Post stimulation, cells were transferred into polypropylene tubes (Becton Dickinson, UK) and were pelleted by centrifugation at 250 x g for 5 minutes and washed twice with 300 µl of PBS. Post washing, cells were re-suspended in 100 µl of PBS and stained using cell surface marker antibodies anti-CD3-FITC (Dako, UK), CD4-PE (eBiosciences, UK) and CD154-APC (eBiosciences, UK) for 20 minutes in the dark at 4°C. Post immunostaining, cells were washed with 300 µl of PBS and were centrifuged at 250 x g for 5 minutes. The cells were re-suspended in 300 µl of PBS for flow cytometric analysis of immunofluorescence using a Cyan ^{TM} ADP (Dako Ltd, UK) and the percentage of CD154^{+ve} CD4 T cells and CD154 expression (MFI) levels were recorded [441].

2.2.8 Flow cytometric detection of intracellular IL10

Post CD3 stimulation (described in 2.2.6), cells were transferred into polypropylene FACS tubes (Becton Dickinson, UK) and washed with 300 µl of PBS and centrifuged at 250 x g for 5 minutes. Post washing, cells were suspended in 100 µl of PBS and were stained with a combination of extracellular surface markers to identify transitional B cells using anti-human CD19 PE (eBiosciences, UK), anti-human CD24 FITC (eBiosciences, UK) and anti-human CD38 PEcy7 (eBiosciences, UK) antibodies or B10 cells using anti-human CD19 FITC (eBiosciences, UK), anti-human CD5 PEcy7 (eBiosciences, UK) and anti-human CD1d PE antibodies (eBiosciences, UK) for 20 minutes in dark at 4°C. Post immunostaining, cells were washed with 300 µl of PBS and were centrifuged at 250 x g for 5 minutes. The supernatant was removed and the cells were re-suspended in 50 µl of Reagent A (Fix and Perm kit, Invitrogen, UK) and were incubated for 30 minutes in the dark

at room temp. Post fixing, the cells were washed with 300 μl PBS and centrifuged at 250 x g for 5 minutes. Pelleted cells were resuspended in 50 μl of Reagent B (Fix and Perm kit, Invitrogen, UK) and anti-human IL10 Alexa fluor 647 antibody (eBiosciences, UK) was added to the cells and they were incubated for 30 minutes in the dark at room temperature. Post incubation, the cells were washed with 300 μl of PBS and centrifuged at 250 x g for 5 minutes. Cells were resuspended in 300 μl of PBS for flow cytometric analysis using a Cyan ™ ADP (Dako Ltd, UK) and the percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} cells and IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} cells as well as IL10 expression (MFI) levels were recorded [441].

2.2.9 Isolation of B cells

B cells were isolated from PBMCs by negative selection using magnetic activated cell sorting (MACS) technology (Human B cell isolation kit; Miltenyi Biotech, Germany). Freshly isolated PBMCs were counted and washed in 10 ml of MACS buffer (Miltenyi Biotech, Germany) at 300 x g for 10 minutes at 4°C. The cell pellet obtained was re-suspended in 40 μl of MACS buffer (per 10^7 cells; Miltenyi Biotech, Germany). To this suspension, 10 μl of B cell Biotin- antibody cocktail (per 10^7 cells; Miltenyi Biotech, Germany) was added and the sample was incubated for 10 min at 4°C with occasional vortexing. Post incubation, 30 μl MACS buffer (per 10^7 cells; Miltenyi Biotech, Germany) and 20 μl B cell microbead cocktail (per 10^7 cells; Miltenyi Biotech, Germany) was added to the cells and they were incubated for 15 min at 4°C. Post incubation, cells were washed in 2ml of MACS buffer (per 10^7 cells) and were centrifuged at 300 x g for 10 minutes at 4°C. The sample was resuspended in 500 μl of MACS buffer.

For magnetic isolation of B cells an LS column (Miltenyi Biotech, Germany) was placed in the magnetic field of a Quadra MACS separator (Miltenyi Biotech, Germany). The column

was prepared by washing with 3ml of ice cold MACS buffer .The PBMC suspension in MACS buffer was then applied to the column, followed by 9 ml (1ml at a time) of MACS buffer. The elute containing purified B cells was collected in a universal tube (Starsted, UK). Cells were pelleted by centrifugation at 300 x g for 10 minutes at 4°C and the cells were resuspended in complete RPMI 1640 medium (described in 2.2.1) at 2.5 x 10^4 cells/ml.

To determine the purity of isolated B cells, 100 μl of the isolated B cells were taken up in a FACS tube (Becton Dickinson, UK) and were stained with anti-human CD3 FITC (Dako , UK) and anti-human CD19 PE (eBiosciences , UK) for 20 minutes at 4°C in the dark. Post incubation, cells were washed with 300 μl of PBS and centrifuged at 250 x g for 5 minutes and taken up in 300 μl of PBS and purity was assessed using Cyan ^{TM} ADP (Dako Ltd, UK). B cells were defined as $CD3^{-ve}$ $CD19^{+ve}$ cells and purity obtained on a routine basis was > 92%.

2.2.10 Stimulation of isolated B cells – CD40L and LPS

Purified B cells were taken up in 200μl of complete RPMI medium (described in section 2.2.1) which was pre-warmed to 37°C and cultured in a 96 well U bottom plate (Becton Dickinson, UK) at 2 x 10^5 cells per well for 48 hr at 37°C at 5% CO_2 with recombinant human CD40L (1μg/ml; Peprotech, UK) or LPS isolated from *Escherichia coli* serotype 0111:B4 (1μg/ml; Sigma-Aldrich, UK). Brefeldin A (10 μg/ml; Sigma Aldrich, UK) was added during the last 6hr of the stimulation. Post incubation, B cells were transferred into polypropylene FACS tubes (Becton Dickinson, UK) washed with 300 μl PBS and were centrifuged at 300 x g for 10 minutes [441]. Cells were stained to measure intracellular IL-10 levels (described in section 2.2.8). Cells were resuspended in 300 μl of PBS for flow cytometric analysis using a Cyan ^{TM} ADP (Dako Ltd, UK) and the percentage of

IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} cells and IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} cells as well as IL10 expression (MFI) levels were recorded.

2.2.11 Quantification of IL10 levels in supernatant

Isolated B cells (1 x 10^6) were taken up in 200µl of pre-warmed complete RPMI medium (described in section 2.2.1). B cells were stimulated in a 96 well U bottom plate (BD biosciences, UK) with either recombinant human CD40L (1µg/ml; Peprotech, UK) or LPS isolated from *Escherichia coli* serotype 0111:B4 (1µg/ml; Sigma Aldrich, UK) for 48 hr at 37°C at 5%CO$_2$. Brefeldin A was not added during the last 6 hr of stimulation. Post stimulation, cells were pelleted by centrifugation at 300 x g for 10 minutes and supernatants were transferred into cryovials and snap frozen in liquid nitrogen and stored at -80°C, until future analysis.

IL10 levels in supernatants were quantified using commercially available Human IL-10 ELISA (Abcam, UK), as per manufacturer's instructions. Firstly, frozen supernatants were thawed on ice and 100 µl of cell culture supernatants were added to 96 well plate coated with anti-mouse IL10. 50µl of secondary biotinylated anti-IL10 was added to each well and the plate was incubated at room temperature for 2hr. Post incubation, liquid was aspirated and the plate was washed three times with 300 µl of washing solution. Post washing, 100µl of Streptavidin –HRP was added to each well and incubated for 30 minutes at room temp. The liquid was aspirated and the plate was washed again three times with 300 µl of washing solution. Post washing, 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added into each well and incubated in dark for 12-15 minutes at room temp. The enzyme substrate reaction was stopped by adding 100µl of stop reaction (H$_2$SO$_4$) into each well. The plate was read on a spectrophotometer (BioTek® Synergy HT, UK) using 450 nm as the primary wavelength within 5 minutes of adding stop solution. The OD values for

individual samples were calculated using a standard curve created by GraphPad Prism®
software (GraphPad Software Limited, USA).

2.2.12 Measuring phosphorylation state of STAT3

PBMCs isolated from healthy young and old donors (described in section 2.2.3) were rested
for 1 hr in 150 µl of complete pre warmed RPMI medium (described in section 2.2.1) in a 96
well U bottom plate (Becton Dickinson, UK) at 37°C at 5% CO_2 . After 1 hr, cells were
stimulated with 0.5µg of purified mouse-anti-human CD40 mAb (per 10^6 cells; BD
biosciences, UK for 0, 5, 15 or 30 minutes in the dark at 4°C. Post stimulation, cells were
transferred into polypropylene FACS tubes (Becton Dickinson, UK) and were washed with
300 µl of ice cold PBS and centrifuged at 250 x g for 5 min. Cells were resuspended in
100µl of PBS and stained with cell surface markers antibodies anti-human CD19-PE
(eBiosciences, UK), anti-human CD24-FITC (eBiosciences, UK) and anti-human CD38-
PEcy7 antibodies (eBiosciences, UK) for 20 minutes in the dark at 4°C. Post
immunostaining, cells were washed in 300 µl PBS and centrifuged at 250 x g for 5 minutes
and were resuspended in 100µl of warm PBS and rested for 30 minutes at 37°C at 5% CO_2.
Post incubation, cells were fixed immediately to maintain phosphorylation state by adding
100µl of 1.6% paraformaldehyde (Sigma Aldrich, UK) for 30 minutes at room temperature
in the dark. Post fixation, cells were washed with 300 µl ice-cold PBS and centrifuged at 250
x g for 5 min. 2 ml of ice-cold methanol (VWR International, UK) was added to and the
cells were incubated for 30 min in the dark at room temperature. Post permeabilisation,
samples were washed with 300 µl of PBS and centrifuged at 250 x g for 5 minutes and pellet
was taken up in 100 µl of cold PBS and stained with anti-STAT3 (pY705) phosphospecific
antibody (BD biosciences, UK) or appropriate phosphoflow Alexa fluor 647 isotype mouse
IgG2b control (BD biosciences, UK) for 30 minutes in the dark at 4°C. Cells were

resuspended in 300 μl of PBS for flow cytometric analysis using a Cyan ^{TM} ADP (Dako, UK) and the percentage of pSTAT3^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} cells as well as pSTAT3 expression (MFI) levels were recorded [441].

2.2.13 Flow cytometry analysis

During flow cytometric analysis using a Cyan ^{TM} ADP (Dako Ltd, UK) 50,000 live lymphocytes were gated; dead cells were excluded on the basis of forward and side scatter. Appropriate isotype controls were used for setting gates. During multicolour immunostaining compensation was performed to ensure that spectral of different fluorochrome conjugated-antibody did not overlap by staining cells individually with each antibody. Flow cytometry data was analysed using Summit v 4.3 software (Dako, USA).

2.2.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism$^{®}$ (Graph Pad, La Jolla, USA) software. Data distribution was checked using the Kolmogorov-Smirnov test. For normally distributed data, a Student's t test analysis was performed to assess differences between two groups. P values of <0.05 were considered significant. To assess the relationship between two variable linear regressions was used.

2.3. Results

2.3.1 Identification of IL10 producing B cells in peripheral blood.

Transitional B cells have been identified previously on the basis of differential expression of CD24 and CD38 [300] and the gating strategy used to identify the different peripheral B cell subsets is shown in Figure 2.1a. Functionally immature CD19^{+ve}CD24^{hi}CD38^{hi} B cells have been reported to comprise the highest percentage of IL10 producing B cells upon CD40 stimulation [301, 305]. These findings were confirmed here as demonstrated by an enrichment of IL10^{+ve} B cells within the CD19^{+ve}CD24^{hi}CD38^{hi} subset in peripheral blood upon CD40 stimulation [Figure 2.1b] [441]. Additionally, studies done on B10 cells have reported an increase in IL10^{+ve} B cells on stimulation via TLR signals [308]. Here for the first time I have shown that LPS stimulation also upregulated the percentage of IL10 producing CD19^{+ve}CD24^{hi}CD38^{hi} cells in humans [Figure 2.1c] [441]. B cells were also cultured in the absence of stimulus and in the presence of BrefeldinA to block IL10 secretion and serve as negative control.

Tedder *et al* reported that CD19^{+ve} CD5^{+ve} CD1d^{hi} B cells have immunoregulatory properties and referred to them as "B10" cells [303]. I have confirmed their presence in the peripheral circulation in humans [gating strategy shown in Figure 2.2a] and shown that B10 cells respond to stimulation via CD40 pathway [Figure 2.2b] and TLR4 ligation to induce IL10 expression [Figure 2.2c].
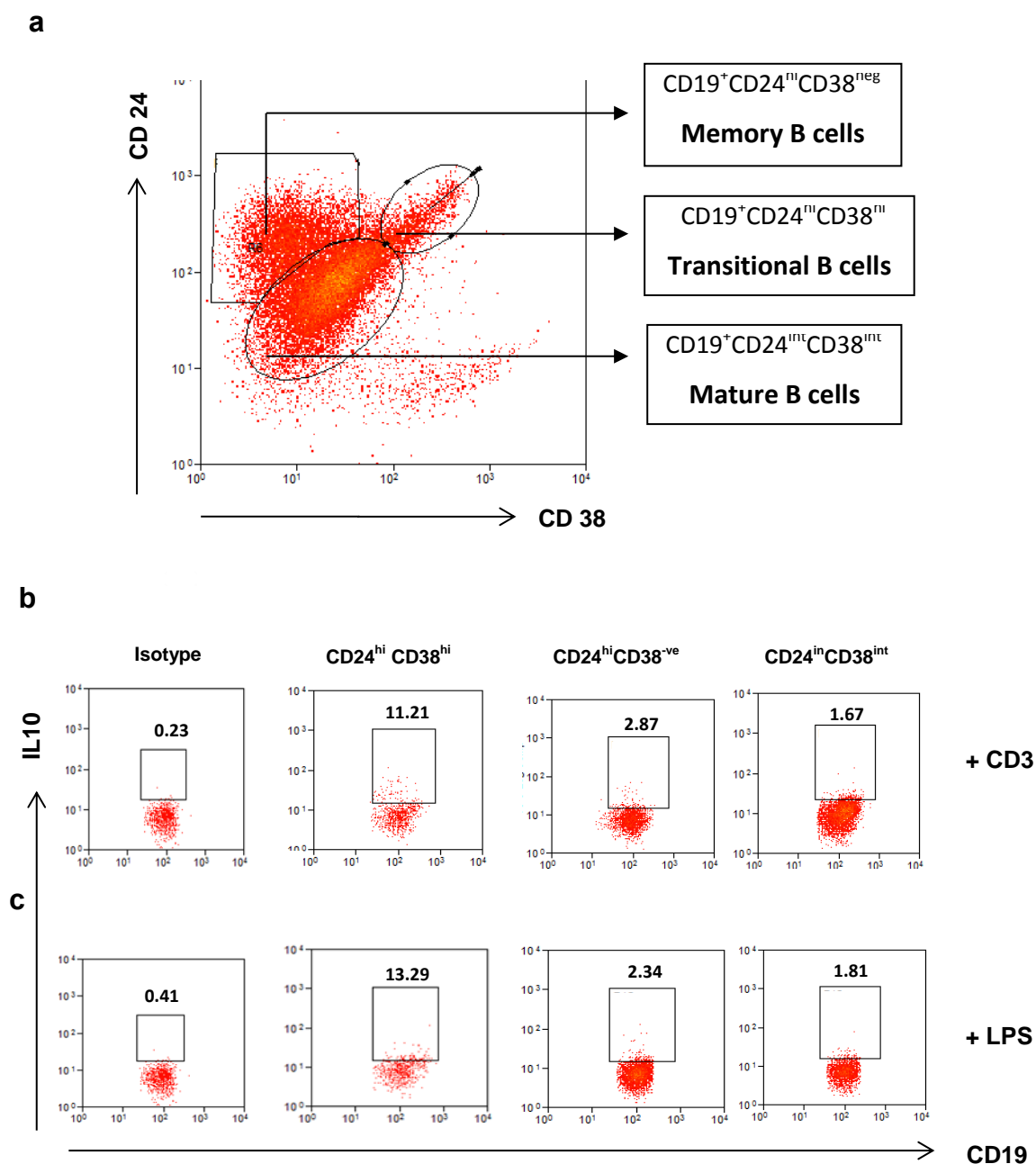
Figure 2.1. CD19^{+ve}CD24^{hi}CD38^{hi} B cells are the main producers of IL10 in B cells.

PBMCs were isolated from peripheral blood, stimulated using anti-CD3 antibody for 72 hr and immunostained for expression of CD19, CD24, and CD38 and intracellularly stained to measure IL10 levels. (a) Representative flow cytometric plot showing the gating strategy used to identify transitional B cells in the B cell pool via expression of CD24 and CD38; (b) IL10 expression in B cell subsets following T cell dependent stimulation via CD3,(c) or T cell independent stimulation via LPS.
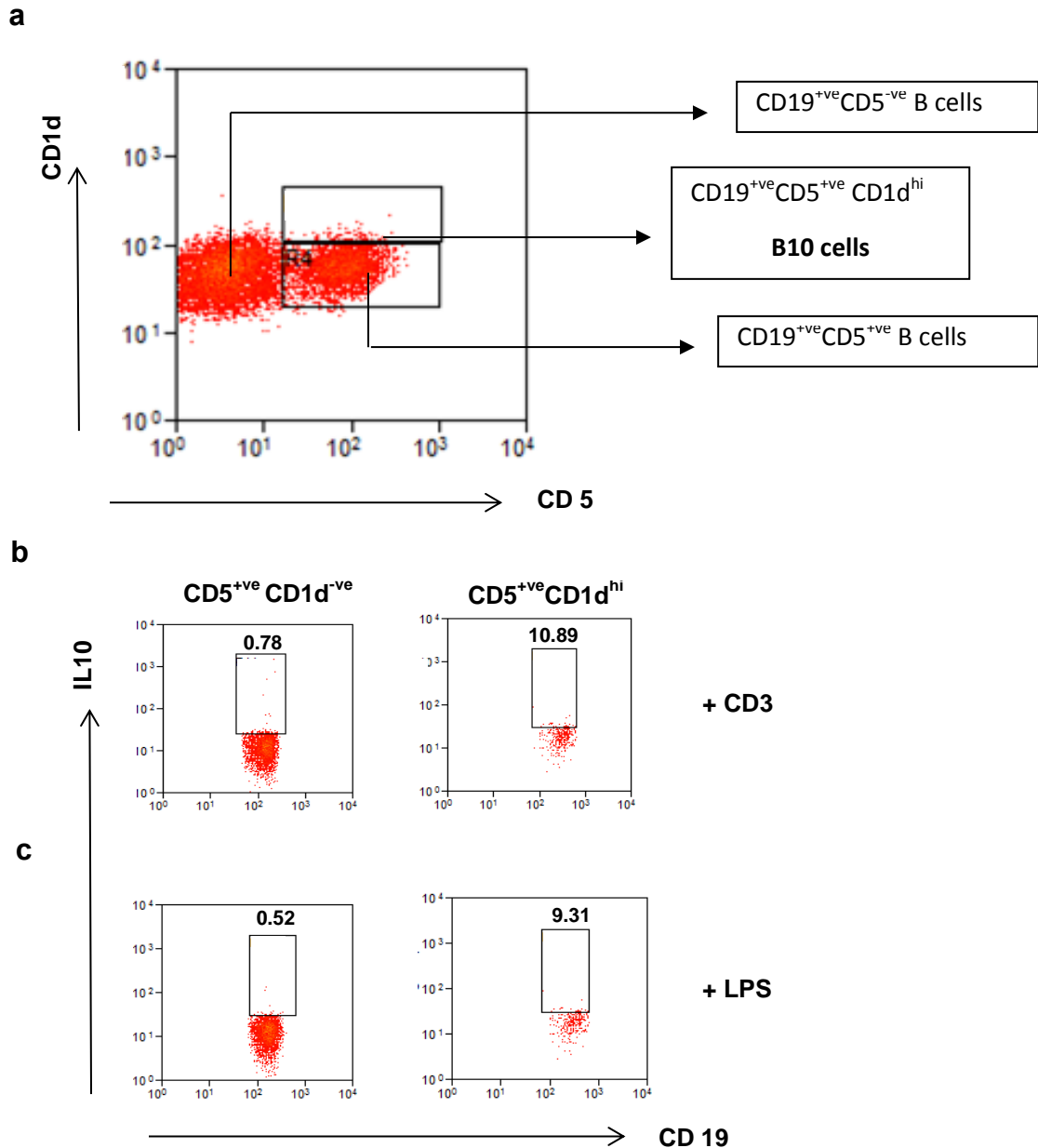
Figure 2.2. IL10 production by CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells.

PBMCs isolated from peripheral blood were stimulated using anti-CD3 antibody or LPS for 72 hr and immunostained for expression of CD19, CD5, and CD1d and intracellularly stained to measure IL10. (a) Representative flow cytometric plot showing the gating strategy used to identify B10 cells in the B cell pool via expression of CD5 and CD1d, (b) IL10 expression in B cell subsets following T cell dependent stimulation via CD3 (c) or T cell independent stimulation via LPS.

2.3.2 Effect of age on the frequency of circulating transitional B cells and B10 cells

On examining the percentage and absolute numbers of B cells in peripheral blood of healthy young and old donors, a significant decline in both percentages, $p < .001$, [Figure 2.3a] and absolute numbers of total B cells, $p = .03$, [Figure 2.3b] was found in older donors. These findings are in broad agreement with previous studies reporting a decline in circulating B cells with age [241, 243, 244].

Previous studies have used two separate set of phenotypic markers to identify B_{regs}. In this chapter, the effect of age on both transitional B cells ($CD19^{+ve}CD24^{hi}CD38^{hi}$) and B10 ($CD19^{+ve}CD5^{+ve}CD1d^{hi}$) subsets of B_{regs} was assessed. A significant decline in the percentage, $p < .001$ [Figure 2.4a] and absolute number of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells in peripheral blood was seen with age, $p < .001$ [Figure 2.4b] [441]. Aditionally, a significant decline in the percentage, $p < .001$ [Figure 2.4c] and total number of B10 cells, $p < .001$ was also found in older adults [Figure 2.4d] [441].

Interestingly, a significant negative correlation, $\beta = -.68$, $p < .001$, $\Delta R^2 = .47$ was found between age and frequency of $CD24^{hi}CD38^{hi}$ cells in B cell pool [Figure 2.5], such that with increasing age the frequency of $CD24^{hi}CD38^{hi}$ B cells declines.
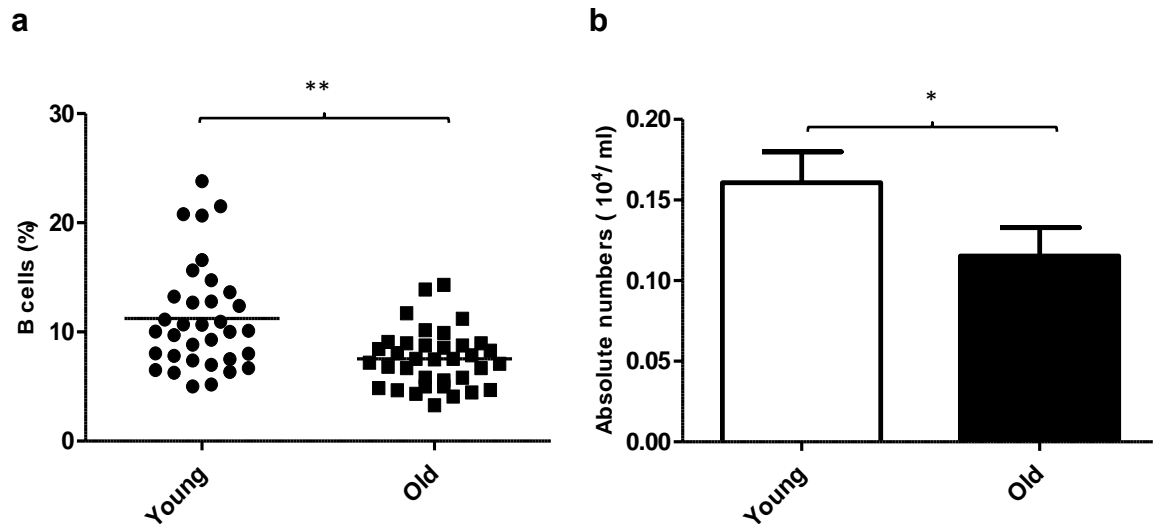
Figure 2.3 B cells in peripheral blood of young and old donors.

(a) Representation of percentage of B cells in peripheral blood of healthy young (n = 34) and old donors (n = 35) .The mean value is indicated by the bar. (b) Representation of the mean absolute number of B cells in peripheral blood of healthy young (n = 15) and old donors (n = 15). Data is expressed as mean ± SEM. * p <.05, ** p <.005.
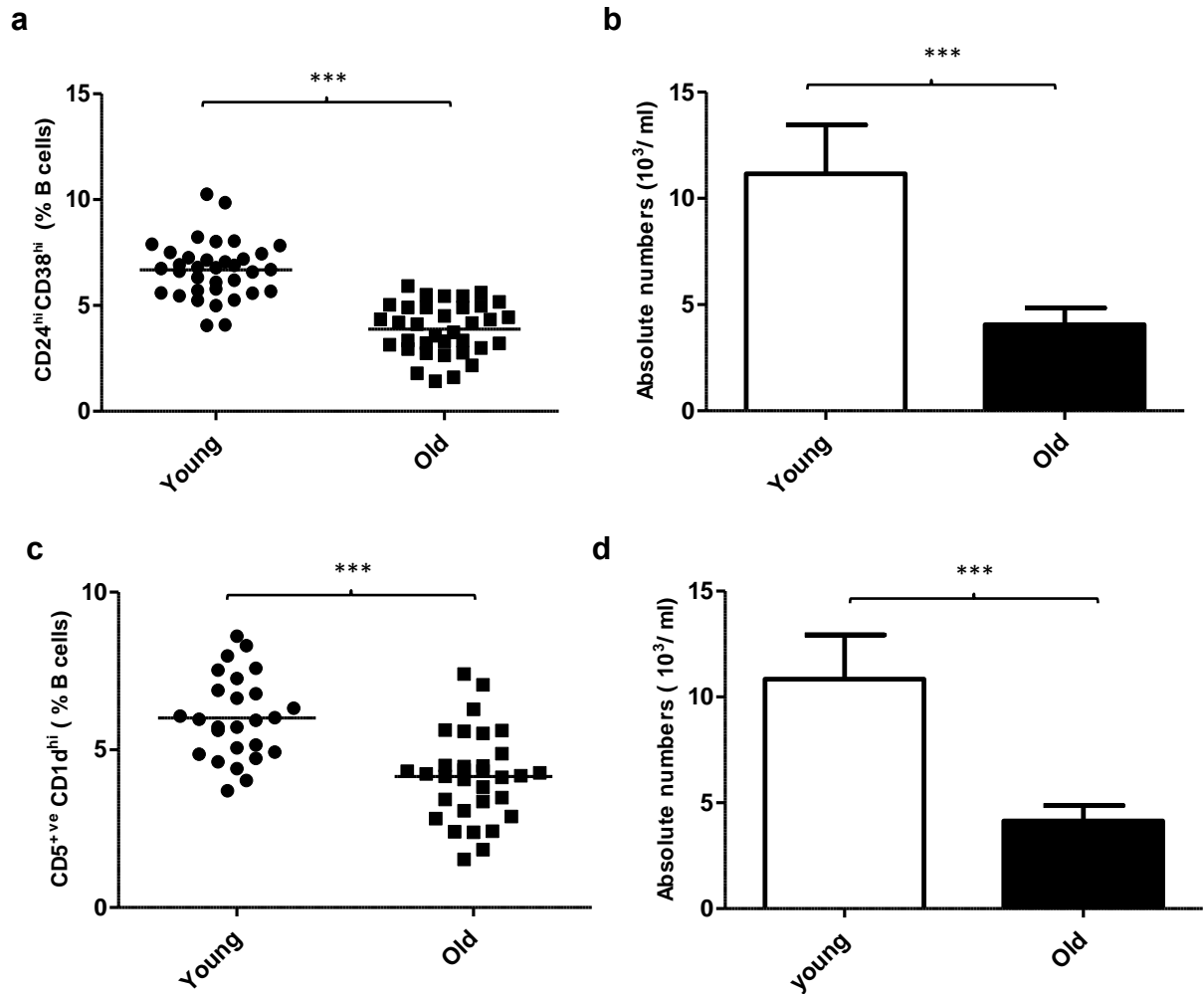
Figure 2.4 Transitional (CD19^{+ve}CD24^{hi}CD38^{hi}) B cells and B10 (CD19^{+ve}CD5^{+ve}CD1d^{hi}) cell frequency in peripheral blood with age.

(a) The percentage of CD19^{+ve}CD24^{hi}CD38^{hi} B cells in the peripheral B cell pool of young donors (n= 24) and old donors (n = 22) in the basal state. The mean value is indicated by the bar. (b) The absolute number of CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of healthy young (n=15) and old donors (n=15). Data is expressed as mean ± SEM. (c) The percentage of CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells in the peripheral B cell pool of young donors (n= 18) and old donors (n = 18) in the basal state. The mean value is indicated by the bar. (d) The absolute number of B10 cells in peripheral blood of healthy young donors (n=15) and old donors (n=15). Data is expressed as mean ± SEM. * p <.05, ** p <.005 , *** p <.001 .
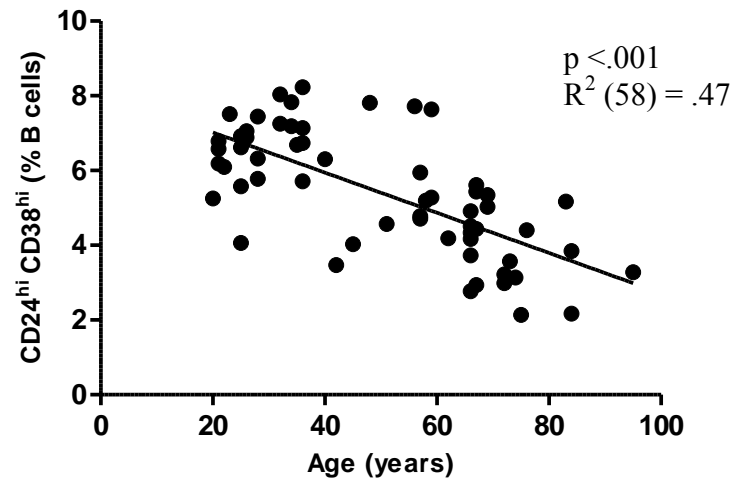
Figure 2.5 Correlation between age and frequency of CD19[+ve]CD24^{hi}CD38^{hi} B cells.

Representation of the correlation between percentage of circulating CD24^{hi}CD38^{hi} B cells and age in healthy individuals (n = 58).

Further , on examining $CD19^{+ve}CD24^{int}CD38^{int}$ (mature) B cells in peripheral blood of healthy young and old donors, a significant decline in their frequency was seen with age, p = .002 [Figure 2.6a] although the absolute number of mature B cells in peripheral blood remained unaltered, p = .12 [Figure 2.6b]. An increase in the frequency of $CD19^{+ve}CD24^{neg}CD38^{+ve}$ (memory) B cells was also detected in old donors, p = .03 [Figure 2.6c], but the absolute number of memory B cell subsets in peripheral blood did not differ between the two groups, p = .89 [Figure 2.6d].

2.3.3 Impaired IL10 induction by transitional B cells and B10 cells in older adults – T cell dependent stimulation

The hallmark of suppressive function of transitional B cells and B10 cells is their ability to produce IL10 [294, 308, 319]. Contact dependent delivery of activating signals by CD4 T cells to the B_{regs} induces IL10 production. To mimic T-B cell interaction, PBMCs were stimulated in the presence of plate bound CD3 monoclonal antibody, a physiologically relevant stimulus capable of upregulation of CD154 expression on CD4 T cells, which in turn induces IL10 production by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells [301].

A time course experiment was performed to determine the optimal time point for IL10 expression by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells on CD3 stimulation. PBMCs were stimulated in a CD3 coated plate for 24, 48 and 72 hrs. Very few $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells expressed IL10 without stimulation (control) and on CD3 stimulation an increase in IL10 expression by transitional B cells at 24 hr, p < .01; 48 hr, p = .01 and 72 hr, p = .001 was seen, with the highest IL10 expression after 72 hr stimulation [Figure 2.7]. Thus, hereafter $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells were stimulated with plate bound CD3 for 72 hr to measure IL10 induction.
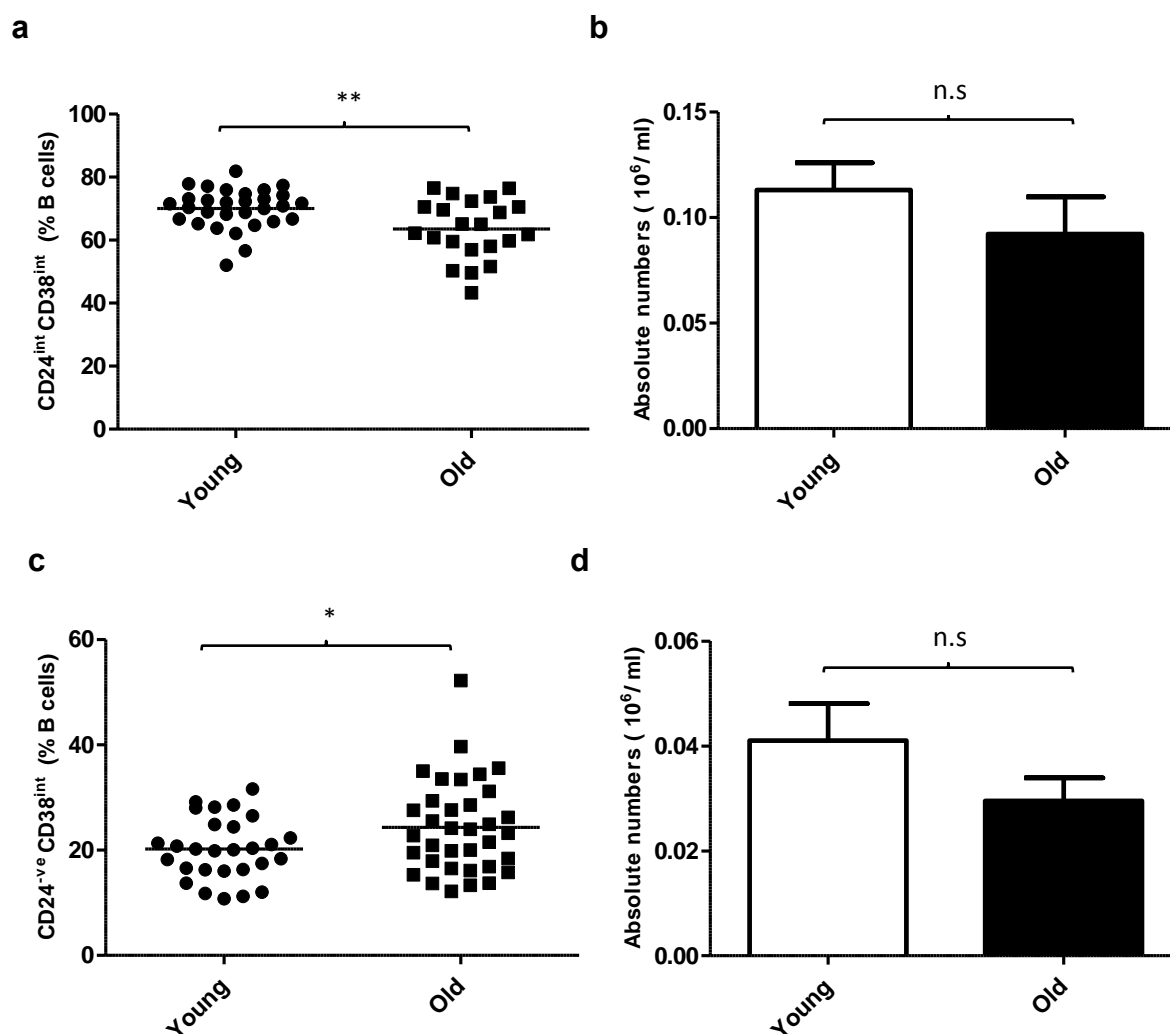
Figure 2.6 Mature (CD19^{+ve}CD24^{int}CD38^{int}) and memory (CD19^{+ve}CD24^{-ve}CD38^{+ve}) B cells in peripheral blood with age.

(a) The percentage of CD19^{+ve}CD24^{int}CD38^{int} B cells in the peripheral B cell pool in young donors (n= 30) and old donors (n = 27) in the basal state. The mean value is indicated by the bar. (b) The absolute number of CD19^{+ve}CD24^{int}CD38^{int} B cells in peripheral blood of healthy young (n=15) and old donors (n=15) in the basal state. Data is expressed as mean ± SEM (c) The percentage of CD19^{+ve}CD24^{-ve}CD38^{+ve} B cells in the peripheral B cell pool of young donors (n= 30) and old donors (n = 27). The mean value is indicated by the bar. (d) The absolute number of CD19^{+ve}CD24^{-ve}CD38^{+ve} B cells in peripheral blood of healthy young (n= 15) and old donors (n = 15). Data is expressed as mean ± SEM * p <.05, ** p <.005.
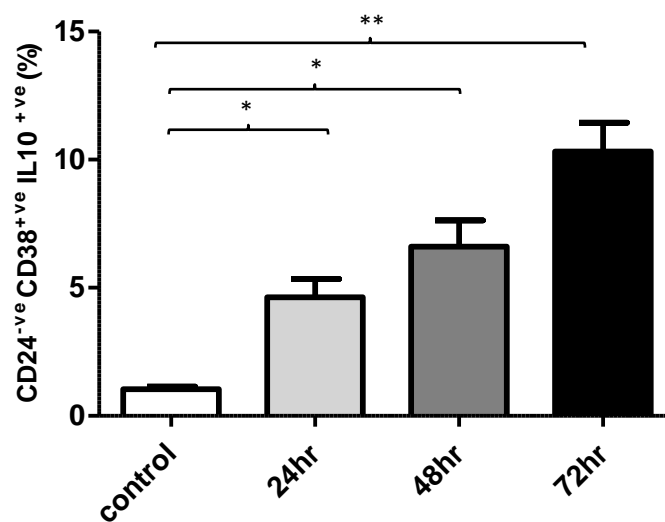
Figure 2.7 Time course experiment showing the frequency of IL10 producing CD19^{+ve}CD24^{+ve}CD38^{+ve} B cells upon CD3 stimulation of whole PBMCs.

The mean frequency of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of healthy young donors on CD3 stimulation of whole PBMCs (n =3). Data is expressed as mean ± SEM. * p <.05 and ** p <.005.

To examine the effect of ageing on immunosuppressive properties of B cells, the percentage of IL10 expressing CD19^{+ve}CD24^{hi}CD38^{hi} B cells and CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells was assessed post-stimulation of PBMCs with plate bound CD3 for 72 hr. A significant decline was observed in the percentage of IL10 expressing CD19^{+ve}CD24^{hi}CD38^{hi}, $p < .001$ [Figure 2.8a] and CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells, $p < .001$ [Figure 2.8c] in old compared to young donors[441]. This decline in IL10 induction by CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age was not affected by gender (data not shown). On comparing the level of IL10 expressed on a per cell basis no significant differences were observed between young and aged donor CD19^{+ve}CD24^{hi}CD38^{hi} B cells, $p = .46$ [Figure 2.8b] or B10 cells, $p = .84$ [Figure 2.8d][441].

Further, on examining IL10 induction by B cells as a whole or CD19^{+ve}CD24^{neg}CD38^{+ve} and CD19^{+ve}CD24^{int}CD38^{int} B cells no differences were observed with age [Table 2.2]. The decrease in IL10 expressing B cell subsets is thus restricted to the transitional B cells and B10 cells.

Variable	Young	Old	p
	(SD)		
IL10^{+ve} B cells (%)	1.37 (1.22)	1.16 (.96)	.54
IL10 production (MFI value)	31.15 (12.99)	30.05 (7.85)	.75
IL10^{+ve} CD19^{+ve}CD24^{neg}CD38^{+ve} (%)	1.61 (1.14)	1.44 (1.23)	.70
IL10 production (MFI value)	20.29 (5.63)	21.09 (6.38)	.61
IL10^{+ve} CD19^{+ve}CD24^{int}CD38^{int} (%)	.95 (.75)	.93 (.63)	.94
IL10 production (MFI value)	21.12 (9.73)	21.62 (7.66)	.88

Table 2.2 IL10 expression by B cell subsets on CD3 stimulation with age.

Interestingly, a significant negative correlation , β = -.62, p <.001, ΔR^2 = .38 between age and percentage of IL10^{+ve}CD24^{hi}CD38^{hi} B cells on CD3 stimulation was observed [Figure 2.9] such that with increasing age the frequency of IL10^{+ve}CD24^{hi}CD38^{hi} B cells declines.

Previous studies on CD19^{+ve}CD24^{hi}CD38^{hi} B cells have routinely included 6h stimulation with PMA and Ionomycin at the end of the 72h anti-CD3 treatment [301]. To test if the defect in IL10 induction by CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age could be overcome by short re-stimulation (6 hr), cells were also similarly treated with PMA and Ionomycin. However, a significant decline in the percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi}, p < .001 [Figure 2.10a] and IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells, p < .001 [Figure 2.10c] remained in older individuals. Further, on comparing the levels of IL10 expressed by the B$_{regs}$, no significant differences were observed in IL10 expression by CD19^{+ve}CD24^{hi}CD38^{hi} cells, p = .17 [Figure 2.10b] or CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells, p = .54 [Figure 2.10d] with age after PMA and Ionomycin treatment.
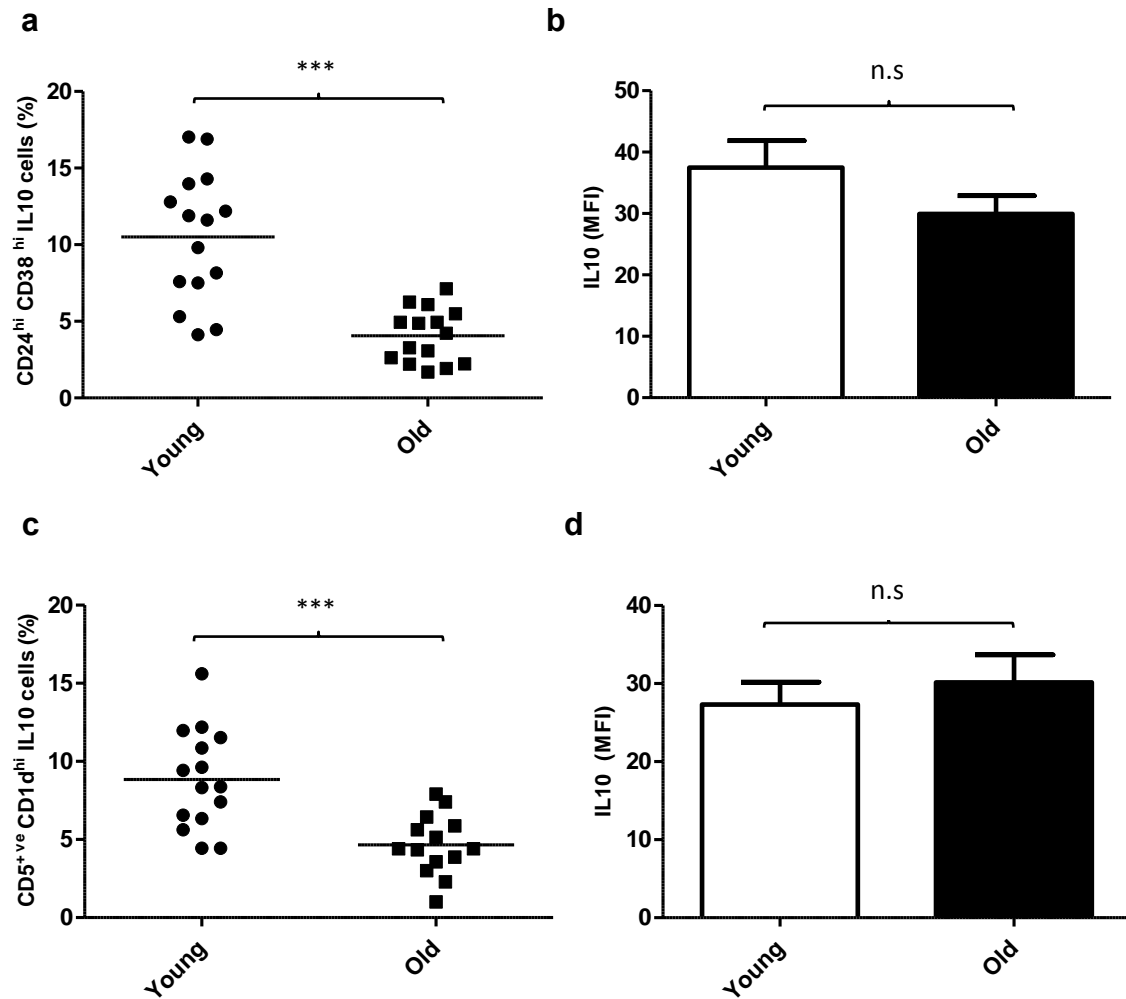
Figure 2.8 IL10 expression by transitional B cells and B10 cells post stimulation with plate bound CD3 in young and old adults.

(a) Percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells on CD3 stimulation for 72 hr in the peripheral B cell pool of young (n= 15) and old donors (n = 15). The mean value is indicated by the bar. (b) Mean amount of IL10 expressed (MFI values) by IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells on CD3 stimulation of PBMCs isolated from young (n = 15) and old donors (n=15). Data is expressed as mean ± SEM (c) Percentage of IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} cells on CD3 stimulation in the peripheral B cell pool of young (n=13) and old donors (n=13). The mean value is indicated by the bar. (d) Mean amount of IL10 (MFI values) expressed by individual IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} cells on CD3 stimulation of PBMCs isolated from young (n=13) and old donors (n=13). Data is expressed as mean ± SEM. *** p <.001.
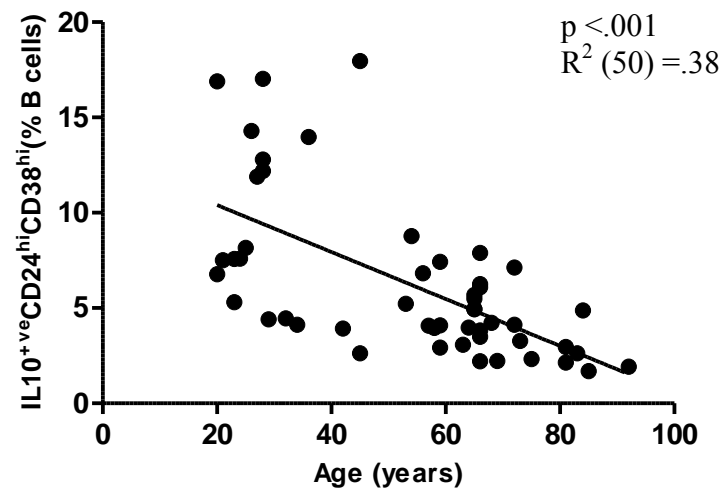
Figure 2.9 Correlation between age and frequency of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells.

Representation of the correlation between percentage of IL10^{+ve}CD24^{hi}CD38^{hi} B cells and age healthy individuals (n = 50).
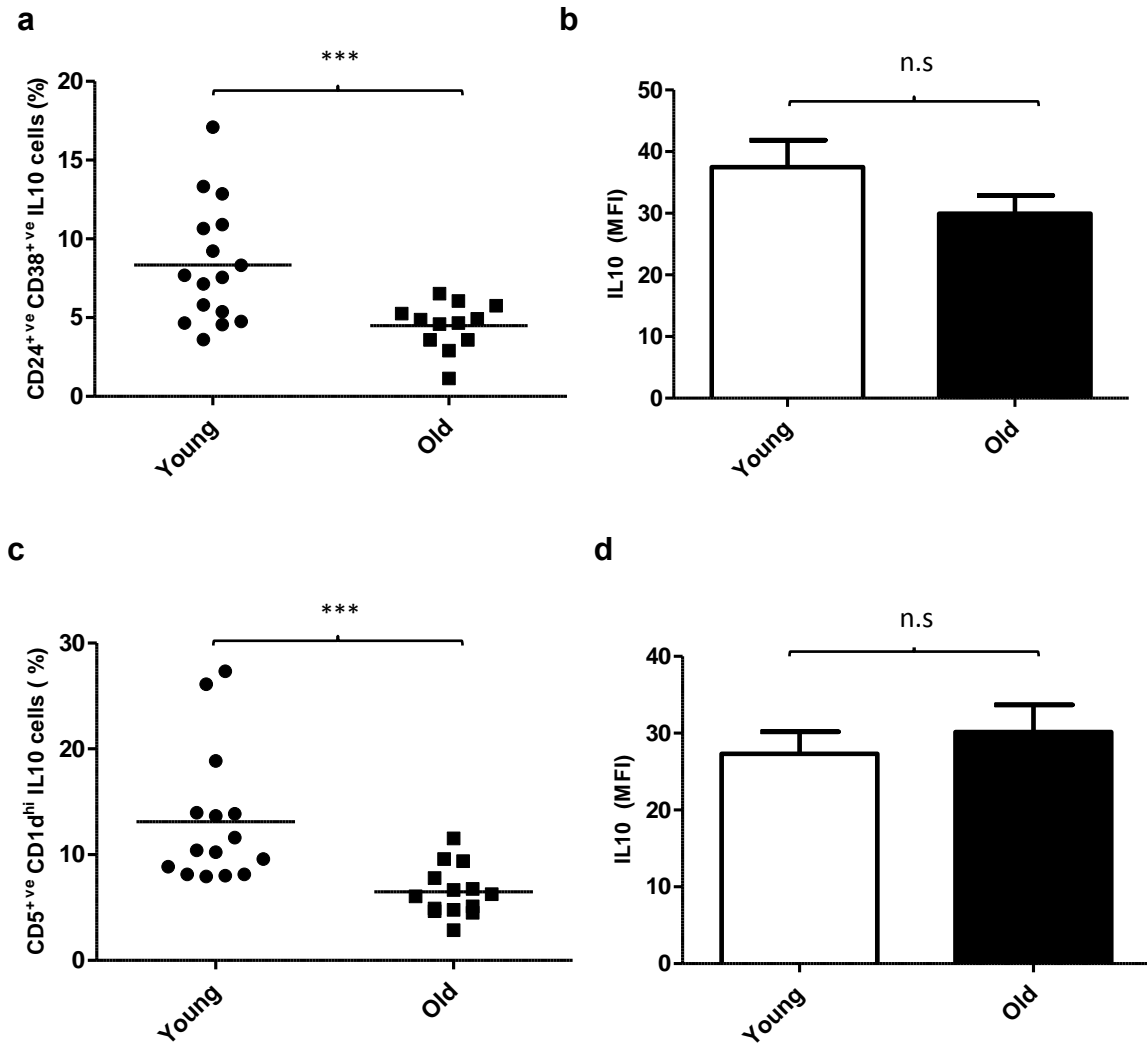
Figure 2.10 IL10 inductions with age by transitional B cells and B10 cells on CD3 stimulation along with short term stimulation with PMA and Ionomycin.

(a) The percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} on CD3 stimulation along with short term stimulation with PMA and Ionomycin (last 6 hr) in the peripheral B cell pool of young (n=14) and old donors (n=16). The mean value is indicated by the bar. (b) Mean amount of IL10 expressed by CD19^{+ve}CD24^{hi}CD38^{hi} B cells on CD3 stimulation plus PMA and Ionomycin stimulation in young (n=14) and old donors (n=16). Data is expressed as mean ± SEM (c) Percentage of IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells on CD3 stimulation along with short term stimulation with PMA and Ionomycin (last 6 hr) in peripheral B cells of young (n=13) and old donors (n=13). The mean value is indicated by the bar. (d) Mean amount of IL10 expressed by CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells on CD3 stimulation plus PMA and Ionomycin in young (n=13) and old donors (n =13). Data is expressed as mean ± SEM. *** p <.001.

2.3.4 CD40 expression on aged and young B cell subsets

CD40 is an integral membrane protein expressed during all stages of B cell differentiation and development [329, 442]. The proportion of CD40[+ve] cells in the B cell pool and the proportion of CD40[+ve]CD19[+ve]CD24^{hi}CD38^{hi} B cells in peripheral blood remained stable with age (p = .52 and p = .77 respectively) [441]. In addition, CD40 expression on total B cells and on CD19[+ve]CD24^{hi}CD38^{hi} B cells also did not differ between young and aged donors (p = .56 and p = .51 respectively) [Table 2.3]. Thus, the impaired ability of IL10 production by CD19[+ve]CD24^{hi}CD38^{hi} cells in healthy older adults on CD3 stimulation was not simply a result of reduced CD40 expression on these cells with age.

Variable	Young	Old	p
	Mean (SD)		
CD40[+ve] B cells (%)	95.48 (2.08)	94.61 (4.58)	.52
CD40 expression (MFI value)	40.53 (19.50)	43.80 (12.59)	.56
CD40[+ve] CD19[+ve]CD24^{hi}CD38^{hi} (%)	94.18 (6.55)	94.86 (7.78)	.77
CD40 expression (MFI value)	41.52 (20.03)	45.83 (16.79)	.51

Table 2.3 CD40 expression by B cell subsets with age in resting state.

2.3.5 Expression of CD40L (CD154) on CD4[+ve] T cells with age

T cells activated via the TCR/CD3 pathway are capable of providing a co-stimulatory signal for induction of IL10 production by CD19[+ve]CD24^{hi}CD38^{hi} B cells via upregulation of CD40 ligand (CD154) expression [443, 444]. It was observed that resting CD4 T cells expressed very low levels of CD154 (0.2-1.5 %), which has been reported previously [445]. On examining the percentage of CD154[+ve] CD4 T cells it was observed that levels were lower in old donors compared to young donors , p = .02 in resting state [Figure 2.11a],

though expression levels of CD40L (CD154) on resting CD4 T cells on a per cell basis showed no significant differences with age, p = .67 [Figure 2.11b].

CD3 activation results in transient upregulation of CD154 expression. A 5-10 fold increase in percentage of CD4 T cells expressing CD40L post-CD3 stimulation (72 hr) was observed, which is similar to previous reports [445]. Activation of T cells revealed an age related decline in CD3-induction of CD40L expressing CD4 T cells, p = .003 [Figure 2.11 c], though the mean expression levels of CD40L on CD3 stimulated CD4 T cells did not significantly differ between young and aged donors, p = .31 [Figure 2.11d]. Thus, suggesting the possibility that age related decline in CD4 T cell ability to provide an activating stimulus to B cells might be one of the factors contributing towards impaired induction of IL10 production by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells with age [441].

2.3.6 Intrinsic defects in $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells with age

To further examine the mechanisms responsible for low induction of IL10 producing cells with age, whether $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells also exhibited any cell-intrinsic defects in response to CD40 stimulation was determined. Recombinant CD40 ligand protein (Peprotech, UK) was used to stimulate isolated B cells as used in another study [306]. A time course experiment was performed to determine the optimal time point for IL10 production by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells on stimulation of isolated B cells with recombinant CD40L for 24 and 48 hrs. Very few $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells produced IL10 in absence of stimulation (control). There was a significant upregulation in IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells on 24 hr stimulation with recombinant CD40L, p = .02 and a further increase in the percentage of IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells after 48 hr stimulation, p = .004 , [Figure 2.12]. Hereafter $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells were stimulated for 48 hr via CD40 pathway to induce IL10 production, which has been reported

previously [319, 446]. CD40 stimulation also induces IL10 production in B10 cells [308]. Similar time course experiments done on isolated B cells revealed maximum IL10 production by $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells on 48 hr stimulations with recombinant CD40L protein (data not shown).

An age associated decline in the percentage of IL10 expressing $CD19^{+ve}CD24^{hi}CD38^{hi}$, p = .002 [Figure 2.13a] and $CD19^{+ve}CD5^{+}CD1d^{hi}$ B cells. p = .003 [Figure 2.13c] was seen in healthy older individuals on stimulation with recombinant CD40L. However, no significant differences were observed in the mean intracellular expression of IL10 by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells, p = .76 [Figure 2.13b] and $CD19^{+ve}CD5^{+}CD1d^{hi}$ B cells, p = .61 [Figure 2.13d] post stimulation with CD40L [441].

2.3.7 IL10 secretion by B cells from older adults is impaired on stimulation via CD40

CD40 generated signals in addition to promotion of IL10 production also induces IL10 secretion by B cells [294, 319]. IL10 secretion by isolated B cells on prolonged stimulation (48 hr) with recombinant CD40L was determined by performing an ELISA to measure IL10 levels in supernatants of cell cultures. A significant decline in IL10 levels in supernatant of CD40 stimulated aged donor B cells on comparison with young donor B cells, p = .04 was found [Figure 2.14] [441]. These data suggest that IL10 secretion by B cells on CD40 stimulation is impaired with age.
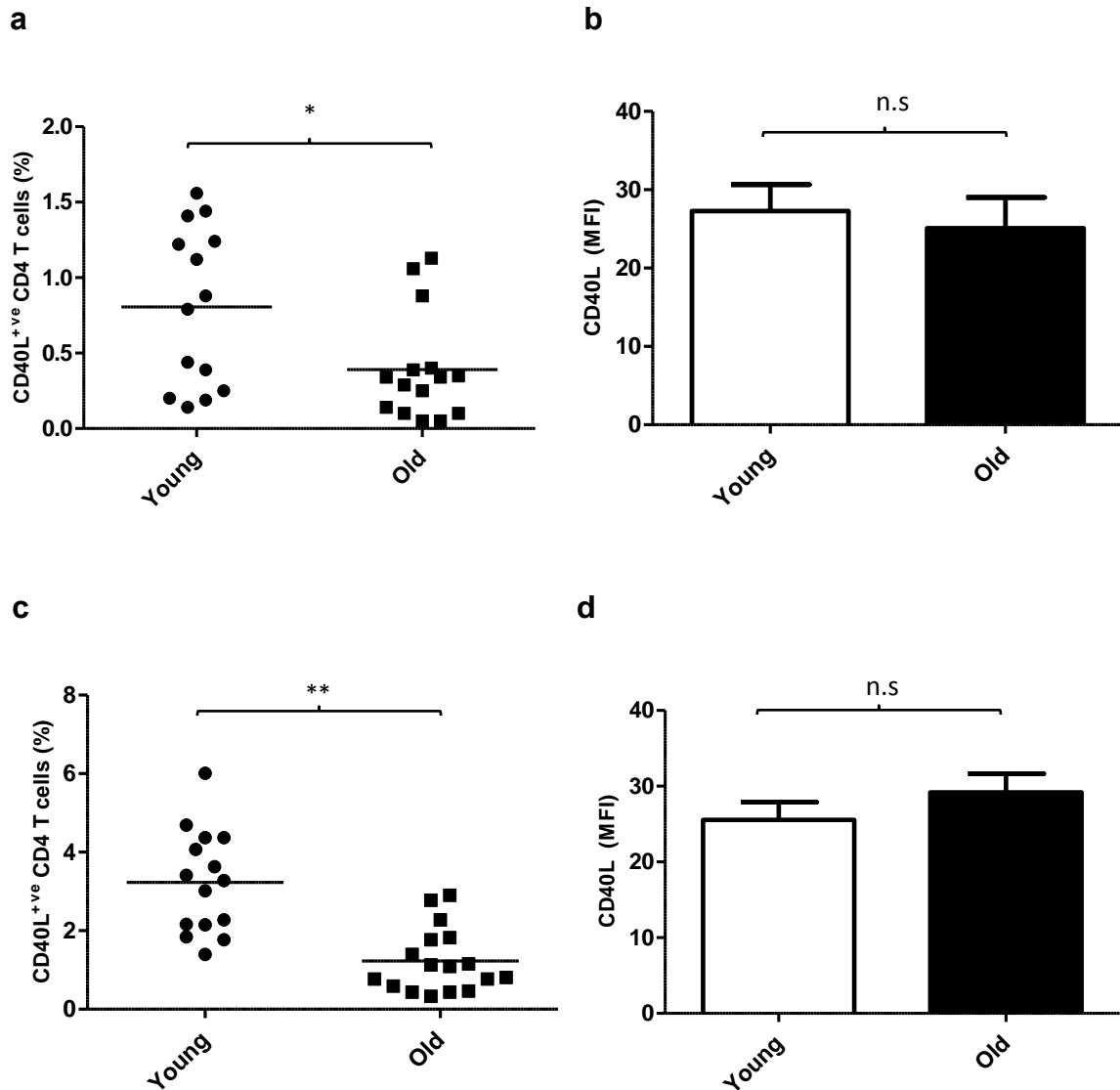
Figure2.11 CD40L expression on CD4 T cells in the resting and activated state with age.

(a) Percentage of CD40L^{+ve}CD4 T cells in young (n=14) and old donors (n=15) in the resting state. The mean value is indicated by the bar. (b) Mean per cell expression of CD40L (MFI value) on CD40L^{+ve} CD4 T cells in young (n=14) and old donors (n=15) in the resting state. Data is expressed as mean ± SEM (c) Percentage of CD40L^{+ve} CD4 T cells post CD3 stimulation in young (n=14) and old donors (n=15). The mean value is indicated by the bar. (c) Mean expression of CD40L (MFI value) on CD40L^{+ve} CD4T cells in young (n=14) and old donors (n=15) post stimulation. Data is expressed as mean ± SEM * p < .05, ** p < .005.
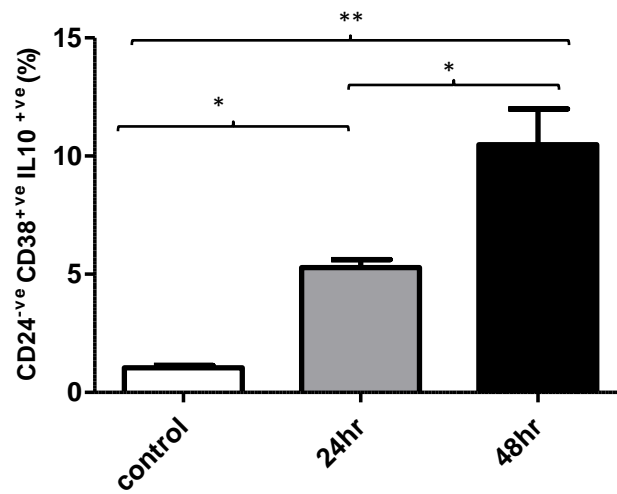
Figure 2.12 Time course of induction of IL10 producing CD19^{+ve}CD24^{+ve}CD38^{+ve} B cells on CD40L stimulation of isolated B cells.

Representation of the mean frequency of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of healthy young donors on stimulation of isolated B cells with recombinant CD40L (n=3). Data is expressed as mean ± SEM. * p <.05, ** p <.005.
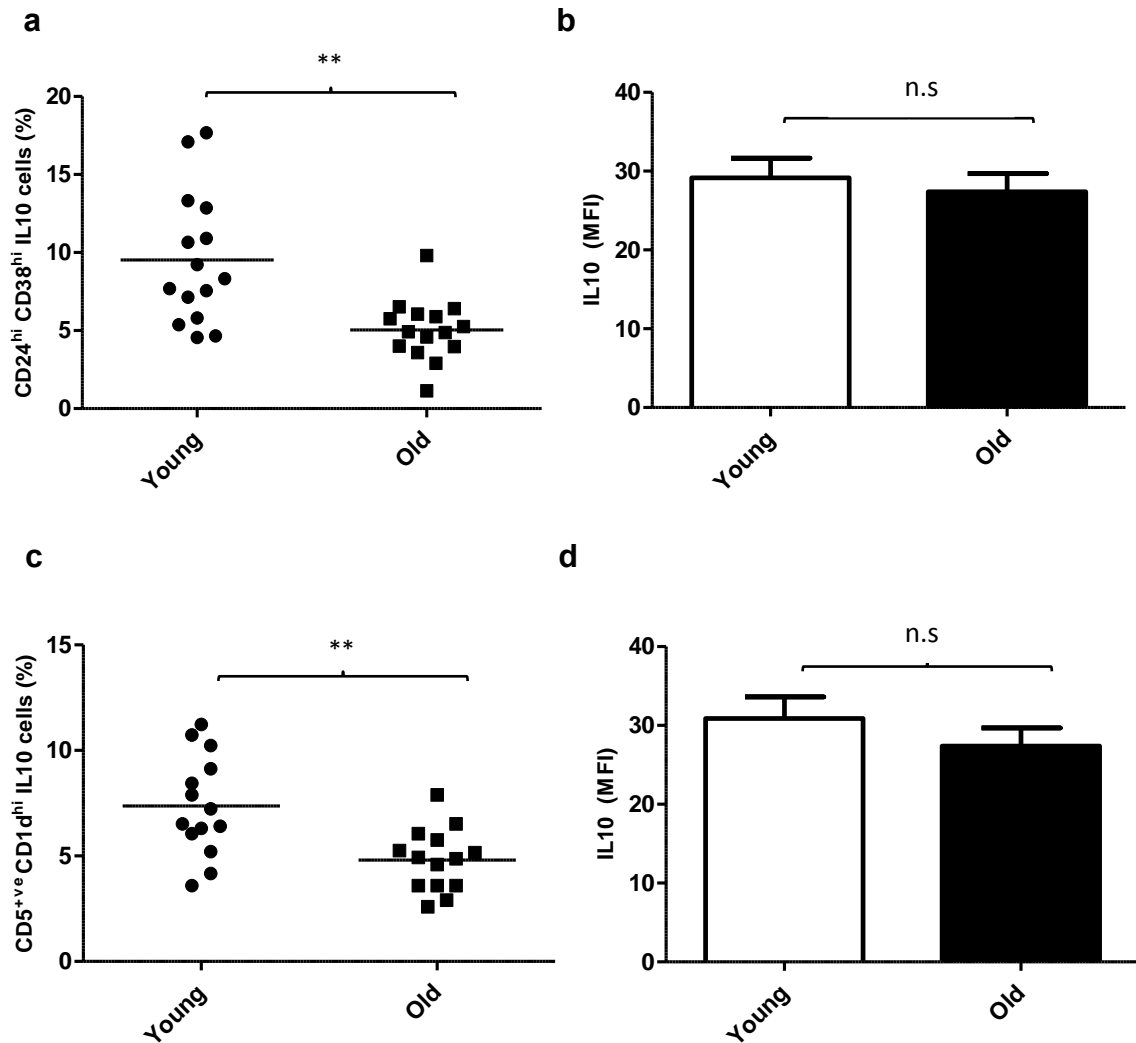
Figure 2.13 CD19^{+ve}CD24^{hi}CD38^{hi} B cells and CD19^{+ve}CD5$^+$CD1d^{hi}B cells from old donors display impaired IL10 induction on CD40 (T cell dependent) stimulation.

(a) Percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells on stimulation of isolated B cells for 48 hr with CD40L in peripheral B cell pool of young (n=15) and old (n=15). The mean value is indicated by the bar. (b) Mean expression of IL10 in CD19^{+ve}CD24^{hi}CD38^{hi} B cells on 48 hr with CD40L in young (n=15) and old donors (n=15). Data is expressed as mean ± SEM. (c) Percentage of IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} cells on stimulation of isolated B cells for 48 hr with CD40L in peripheral B cell pool with age in young (n=15) and old donors (n=15). The mean value is indicated by the bar. (d) Mean expression of IL10 in CD19^{+ve}CD5^{+ve}CD1d^{hi} on 48 hr with CD40L in young (n=15) and old donors (n=15). Data is expressed as mean ± SEM. * p <.05, ** p <.005.
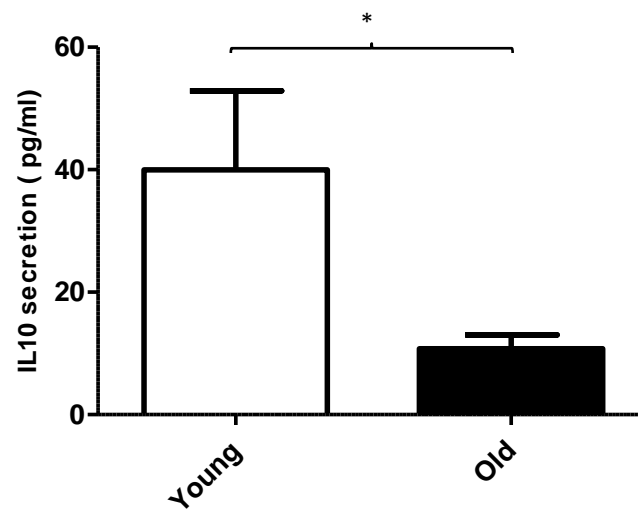
Figure 2.**14 IL10 secretion by B cells on CD40 (T cell dependent) stimulation with age.**

Representation of the mean IL10 concentration in cell culture supernatants post 48 hr stimulations with CD40L in B cells isolated from young (n=16) and old donors (n=15). Data is expressed as mean ± SEM * p <.05.

2.3.8 Impaired STAT3 signalling after CD40 stimulation in CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age.

CD40 signalling results in the activation of multiple signalling cascades , including nuclear factor kappa B (NFkB), mitogen activated protein kinase (MAPK) and signal transducers and activators of transcription factor 3 (STAT3) [334]. CD40- mediated STAT3 activation is known to induce IL10 gene expression**.** Due to the very low numbers of CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood , STAT3 phosphorylation at tyrosine residue Y705 was assessed on a single cell basis using phosphoflow cytometry rather than western blotting, immunoprecipitation or immunofluorescence microscopy that have been used previously [301, 447]. PBMCs isolated from young and old donors were stimulated using an agonistic anti-CD40 antibody for 0, 5, 15 or 30 minutes. A significant lower percentage of CD19^{+ve}CD24^{hi}CD38^{hi} cells expressing phosphorylated STAT3 was seen in old donors compared with young subjects cells at 5 min , p = .004; 15 min , p = .03; and 30 minutes , p= .04 post-stimulation [Figure 2.15] [441].   Protein levels (MFI value) of phosphorylated STAT3 between young and aged donor cells after CD40 stimulation showed no significant differences at any time point [Table 2.4].

Variable	Young	Old	p
	Mean	(SD)	
p STAT3 expression (0 min)	52.35 (19.18)	48.31 (18.98)	.74
p STAT3 expression (5 min)	98.83 (21.50)	87.44 (19.80)	.53
p STAT3 expression (15 min)	136.76 (26.14)	102.19 (22.81)	.40
p STAT3 expression (30 min)	84.79 (26.99)	68.89 (24.64)	.74

Table 2.4 The expression levels phosphorylated STAT3 (MFI) by transitional B cells in young and aged donors post CD40 stimulation.
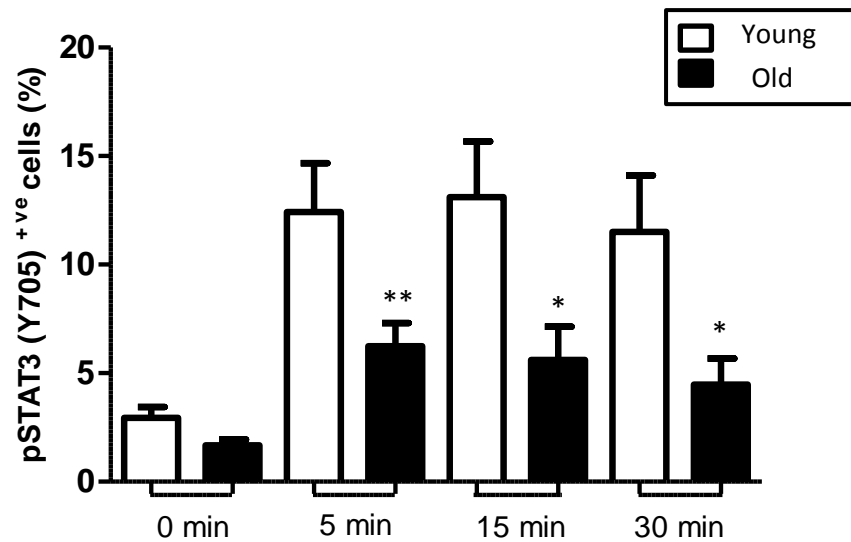
Figure 2.15 Reduced STAT3 phosphorylation in transitional B cells in response to CD40 stimulation with age.

PBMCs were isolated from young (n = 5) and old donors (n = 5) and stimulated with an agonistic anti-CD40 antibody after which cells were fixed and stained for phosphorylated STAT3 (pY705) at the time points shown. Data are represented as percentage of CD19^{+ve}CD24^{hi}CD38^{hi} B cells expressing pSTAT3. Data is expressed as mean ± SEM * p <.05, ** p <.005.

2.3.9 Age associated alteration in IL10 production by B cells – T cell independent stimulation

Activation of B cells via TLR agonists, including LPS (TLR4) and CpG oligonucleotides (TLR9) induces IL10 production [338, 448]. Therefore, whether age associated defects in IL10 production upon stimulation also extended to T cell-independent (LPS) stimulation was assessed.

Time course experiments showed a significant upregulation in IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells upon 24 hour stimulation with LPS compared to B cells in culture without stimulus, p = .003, with a further increase after 48 hr stimulation, p < .001 , [Figure 2.16]. Thus, 48 hr stimulation with LPS was chosen to induce IL10 production, which has been reported previously [319, 446]. Similar time course experiments done on isolated B cells revealed maximum IL10 production by $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells on 48 hr stimulation with LPS (data not shown).

A comparison of B cell responses by cells isolated from young and old donors showed an age associated decline in the percentage of IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ cells, p<.001 [Figure 2.17a] and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells, p <.001 [Figure 2.17c] on LPS stimulation. However, no significant differences were observed in the mean values of IL10 produced by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells, p = .48 [Figure 2.17b] and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells, p = .52 [Figure 2.17d] on stimulation with LPS [441].

Thus, B_{regs} show impaired IL10 production on stimulation via CD40 pathway as well as TLR pathway with advancing age.
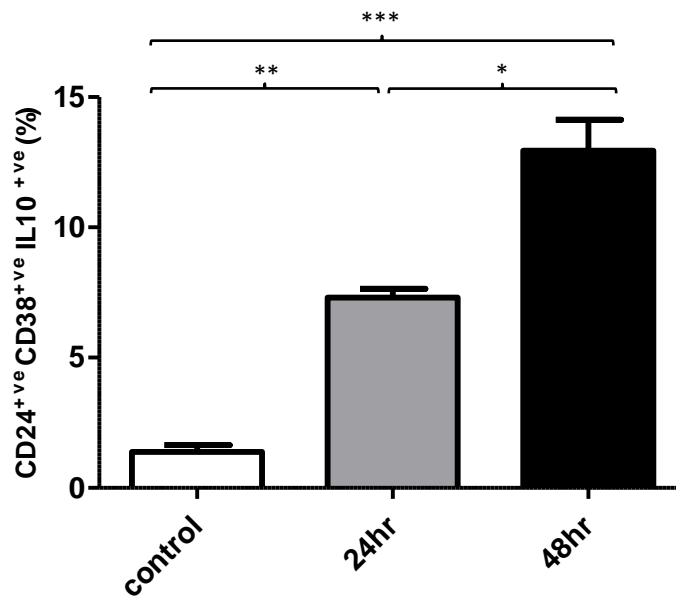
Figure 2.16 Time course experiment showing the frequency of IL10 producing CD19^{+ve}CD24^{+ve}CD38^{+ve} B cells frequency on LPS stimulation of isolated B cells.

Representation of the mean frequency of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of healthy young donors on stimulation of isolated B cells with recombinant LPS (n =3). Data is expressed as mean ± SEM * p <.05, ** p <.005 and *** p <.001.
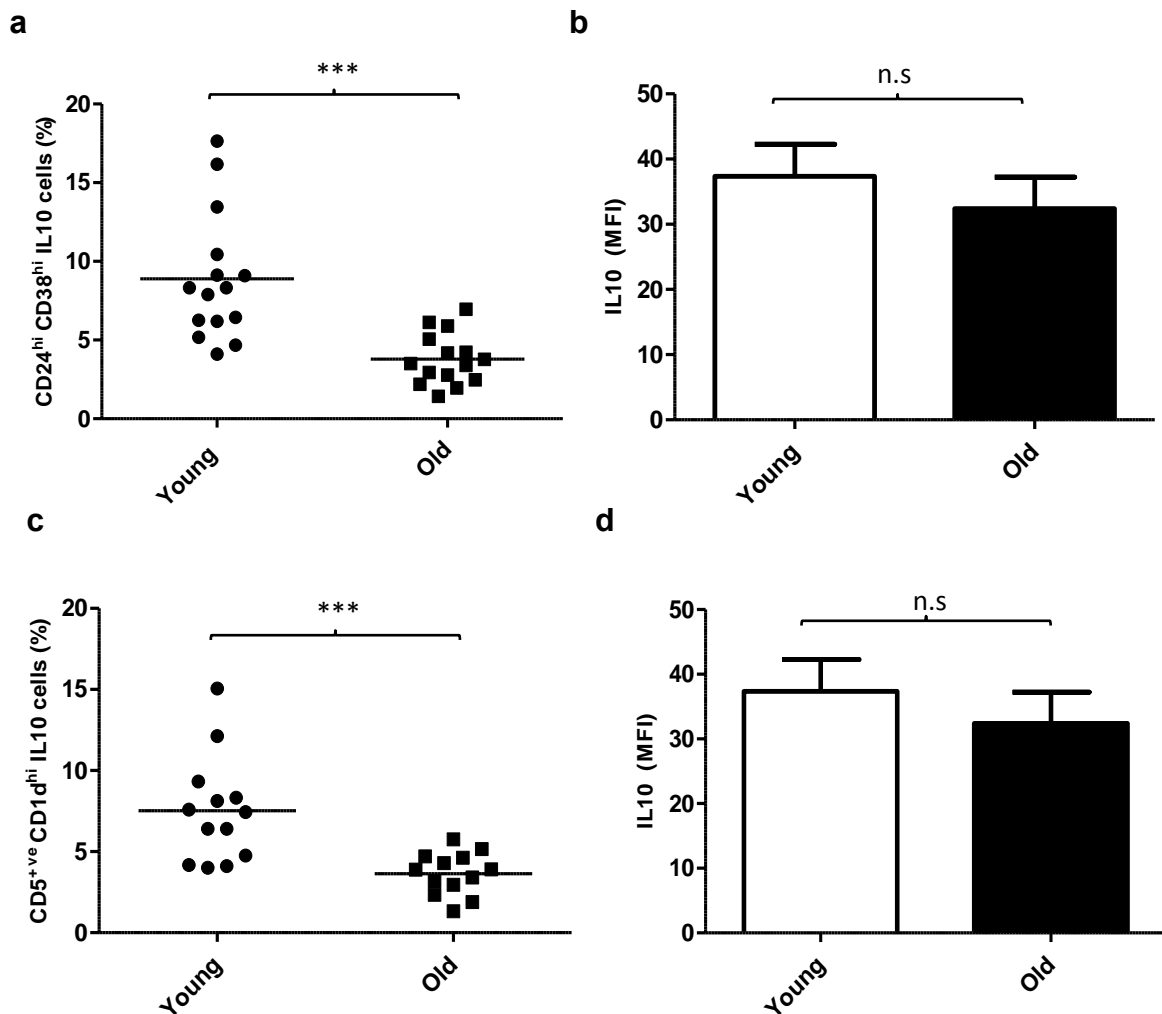
Figure 2.17 CD19^{+ve}CD24^{hi}CD38^{hi} B cells and CD19^{+ve}CD5^{+ve}CD1d^{hi}B cells from old donors display impaired IL10 expression upon LPS stimulation

(a) Percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells on stimulation of isolated B cells for 48 hr with LPS in peripheral B cell pool of young (n=15) and old donors (n=15). The mean value is indicated by the bar. (b) Mean amount of IL10 expressed by CD19^{+ve}CD24^{hi}CD38^{hi} B cells on stimulation with LPS in peripheral blood of young (n=15) and old donors (n=15). Data is expressed as mean ± SEM .(c) Percentage of IL10^{+ve}CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells on stimulation of isolated B cells with LPS in peripheral B cell pool of young (n=15) and old (n =15). The mean value is indicated by the bar. (d) Mean amount of IL10 expressed by CD19^{+ve}CD5^{+ve}CD1d^{hi} B cells on LPS stimulation in peripheral blood of young (n=15) and old donors (n=15). Data is expressed as mean ± SEM. *** p <.001.

Further, on examining IL10 production after LPS stimulation by B cells as a whole, $CD19^{+ve}CD24^{neg}CD38^{+ve}$, or $CD19^{+ve}CD24^{int}CD38^{int}$ B cells no differences were found with age [Table 2.5].

Variable	Young	Old	p
	Mean	(SD)	
$IL10^{+ve}$ B cells (%)	.59 (.26)	.43 (.18)	.51
IL10 production (MFI value)	34.25 (12.56)	32.05 (14.67)	.75
$IL10^{+ve}$ $CD19^{+ve}CD24^{neg}CD38^{+ve}$ (%)	1.71 (.47)	1.31 (.39)	.76
IL10 production (MFI value)	24.12 (15.67)	22.62 (13.98)	.68
$IL10^{+ve}$ $CD19^{+ve}CD24^{int}CD38^{int}$ (%)	0.91 (.32)	0.86 (.48)	.74
IL10 production (MFI value)	23.71 (16.18)	26.09 (15.45)	.44

Table 2.5 IL10 expression by B cell subsets on LPS stimulation with age.

2.3.10 IL10 secretion by B cells in older adults is impaired on stimulation via LPS

In addition to IL10 intracellular expression, IL10 secretion by aged B cells on LPS stimulation was determined. Even though the IL10 levels in B cell culture supernatants appear low, these levels are similar to findings reported by others [306]. Significantly lower IL10 levels were found in the supernatant of LPS stimulated B cells from old donors in comparison with young donor B cells, p = .04 [Figure 2.18] [441]. Suggesting, that the ability of B cells to secrete IL10 on LPS stimulation is impaired with age.

2.3.11 Age associated alteration in TLR4 expression on B cells

On comparing TLR4 expression on the three separate B cell subsets, it was observed that the percentage of $CD24^{hi}CD38^{hi}$ B cells expressing TLR4 was higher compared with $CD24^{int}CD38^{int}$, p <.001 and $CD38^{-ve}CD24^{+ve}$ B cells, p <.001 [Figure 2.19a] in the resting state.

Similarly, TLR4 expression levels (MFI) in CD24^{hi}CD38^{hi} B cells was also higher compared with CD24^{int} CD38^{int}, p = .003 and CD38^{-ve} CD24^{+ve} B cells, p = .004 [Figure 2.19b].

On further analysis, significant differences were found in the percentage of TLR4^{+ve} CD24^{hi}CD38^{hi} B cells, p <.01 [Figure 2.19c], but not in TLR4 expression levels (MFI) on a per cell basis, p = .14 [Figure 2.19d] in older adults in the resting state [441]. On further analysis, no differences were observed in frequency of TLR4^{+ve} CD24^{int} CD38^{int} or in TLR4^{+ve} CD38^{-ve} CD24^{+ve} B cells or in TLR4 expression levels by these two B cell subsets with age (data not shown).

2.3.12 CD80 and CD86 expression on B cell subsets with age

The suppressive activity of B cells is not exclusively IL10 dependent [449, 450], engagement of CD80/CD86 with CD28 and CTLA4 expressed on T cells has been proposed as an additional mechanism of suppression by B$_{regs}$ [301]. On comparing CD80 and CD86 expression on the three separate B cell subsets in the resting condition, it was observed that the percentage of CD19^{+ve}CD24^{hi} CD38^{hi} B cells expressing CD86 was higher compared with CD24^{int} CD38^{int} , p = .02 and CD38^{-ve} CD24^{+ve} B cells , p = .02 [Figure 2.20a]. However, CD86 expression levels (MFI) on CD19^{+ve}CD24^{hi} CD38^{hi} B cells did not differ from CD24^{int} CD38^{int}, p = .89 and CD38^{-ve} CD24^{+ve} B cells, p = .91(data not shown). No significant differences were observed between the percentages of CD19^{+ve}CD24^{hi} CD38^{hi} B cells expressing CD80 in comparison with CD24^{int} CD38^{int}, p = .98 and CD38^{-ve} CD24^{+ve} B cells, p = .87 [Figure 2.20b]. Similarly, CD86 expression levels (MFI) on CD24^{hi} CD38^{hi} B cells did not differ from CD24^{int} CD38^{int}, p = .62 and CD38^{-ve} CD24^{+ve} B cells, p = .49 (data not shown).

Further, on examining the effect of age on CD80 and CD86 expression, no significant differences were found between the percentage of CD19^{+ve}CD24^{hi}CD38^{hi} cells expressing

CD80 , p = .51 [Figure 2.21a] or CD86 , p = .95 [Figure 2.21c] between young and old donors. Similarly, no significant differences were found in cell surface expression levels (MFI) of CD80, p = .95 and CD86, p = .56 on $CD19^{+ve}CD24^{hi}CD38^{hi}$ cells [Figure 2.21b and d] with age [441].

2.3.13 CTLA4 and CD28 expression on T cells with age

Two known ligands for B7 molecules, CD28 and CTLA4 are expressed on T lymphocytes [451]. On examining the expression of CTLA4 on CD4 T cells with age, a significantly higher percentage of $CTLA4^{+ve}$ CD4 T cells was found in peripheral blood of old donors , p = .006 [Figure 2.22a], although CTLA4 expression levels (MFI) remained unaltered , p = .96 [Figure 2.22b] with age. These findings are consistent with previous reports [452, 453]. CD28 is constitutively expressed on the majority of T cells [454], but a significant reduction in the percentage of $CD28^{+ve}$ CD4 T cells was found in our subjects with age , p <.001 [Figure 2.22c] which is consistent with the literature [195, 197]. No significant differences were found in CD28 expression levels (MFI) on CD4 T cells between young and aged donors, p = .61 [Figure 2.22d] [441].
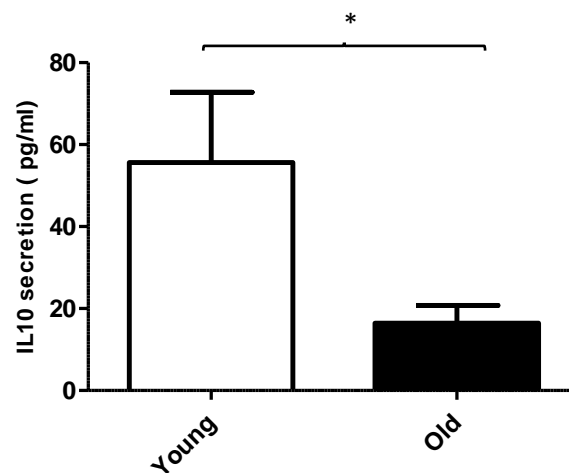
Figure 2.18 IL10 secretion by B cells in old donors on LPS (T cell independent) stimulation.

Representation of the mean IL10 concentration in cell culture supernatants post 48 hr stimulation with LPS done on B cells isolated from young (n=16) and old donors (n=15). Data is expressed as mean ± SEM. * p < .05.
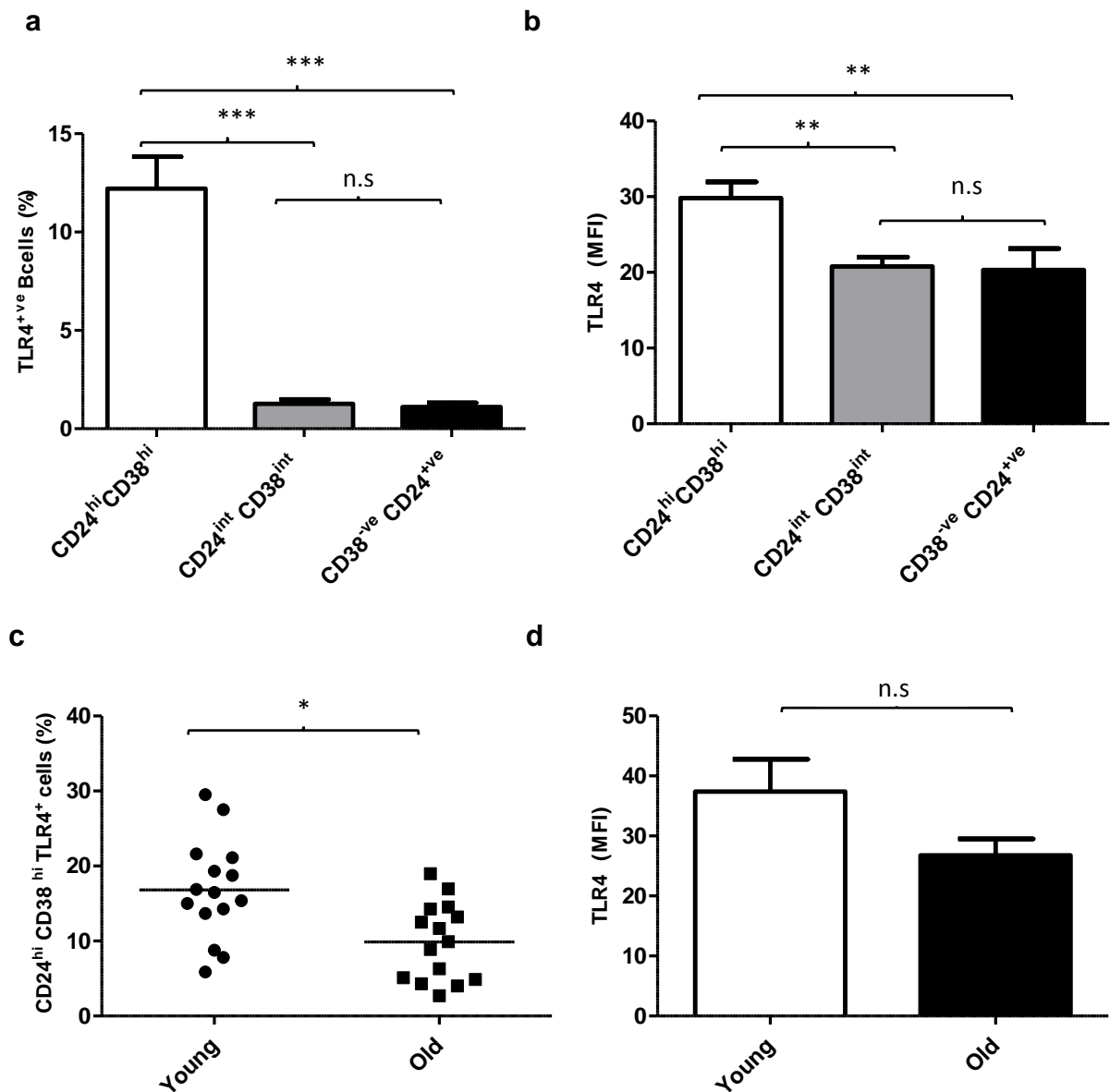
Figure 2.19 TLR4 expression on B cell subsets and effect of ageing.

a) Frequency of TLR4^{+ve}CD24^{hi}CD38^{hi} B cells, TLR4^{+ve}CD24^{int}CD38^{int} B cells and TLR4^{+ve}CD24^{-ve}CD38^{+ve} B cells in peripheral blood of young donors (n=6) in the resting state. (b) Mean TLR4 expression levels (MFI) on CD19^{+ve}CD24^{hi}CD38^{hi} B cells, CD19^{+ve}CD24^{int}CD38^{int} B cells and CD19^{+ve}CD24^{-ve}CD38^{+ve} B in PBMCs of young donors (n=6). Data is expressed as mean ± SEM (c) Percentage of TLR4^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral of young (n=15) and old donors (n =16) in the resting state. The mean value is indicated by the bar. (d) Mean TLR4 expression levels (MFI) on CD19^{+ve}CD24^{hi}CD38^{hi} B cells in young (n=15) and old donors (n=16). Data is expressed as mean ± SEM.* p <.05, **p <.005, ***p <.001.
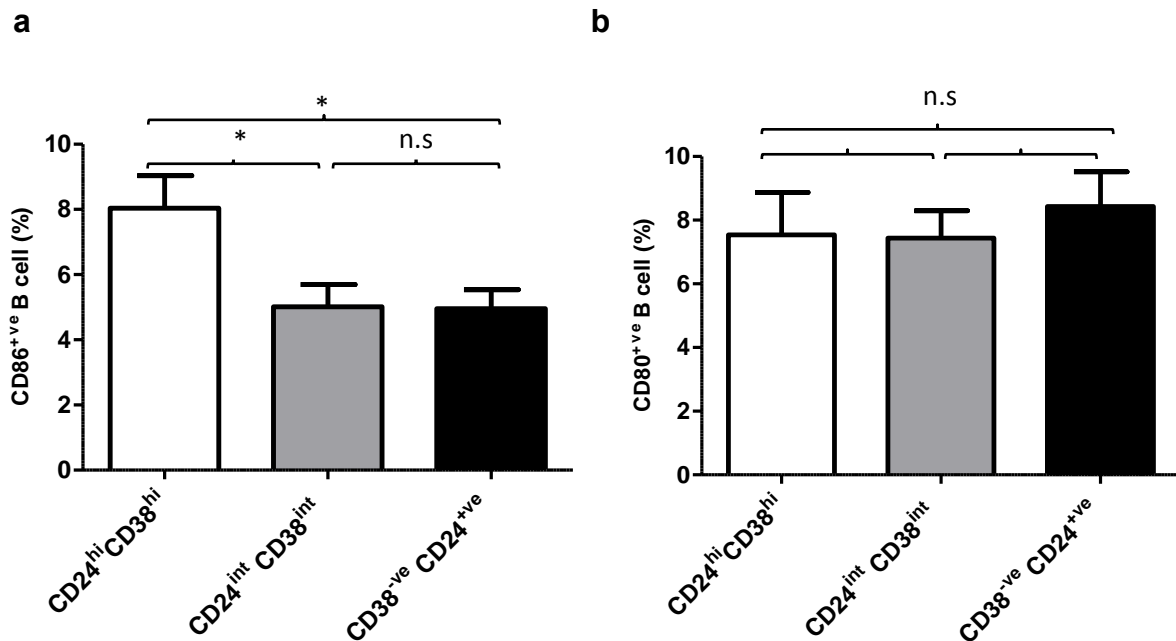
Figure 2.20 CD80 and CD86 expression on B cell subsets (resting state).

(a) Percentage of CD86^{+ve}CD24^{hi}CD38^{hi} B cells, CD86^{+ve}CD24^{int}CD38^{int} B cells and CD86^{+ve}CD24^{-ve}CD38^{+ve} B cells in peripheral blood of young (n=6) in the resting state. (b) Percentage of CD80^{+ve}CD24^{hi}CD38^{hi} B cells, CD80^{+ve}CD24^{int}CD38^{int} B cells and CD80^{+ve}CD24^{-ve}CD38^{+ve} B cells in peripheral blood of young (n= 6) in the resting state. Data is expressed as mean ± SEM. * p <.05.
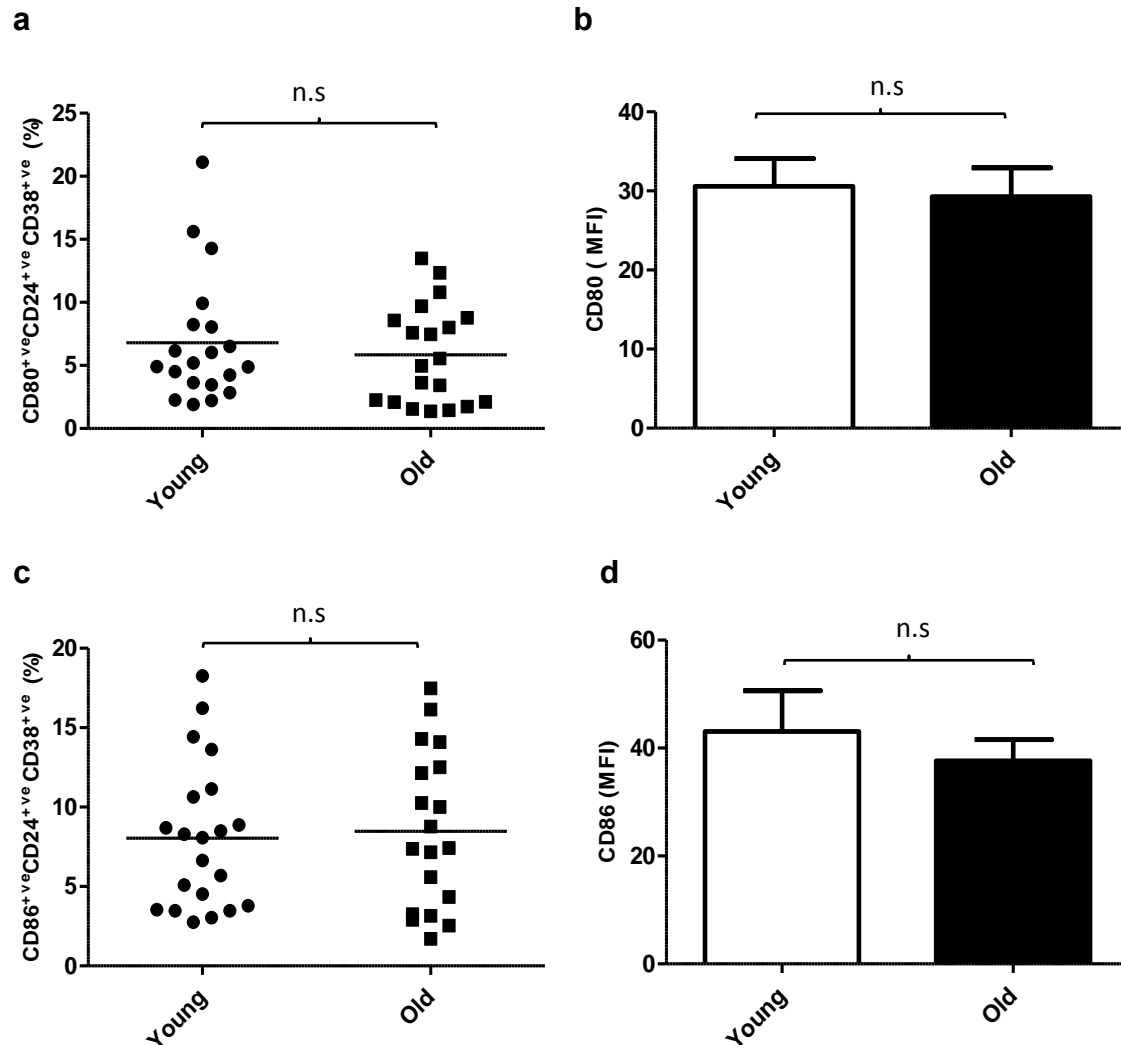
Figure 2.21 CD80 and CD86 expression on CD19^{+ve}CD24^{hi}CD38^{hi} B cell with age.

 (a) Frequency of CD80^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of young (n=20) and old donors (n=20) in the resting state .The mean value is indicated by the bar. (b) Mean CD80 expression levels (MFI) on CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of young (n=20) and old donors (n=20). Data is expressed as mean ± SEM. (c) Frequency of CD86^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of young (n=20) and old donors (n=20) in the resting state .The mean value is indicated by the bar. (d) Mean CD86 expression levels (MFI) on CD19^{+ve}CD24^{hi}CD38^{hi} B cells in peripheral blood of young (n=20) and old donors (n=20). Data is expressed as mean ± SEM.
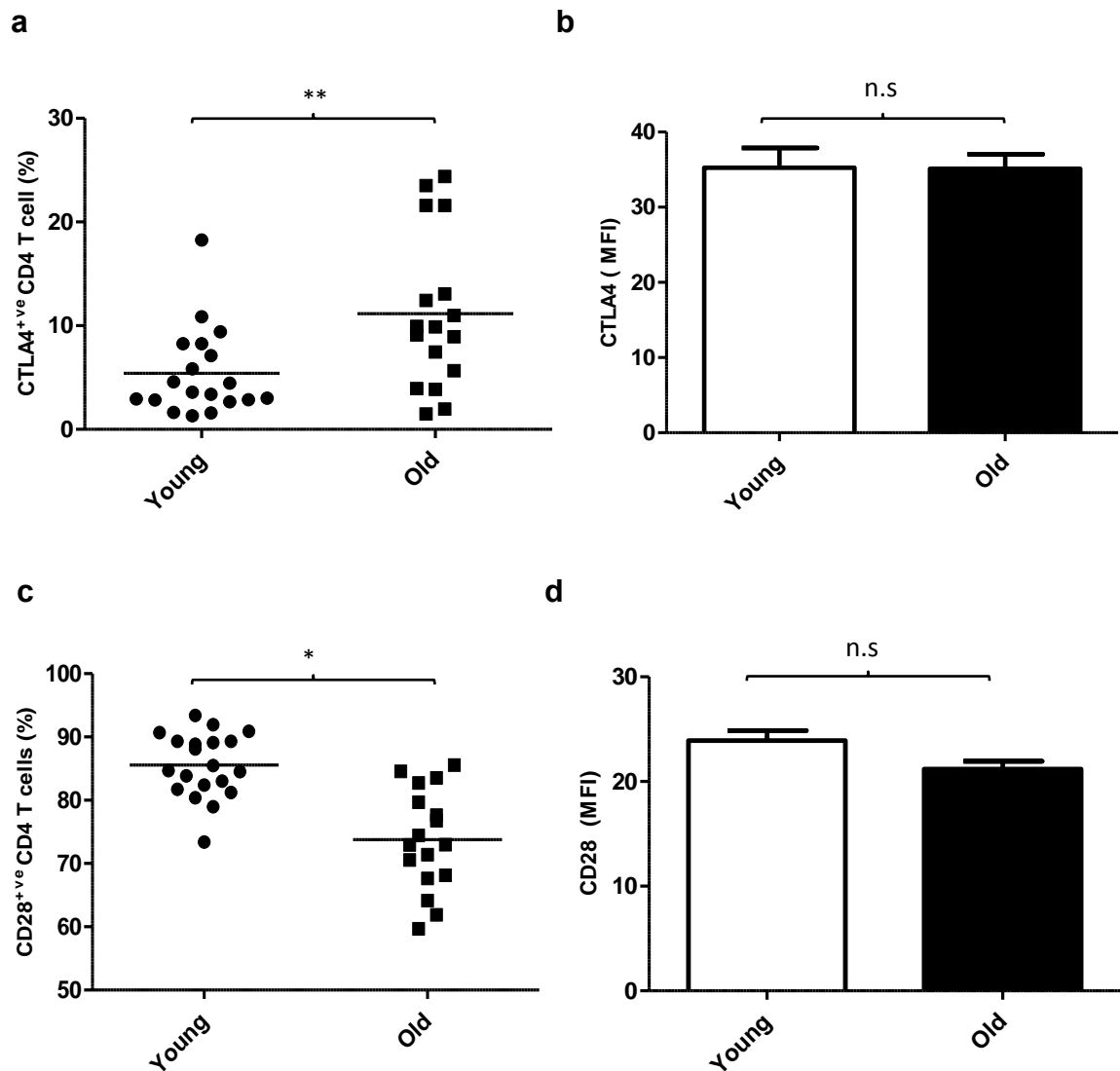
Figure 2.22 CTLA4 and CD28 expression on resting CD4 Tcells with age.

(a) Percentage of CTLA4^{+ve} CD4 T cells in peripheral blood of young (n=20) and old donors (n=20). The bar indicates the mean value. (b) CTLA4 expression levels (MFI) on CD4 T cells of young (n=20) and old donors (n=20). Data is expressed as mean ± SEM. (c) Percentage of CD28^{+ve} CD4 T cells in peripheral blood of young (n=20) and old donors (n=20). The bar indicates the mean value. (d) CD28 expression levels (MFI) on CD4 T cells in peripheral blood of young (n=20) and old donors (n=20). Data is expressed as mean ± SEM. * p <.05 and ** p <.001.

2.3.14 CD19$^+$CD24^{hi}CD38^{hi} B cells and autoimmunity

As previously described, the incidence of autoimmunity increases with age (section 1.5). A significant fraction of older adults (approximately 25%) have detectable levels of low-affinity autoantibodies in their blood [265], such as rheumatoid factor (RF), which are present in very few (only 5%) of young healthy individuals [455]. On testing serum RF levels in healthy young and aged individuals a significant increase in serum RF levels was found in older adults, p<.001 [Figure 2.23a] [441].

In this chapter, functional defects in CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age have also been reported. Additionally, a significant negative correlation was seen between serum RF antibody titre levels and the frequency of IL10^{+ve} CD19^{+ve}CD24^{hi}CD38^{hi} B cells on CD3 stimulation (72 hr), p = .01 [Figure 2.23b]. Taken together these data suggest that age associated defects in IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} B cells, might be contributing towards impaired immune suppression auto-antigens production, resulting in elevated serum autoantibody (rheumatoid factor) levels with age [441].
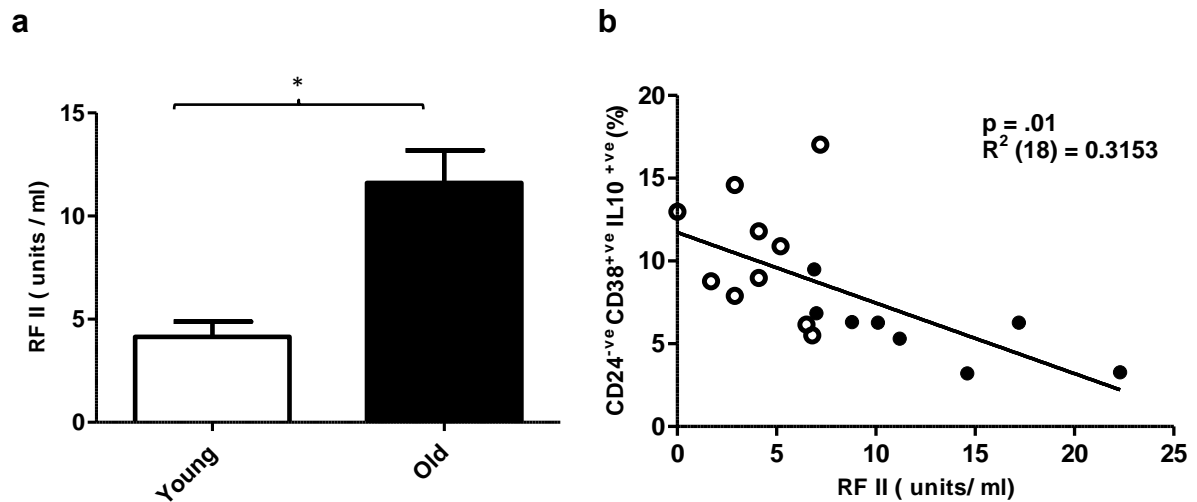
Figure2.23. **Autoantibody levels increase with age and correlate with reduced IL10 production by CD19⁺CD24^hi CD38^hi B cell in older adults**.

(a) Serum levels of rheumatoid factor (RF) in healthy young (n=10) and old donors (n=10). Data is expressed as mean ± SEM.* p <.05. (b) Serum RF values were plotted against frequency of $IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi}$ B cell upon CD3 stimulation in young (open circles) and old (closed circles) donors (n = 20).

2.4. Discussion

The delicate balance between inflammation and immune suppression is dependent upon communication between effector and regulatory components of the immune system and impairment of this relationship results in autoimmunity. Advancing age is associated with remodelling of the immune system [5] that predisposes older adults to the development of autoimmune disorders ,such as rheumatoid arthritis [263]. These age associated changes include a shift in T cell responses towards a Th2 phenotype [209], raised levels of pro-inflammatory cytokines, termed "inflammaging" [456] and reduced T_{regs} immune suppressive activity in older adults [225].

Over the past decade, the paradigm of T_{regs} as the sole cell type involved in regulation of immune responses has been challenged and a role of B cells in regulating immune responses during autoimmunity and inflammatory disorders has been suggested [301, 306]. Furthermore, impairment in IL10 production by B cells occurs in autoimmune diseases, such as SLE [301] and multiple sclerosis (MS) [446]. Interestingly, treatment of multiple sclerosis patients with mitoxantrone, an approved chemotherapy for aggressive MS, results in increased B cell IL10 production and lower production of proinflammatory cytokines (e.g. TNFα and lymphotoxin) [457].

Advancing ageing is accompanied by profound changes in B cell generation (discussed in section 1.4.2). In this chapter, a decline in frequency and absolute numbers of transitional $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ 'B10' cells in peripheral blood with advancing age was revealed. These findings are in line with a previous report according to which a decline in transitional B cells was reported in aged mice [237].

IL10 production has been characterised as the hallmark of the suppressive function of transitional B cells [301, 319] and the B10 subset [306]. CD40 mediated activation is essential for IL10 production by both transitional B cells [294, 301] and B10 cells [306] . In this chapter, a reduction in IL10 producing cells was reported in both subsets of B_{regs} post CD3 stimulation occurs in healthy old donors, which could not be rescued by providing an additional stimulus of PMA and Ionomycin. Further, to investigate the cause of impaired IL10 production by B_{regs} of older adults the stability of CD40 expression with age was determined.   However, CD40 expression remained relatively stable with age, which is consistent with previous finding [247, 458]. Instead, the study revealed impairments in T cell helper activity with age, specifically a significant drop in the frequency of CD4 T cells expressing CD40L in older individuals post CD3 stimulation. These impairments in CD40L upregulation by CD4 T cells on stimulation have been previously seen in mice [205, 207] as well as humans [459]. Interestingly, reduced CD40L expression on CD4 T cells with age has also been associated with increased levels of dual-specific phosphatase 4 (DUSP4) in CD4 T cells from old donors [460]. T cell activation complexes are recruited to plasma membrane microdomains termed 'lipid rafts', these domains are primarily composed of cholesterol [461]. Altered physiochemical status of plasma membrane (higher cholesterol level) has been reported in the T cells of aged subjects, resulting in lower membrane and lipid raft fluidity, which might affect lipid raft formation [462]. In addition, although not investigated here, impaired coalescence of lipid rafts in T cells of older adults following stimulation via CD3 has been reported [463]. Thus, age-associated impaired ability of CD4 T cells to upregulate CD40L expression on CD3 activation might be a result of signal transduction defects in T cells of aged individuals or increased levels of DUSP4, which together contribute to reduced IL10 production by B_{regs}.

Defects reported in IL10 production by aged B_{regs} might also be due to intrinsic defects in these immunosuppressive B cell subsets [464, 465]. The data reported here show that age-related intrinsic B cell defects exist, with reduced STAT3 phosphorylation post stimulation via CD40 pathway [441]. Age-associated intrinsic defects in B cells have also been reported in mice. Adoptive transfer studies in severe combined immunodeficient (SCID) mice revealed that antibodies produced by B cells from young mice, when paired with T cells from aged donors, had a reduced rate of somatic mutation and V_H gene usage. However, when aged B cells were transferred with young T cells somatic mutation still failed to accumulate normally [466]. Another study highlighting intrinsic defects in B cells with age reported a decrease in transcription factor E47 expression on anti-CD40 stimulus, resulting in decreased class switching in aged murine B cells [464, 465]. The data in this thesis show that age-related intrinsic B cell defects extend to $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells which might be a contributing factor towards the reduced ability of aged $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells to produce IL10[441]. This data is also consistent with a previous report of impaired STAT3 signalling with age [467]. Additionally, $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells from SLE patients display impaired IL10 production on CD40 activation which correlated with lower levels of STAT3 phosphorylation [301]. Thus, impairment in downstream CD40 signalling in aged transitional B cells and reduced T cell helper activity might together be responsible for impairment in IL-10 production upon stimulation via CD40 in older subjects[441].

Microbial products are also capable of inducing IL10 production in B cells [308]. For the first time in this chapter, an age-associated significant decline in IL10 production by both $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells on LPS stimulation was shown[441]. Interestingly, there have been previous reports of age-associated defects in TLR induced cytokine production in monocytes, macrophages and dendritic cells [88, 117],

including reduced IL10 production by DCs with age [468]. This thesis found a reduction in the proportion of TLR4 expressing CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age [441], which has been previously reported in monocytes and DCs [93, 469] and might be a factor contributing towards reduced responsiveness towards LPS. B cells also utilise RP105 to recognise and respond to LPS [470], and RP105 deficient mice have been shown to display impaired responses to LPS stimulation [471]. Interestingly, a recent study reported a reduced percentage of RP105 (CD180) expressing B cells in older adults [472], which might also be a contributor towards impaired LPS responsiveness in B cells with age. Also, reduced STAT3 signalling in DC with age has been associated with reduced IL10 production on LPS stimulation [468] and could contribute to altered IL10 production on LPS stimulation by B$_{regs}$ in older adults.

Finally in this chapter, a link between the age-associated reduced immunosuppressive B cell function and increase in age-related autoimmunity is proposed, supported by data showing a negative correlation between IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} and serum RF levels in healthy subjects [441]. Previous findings have reported an exacerbated disease phenotype and enhanced mortality in IL10 gene deficient lupus prone mice and administration of rIL-10 reduced autoantibody production, highlighting the importance of IL10 in down modulating autoimmunity [473]. Importantly, B cell derived IL10 is known to play a crucial role in maintaining suppressive function of T$_{regs}$ during autoimmunity [474, 475]. Mice lacking IL10 producing B cells develop an exacerbated antigen induced arthritis, which is also associated with reduced numbers of T$_{regs}$ and increased Th1 and Th17 cells [476]. Additionally, B7 expression by B cells is also required for their regulatory function, mediating peak expression of IL10 and foxp3 [477]. However no difference in levels of co-stimulatory molecules (CD80 and CD86) on CD19^{+ve}CD24^{hi}CD38^{hi} was seen here in resting

state with age, suggesting that the ability to mediate contact dependent regulation of T_{reg} activity is intact with age[441].

The reduced functioning of $CD19^{+ve}CD24^{hi}CD38^{hi}$ and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells seen here might contribute not only to loss of T_{reg} function with age [225], helping to promote autoimmunity, but also to the age-related increase in systemic inflammation. In addition to effects on T cell differentiation and cytokine production, IL10 can also modulate the inflammatory function of monocytes. The raised level of basal monocyte cytokine output with age has been reported in both aged mice [25] and humans [456]. Interestingly, reduced regulatory B cell function could be an additional factor contributing towards this age-related phenomenon.

Furthermore, the success of therapeutic manipulation of B cells in autoimmune disease, notably the reduction in disease severity following treatment of RA patients with anti-CD20 (Rituximab), is still poorly understood. B cell depletion has been reported to have a beneficial effect during autoimmune disorders [478, 479]. However, the reduction in B cell numbers seen after anti-CD20 therapy, occurs in the absence of a reduction in autoantibody levels [480], due to failure to deplete long lived plasma cells. Interestingly, there have been a few cases of exacerbation of disease symptoms on rituximab treatment, for instance B cell depletion during ulcerative colitis leads to severe disease exacerbation, depletion of mucosal B cells was associated with suppression of local IL10 production [481, 482]. A possible cause for reduction in IL10 levels might be depletion of B cells with immunosuppressive properties. Rituximab therapy has however been associated with increased IL10 mRNA in macrophages, most probably via Fc ligation, providing an alternative source of the regulatory cytokine [483]. Additionally, it has been suggested that re-population of the B cell compartment post rituximab treatment is accompanied by an increase in ratio of peripheral

transitional B cells to memory B cells [484, 485]. Examination of the B cell cytokine profile twelve months after B cell depletion revealed that the newly developed circulating B cells secreted higher IL10 levels than B cells at baseline[446]. Together, suggesting a possible role of transitional B cells in mediating the beneficial effects of B cell depletion therapy.

Interestingly, a novel B cell subset has been characterised recently by the Marrack group and suggested to play a role in age-related autoimmunity: the $CD11c^+$ B cell increases in frequency in aged female mice leading to the name "age-associated B cell (ABC)". These cells are also detected in young lupus-prone mice and elderly women with autoimmune disease [287]. Exactly how they are involved in autoimmunity is not known, but as they express high levels of MHC class II, they may play a role in presenting self-antigen. In mice, the development of ABCs is dependent upon signalling through TLR7 and importantly their depletion in vivo leads to a reduction in serum autoantibodies [287]. Although the presence of ABCs was not determined here, it is interesting to postulate that their age-related increase could be accompanied by a decline in B_{regs} and represent a remodelling of the B cell pool towards autoimmune enhancing cells.

In summary, the findings in this chapter demonstrate that the frequency of $CD19^{+ve}CD24^{hi}CD38^{hi}$ and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells and their ability to produce IL10 after ex-vivo maturation/stimulation declines with age and that this correlates with an age-related increase in serum autoantibody (RF) in healthy older adults. Reduced induction of IL10 production was due to both compromised intrinsic B cell signalling via CD40 pathway and reduced expression of CD40L on CD4 T cells [441].

Chapter 3

Investigating the synergistic effects of physical and psychological stress on innate immune functioning

3.1 Introduction

3.1.1 Background

It is well documented that chronic stress is a global immune suppressor [352, 486] resulting in impairments in neutrophil bactericidal properties [359, 360], monocyte functioning [487-489] and NK cell cytotoxicity [490-492]. Hip fracture is a major health issue in old age [413] and has been previously associated with a high prevalence of depressive symptoms post-surgery [439]. Importantly, development of depressive symptoms in hip fracture patients has health implications including; increased risk of infections, reduced survival [435], impaired recovery and a retarded ability to regain pre-fracture levels of physical functioning [438] . A previous study done in our group has reported suppression of neutrophil superoxide production after hip fracture in older adults, which was accompanied by a high incidence (37%) of post-trauma bacterial infections [359]. However, the factors that influenced recovery after hip-fracture and whether the physical stress of the hip-fracture was the only stressor affecting neutrophil function remains unexplored. In addition whether other aspects of innate immunity are affected in this patient group was not investigated.

The nervous, endocrine and immune system interact with one another. A hallmark of the neuroendocrine response to stress is activation of the HPA axis [362, 363], resulting in elevated cortisol output and flatter diurnal cortisol secretion slope in chronically stressed individuals [493, 494]. Human ageing has also been associated with increased activation of the HPA axis [495, 496]. Cortisol is known to exert an immunosuppressive effect on a range of innate immune cells including; neutrophils [411, 497], monocytes [498] , dendritic cells [499, 500] and NK cells[501-503]. The previous study done in our group also reported a raised cortisol:DHEAS ratio in older adults after hip fracture which was not seen in young fracture patients [411] due possibly to the inability to increase serum DHEAS in the older patients. Whether this ratio

is even further skewed in the presence of depressive symptoms and might contribute to poor outcome in patients with depressive symptoms is unknown and will be explored in this chapter.

Ageing is accompanied by an increase in pro-inflammatory cytokines [17, 291]. Similarly, an elevated level of pro-inflammatory cytokines has also been reported during exposure to chronic psychological stressors including ; caregiving [504, 505], abusive relationships [506], chronic work stress [507], post-traumatic stress disorder [508] and depressive symptoms [509, 510]. On exposure to antigen challenge, an exacerbated inflammatory response has also been reported in chronically stressed older individuals [511, 512].

3.1.1 Aims

In this chapter, we sought to test the hypothesis that psychological stress, specifically the presence of depressive symptoms, would act additively with the physical stress of hip fracture to amplify the effect of ageing upon innate immunity.

(a) To examine the combined effects of both hip fracture and depressive symptoms on physical and cognitive frailty in older adults.

(b) To test if the psychological stress of depressive symptoms could act additively with the physical stress of hip fracture to amplify the effect of ageing upon immunity, with specific reference to neutrophil, monocyte and NK cell function.

(c) Determine the effect of emergence of depressive symptoms in hip fracture patients on activation of the HPA axis.

(d) Test for correlations between depressive symptoms and inflammatory status in hip fracture patients.

(e) To test the longevity of the decline in physical and cognitive function and immune status in hip fracture patients by repeating all tests six months post-surgery.

3.2 Materials and Methods

3.2.1 Antibodies

Those antibodies used in this chapter that were not described in chapter 2 are shown in Table 3.1.

Antibody	Dilution	Company
Anti-human CD14 Pac blue (clone : M5E2)	1/100	Biolegend
Anti-human CD16 FITC (clone: eBio CB16)	1/25	eBiosciences
Anti-human HLADR PE (clone : MCA1879)	1/50	AbD Serotec
Anti-human CD56 PE (clone : C5.9)	1/100	Dako
Anti-human CD3 Pacific blue (clone : UCHT1)	1/100	Biolegend
Anti-human CD57 FITC (clone : HCD57)	1/50	Biolegend
Anti-human LFA1 (CD11a) FITC (clone : MHM24)	1/20	Dako
Anti-human NKG2D PEcy7 (clone: 1D11)	1/10	eBiosciences
Anti-human CD107a FITC (clone : eBio H4A3)	1/20	eBiosciences
Anti-human CD69 FITC (clone: FN50)	1/10	eBiosciences
Annexin V FITC	1/100	BD
Anti-human TNF α PE (clone : MAB11)	1/50	Biolegend
Anti-human Perforin (clone : Dg9)	1/10	Biolegend
Anti-human Granzyme (clone : GB11)	1/10	Biolegend

Table 3.1.List of primary and secondary antibodies used for flow cytometry.

3.2.2 Participants

101 older hip fracture patients were recruited from five hospitals in Birmingham, UK including; Queen Elizabeth hospital, Russell Hall's hospital, West Heath hospital, Good hope hospital and Heartlands hospital, between 2010 and 2012. Inclusion criteria were that participants had to be aged 60 years and over with a hip fracture sustained 4-6 weeks

previously but with no chronic immune-related disorders (e.g. cancer, diabetes) or taking any regular medications that might modify immunity (e.g.immunosuppressant). Additionally, patients must not have had any diagnosis of depressive symptoms by a physician prior to age 50 years or be taking or have previously taken anti-depressant medication, in order to pick up patients with depressive symptoms emerging post-hip fracture rather than those with a prior history of and thus propensity to depressive symptoms. Participants started on anti-depressant treatment, therapy, or any change that would mean they no longer met the inclusion criteria between week 6 and month 6 testing sessions were excluded. Forty three healthy older adults were also recruited from the community as healthy controls via invitation letters to the Birmingham 1000 Elders cohort of older adults involved in current research at the University of Birmingham. These controls also had to meet the inclusion criteria above but not have had a hip fracture. All participants were Caucasian. The study was approved by South Birmingham Local Research Ethics Committee and all participants provided written informed consent (study ref: 09/H1203/80).

3.2.3 Study design

The study was a prospective case-control design with three groups of older adults: hip fracture patients with or without depressive symptoms and healthy older adults. Consent was gained whilst patients were still in hospital. All participants completed questionnaires, structured interviews and provided a blood sample (40 ml) 4-6 weeks and six months after hip fracture. Control participants completed a depressive symptoms and anxiety symptoms scale and basic demographic information was collected when attending the University for one-off blood sampling. Blood samples were taken between 08.00 and 11.00 to minimise any effect of diurnal variations in steroid or cytokine levels. None of the patients had an infection at the time of blood sampling. Interviews were performed either in the hospital or

in the participant's home. Assays for phagocytosis and superoxide production and NK cell cytotoxicity were performed on the same day as blood sampling. PBMCs and serum were frozen for later analysis [513].

3.2.4 Interview and Questionnaires

The following interviews, questionnaires and assessments for physical and cognitive function were carried out by Dr Jane Upton. Standard socio-demographic and health behaviour information, including date of birth, sex, diet, physical activity, sleep quantity and occupational classifications were taken as a part of a questionnaire pack presented during the testing session. All medications, prescriptions and over-the-counter medications were recorded by the interviewer. Health behaviours were assessed using questions adapted from the Whitehall II study [514].

In this thesis, we did not clinically diagnose hip fracture patients for depressive disorder, but instead we have examined the presence of depressive symptoms by means of structured interviews using standardised psychometric questionnaires. Depressive symptoms were evaluated by a Geriatric Depression Scale (GDS) [515], a reliable and valid self-rating depressive symptoms screening scale for elderly populations. In this thesis, the term "depressive symptoms" was defined as a GDS score greater than or equal to 6 [401]. The Hospital Anxiety and Depression Scale (HADS) was also used to measure depressive symptoms and anxiety [516]. The scale contains 14 items, with seven assessing aspects of depressive symptoms and seven assessing anxiety. Healthy control participants completed the HADS depressive symptoms sub-scale in order to check that they did not have significant depressive symptoms. A cut-off of ≥ 8 has previously been used to indicate possible depression [517].

3.2.5 Assessing physical frailty

Physical frailty was assessed using a variety of tools validated in the patient group under study as follows:

Activities of daily living: The Oxford Hip Score (OHS), a 12-item questionnaire to assess functional ability with respect to activities of daily living (ADL) and pain in patients undergoing hip replacement surgery [518]. Each item has 5 possible responses with a score ranging from 0-4, with 4 being the best outcome.

Handgrip (upper body) strength: Handgrip strength, an index of upper body strength was measured using a hydraulic hand dynamometer (Lafayette Instrument, UK). The participant was asked to perform a maximal voluntary contraction (MVC), standing with the dynamometer in one hand and gripping the dynamometer as hard as they could for 3 seconds. This was repeated 3 times for each hand. A rest of 30 seconds was given between each trial to avoid the effects of muscle fatigue. The average of the 3 trials for each hand was considered to be the maximum voluntary handgrip strength.

Timed Up and Go (TUG) Test: The timed "Up & Go" test is used for quantifying functional mobility [519]. The TUG test measures, in seconds, the time taken by an individual to stand up from a standard arm chair, walk a distance of 3 metres, turn, walk back to the chair and sit down.

Berg balance scale : The Berg Balance Scale (BBS) is a scale developed to measure balance among older people in a clinical setting and assess the performance of functional tasks [520]. The scale involves 14 tasks that include balance activities such as sitting with arms folded, rising, standing, transferring between one surface and another, picking up objects from the floor, turning around in a full circle and standing on one leg. Each task was scored on a 5 point ordinal scale ranging from 0 to 4 (0= unable to perform, 4= independent) and the aggregate score ranges from 0 to 56 [520].

3.2.6 Assessing Cognitive function

The Mini Metal State Examination (MMSE) is a tool that has been used to detect cognitive defects seen in syndromes of dementia and delirium and for measuring cognitive changes over time [521] and has been used in this study to assess cognitive impairments in hip fracture patients. MMSE is an 11 question measure that tests five areas of cognitive function: orientation, registration, attention and calculation, recall and language. Cognitive impairment assessed using MMSE is graded by a score ranging from 0 to 30. In this thesis, a score of below 17 is characterised as 'severe cognitive impairment', a score ranging between 18 and 23 is an indicator of 'mild impairment' and scores above 24 indicate 'no cognitive impairment'.

3.2.7 Measuring neutrophil and monocyte phagocytosis

Phagocytosis was measured in whole blood using a commercially available kit (Phagotest kit, Orpegen Pharma, Germany). All the reagents were provided in the kit and the experiments were performed according to manufacturer's instructions. For each patient sample, 100 μl of peripheral whole blood was aliquoted into two FACS tubes (Becton Dickinson, UK). 20 μl of FITC labelled opsonised *E.coli* was added to both tubes, one tube was incubated at 37°C for 10 minutes to allow phagocytosis and the control tube was kept on ice for 10 minutes. Following incubation, both tubes were simultaneously kept on ice and 100 μl of quenching solution was added to each tube to stop the reaction. Samples were then washed twice using 2 ml of washing solution and were centrifugation at 250 x g for 5 minutes at 4°C. Post washing, red blood cells were lysed by suspending the pellet obtained after centrifugation in 2 ml of lysing solution for 20 minutes at room temperature. Following the lysis step, the cell suspension was centrifuged at 250 x g for 5 minutes at 4°C and the pellet was washed using 3 ml of wash solution and cells were spun down at 250 x g for 5

minutes. After washing, cells were resuspended in 200 µl of DNA staining solution and analysed immediately using Cyan^{TM} ADP flow cytometer (Dako Ltd , UK) [522].

Data were analysed using Summit V 4.3. The side scatter vs forward scatter plot allows recognition of neutrophils and monocytes (characterised by their size and granularity relative to other blood cell populations). Separate gates were set on neutrophils and monocytes and their FITC fluorescence intensity was measured on a separate histogram. Negative controls enabled measurement of the background fluorescence.

The phagocytic index was used as a measure of the phagocytic capabilities of neutrophils and monocytes. The phagocytic index was calculated as the percentage of cells that have ingested bacteria, multiplied by the mean fluorescent intensity (MFI), this figure was then divided by 100.

$$\text{Phagocytic index} = \frac{(\% \text{ of cells with ingested bacteria x MFI})}{100}$$

3.2.8 Measuring neutrophil and monocyte superoxide production

Superoxide production was measured in whole blood using a commercially available kit (Phagoburst kit, Orpegen Pharma, Germany). The experiments were performed according to manufacturer's instructions. Firstly, fluorogenic substrate dihydrorhodamine (DHR) 123 was prepared by reconstituting the substrate disk with 1 ml of washing solution for 30 minutes prior to the assay. For each sample, 100 µl of whole blood was added to four separate FACS tubes (Becton Dickinson, UK) and four treatment conditions were carried out: 20 µl of wash solution (negative control); 20 µl of f MLP (low control); 20 µl of PMA (high control) or 20 µl of opsonised *E.coli* (test). The tubes were incubated at 37°C for 10 minutes in a water-bath then 20 µl of fluorogenic substrate dihydrorhodamine (DHR) 123

was added and the samples were incubated at 37°C for further 10 minutes. The reaction was stopped by adding 1 ml of lysis solution to cell suspension and incubated for 20 minutes at room temperature. Following lysis, the cells were washed using 3 ml of wash buffer and centrifuged at 250 x g for 5 minutes at 4°C. Post washing, 200 µl of DNA staining solution was added to cell suspensions. Oxidation of substrate DHR123 was analysed by flow cytometry using a Cyan^{TM} ADP flow cytometer (Dako Ltd, UK). The superoxide production is indicated by the MFI of the monocyte and neutrophil population [522].

3.2.9 Immunostaining for phenotypic analysis of monocytes

Isolated PBMCs (described in section 2.2.3) were resuspended in PBS (1 x 10^6/ml) were transferred into polypropylene tubes (Becton Dickinson, UK) for immunostaining with a combination of fluorochrome conjugated antibodies including; anti-human CD14-Pacific blue (Biolegend, UK), anti-human CD16-FITC (eBiosciences, UK), HLADR-PE (AbD Serotec, UK), anti-human CD80-APC (Biolegend, UK) and anti-human CD86-APC (Biolegend, UK) for 20 minutes at 4°C in the dark. Following incubation, cells were washed twice in 300 µl of PBS and centrifuged at 250 x g for 5 minutes. Cells were resuspended in 300 µl of PBS for flow cytometric analysis using a Cyan ^{TM} ADP (Dako Ltd, UK). Gating strategy used to identify monocyte subsets has been shown in Figure 3.1.

3.2.10 Monocyte stimulation - LPS

PBMCs were resuspended in complete RPMI medium (Sigma Aldrich, UK) at a concentration of 1 x 10^6/ml and 200µl aliquots were incubated in a 96 well U bottom plate (Becton Dickinson, UK) with LPS isolated from *Escherichia coli* serotype 0111:B4 (1µg/ml;
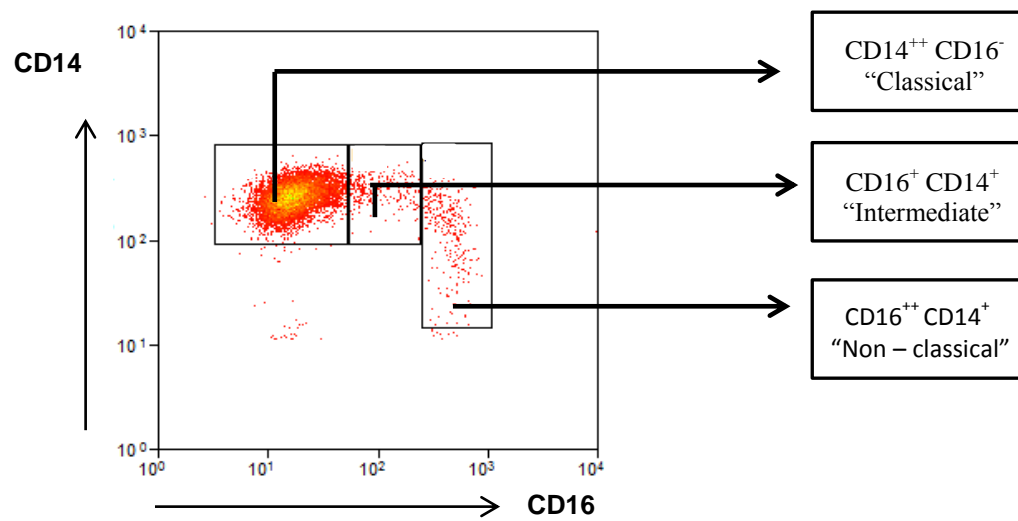
Figure 3.1 Monocyte subset gating strategy.

Representative flow cytometric plot showing the gating strategy used to identify different monocyte subsets on the basis of expression of CD14 and CD16.

Sigma-Aldrich, UK) for 4 hr at 37°C in a humidified 5% CO_2 atmosphere. Brefeldin A (1µg/ml; Sigma-Aldrich, UK) was added to one set of cells. Post incubation, cells were pelleted by centrifugation at 250 x g for 5 minutes. The supernatants of the cells stimulated in the absence of Brefeldin A were transferred into cryovials and snap frozen in liquid nitrogen and stored at -80°C for future analysis. Cells stimulated in the presence of Brefeldin A were transferred into polypropylene tubes (Becton Dickinson, UK) for immunostaining with anti-human CD14-Pacific blue (Biolegend, UK) for 20 min in the dark at 4°C. Post immunostaining, cells were washed using 300 µl of PBS and centrifuged at 250 x g for 5 minutes. Pelleted cells were resuspended in 50 µl of Reagent A (Fix and Perm kit, Invitrogen Ltd, UK) and were incubated for 30 minutes in the dark at room temperature. Post fixing, cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes. The pelleted cells were resuspended in 50 µl of Reagent B (Fix and Perm kit, Invitrogen Ltd, UK) and anti-human TNFα-PE antibody was added (Biolegend, UK) for 30 minutes and cells were incubated in dark at room temperature. Finally, the cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes. Cells were resuspended in 300 µl PBS for flow cytometric analysis on a Cyan ^{TM} ADP (Dako Ltd, UK) and the percentage of TNFα expressing monocytes as well as TNFα expression (MFI) levels were recorded.

TNFα levels in supernatants were quantified using commercially available human TNFα ELISA (Abcam, UK), as per manufacturer's instructions. Firstly, frozen supernatants were thawed on ice and 100 µl of cell culture supernatants were added to 96 well plate coated with anti-mouse TNFα. 50µl of secondary biotinylated anti-TNFα was added to wells and the plate was incubated at room temperature for 2hr. Post incubation, liquid was aspirated and the plate was washed three times with 300 µl of washing solution. Post washing, 100 µl of Streptavidin –horseradish peroxidase (HRP) was added to each well and incubated for 30

minutes at room temp. The liquid was aspirated and plate was washed again three times with 300 µl of washing solution. 100 µl of 3, 3', 5,5'-tetramethylbenzidine (TMB) substrate solution was added into each well and incubated in the dark for 12-15 minutes at room temperature. The enzyme substrate reaction was stopped by adding 100 µl of stop reaction (H_2SO_4) into each well. The plate was read on a spectrophotometer (BioTek® Synergy HT, UK) using 450 nm as the primary wavelength within 5 minutes of adding stop solution. The OD values for individual samples were calculated using a standard curve created by GraphPad Prism® software (GraphPad Software Limited, USA).

3.2.11 Immunostaining for phenotypic analysis of NK cells

100 µl of PBMCs (1 x 10^6 / ml) re-suspended in PBS were transferred into polypropylene tubes (Becton Dickinson, UK) for immunostaining with combinations of antibodies: anti-human CD3-Pacific blue (Bio legend, UK) ,anti-human CD56-PE (Dako, UK) , anti-human CD11a-FITC (Dako, UK), anti-human CD16-FITC (eBiosciences, UK) and anti-human CD57-FITC (Biolegend, UK) or NKG2D-PEcy7 (eBiosciences, UK) for 20 minutes in the dark at 4°C. Post incubation, cells were washed twice using 300µl of PBS and were centrifuged at 250 x g for 5 minutes at 4°C. Cells were resuspended in 300 µl of PBS for flow cytometric analysis using Cyan ^{TM} ADP (Dako , UK). Results were expressed both as the percentage of cell surface marker positive NK cells and mean expression levels of cell surface markers (MFI).

3.2.12 Isolation of NK cells

NK cells were isolated from PBMCs by negative selection using MACS technology (Human NK cell isolation kit; Miltenyi Biotech, Germany). Freshly isolated PBMCs were counted and washed in 10 ml of MACS buffer (Miltenyi Biotech, Germany) and centrifuged at 300 x g for 10 minutes at 4°C. The pellet obtained was re-suspended in 40 µl of MACS buffer (per

10^7 cells; Miltenyi Biotech, Germany) and to this suspension 10 µl NK cell Biotin- antibody cocktail was added (per 10^7 cells ; Miltenyi Biotech, Germany) and the cells incubated for 10 min at 4°C with occasional vortexing. Post incubation 30 µl of MACS buffer (per 10^7 cells; Miltenyi Biotech, Germany) and 20 µl of NK cell microbead cocktail (per 10^7 cells; Miltenyi Biotech, Germany) was added to the cells and incubated for 15 min at 4°C. Cells were then washed in 2ml of ice cold MACS buffer (per 10^7 cells; Miltenyi Biotech, Germany) and centrifuged at 250 x g for 10 minutes at 4°C. The pellet was resuspended in 500 µl of ice cold MACS buffer.

For magnetic isolation of NK cells, an LS column (Miltenyi Biotech, Germany) was placed in the magnetic field of a Quadra MACS separator (Miltenyi Biotech, Germany). The column was prepared by washing with 3ml of ice cold MACS buffer (1 ml at a time). The PBMC suspension in MACS buffer was then applied to the column, followed by 9 ml of ice cold MACS buffer (1ml at a time). The elute containing purified NK cells was collected in a universal tube (Starsted, UK) and NK cells were pelleted by centrifugation at 300 x g for 10 minutes at 4°C. The cells were resuspended in complete RPMI 1640 (Sigma Aldrich, UK) at 1 x 10^6 cells/ml.

To determine the purity of isolated NK cells, 100 µl of the isolated NK cells were taken up in a FACS tube (Becton Dickinson, UK) and were stained with anti-human CD3 Pacific blue (Biolegend, UK) and anti-human CD56 PE (Dako, UK) for 20 minutes at 4°C in the dark. Post incubation cells were washed using 300µl of PBS and centrifuged at 250 x g for 5 minutes. Cells were then taken up in 300 µl of PBS and purity was assessed using Cyan^{TM} ADP (Dako Ltd, UK). NK cells were defined as CD3^{-ve} CD56^{+ve} cells and purity obtained on a routine basis was > 92%.

3.2.13 NK cell killing assay and assessment of effector-target conjugate formation

The MHC class I deficient NK- sensitive cell line K562 (American Type Culture Collection (ATCC), UK), were maintained in complete RPMI 1640 medium in 75 cm^2 cell culture flasks (Starsted, UK) at 37°C in a humidified 5% CO_2 atmosphere and were used as the target population for the NK cell cytotoxicity assays. K562 cell cultures were maintained by replacement of RPMI medium on the day preceding the assay and K562 cells were replaced on a monthly basis.

NK cell cytotoxicity against K562 cells was assessed by two-colour flow cytometry using an adapted version of the protocol described by Godoy-Ramirez *et al* [523]. 100μl of isolated NK cells (1x 10^6 /ml) were dispensed in to wells of a 96-well round bottomed plate (Becton Dickinson, UK) containing 100μl of K562 cells (1 x 10^5 / ml) resulting in a final effector (E) to target (T) cell ratio of 10:1. Due to the limited amount of blood available from hip fracture patients only one E: T ratio (10:1) could be performed, though the assay was validated over a range of ratios initially (20:1, 10:1, 5:1 and 2.5:1). The effector-target cell contact was increased by centrifugation at 50 x g for 1 minute and samples were incubated at 37°C in a humidified 5% CO_2 atmosphere for 2 hours. To account for spontaneous lysis of target cells during this period, 100μl of K562 cells were incubated in the absence of effector cells. Post incubation, cells were pelleted by centrifugation at 250 x g for 5 minutes, the supernatant was removed and cells were re-suspended in 100 μl PBS and transferred into FACS tubes (Becton Dickinson, UK) and stained with anti- human CD56-PE (Dako, UK) for 20 minutes in the dark at 4°C to identify NK cells. Post incubation, cells were washed with 300μl of PBS and centrifuged at 250 x g for 5 minutes and the subsequent pellet was re-suspended in 90μl PBS. To detect cell death, 10μl of sytox blue cell stain (pre-diluted

1:800 in PBS; Invitrogen, UK) was added to the cells and incubated in the dark for 5 minutes at 4°C and analysed by flow cytometry using Cyan ^{TM} ADP (Dako Ltd, UK).

Post-culture immunostaining and scatter profiles were used to distinguish between effector (NK cells), target cells (K562 cells) and conjugates of both cells. The NK-target conjugates were identified as K562 cells positive for CD56 expression [Figure 3.2]. To assess the percentage of lysed target cells within the sample, NK cells and K562 cells were distinguished from one another via staining with anti-human CD56 PE antibody. The percentage of dead K562 cells was determined using sytox dye and the percentage of specific lysis was calculated using the following equation:

$$\% \text{ Specific lysis} = \left[\frac{(\text{test} - \text{spontaneous})}{2000}\right] \times 100$$

In this equation, test represents the percentage of target cells lysed by NK cells and spontaneous represents the percentage of target cells lysed in culture in the absence of NK cells.

3.2.14 Measurement of perforin and granzyme expression in NK cells

100 μl of PBMCs (1x 10^6/ ml) were transferred into FACS tubes (Becton Dickinson, UK) and were stained for extracellular surface markers to identify NK cells using antibodies anti-human CD3-Pacific blue (Biolegend, UK) and anti-human CD56 PE (Dako, UK) for 20 min in the dark at 4°C. Post incubation, cells were washed using 300 μl of PBS and centrifuged at 250 x g for 5 minutes. Pelleted cells were re-suspended in 50 μl of Reagent A (Fix and Perm kit, Invitrogen Ltd, UK) and incubated for 30 minutes in dark at room temp. Post fixing, the cells were washed with 300 μl PBS and centrifuged at 250 x g for 5 minutes. Pelleted cells were resuspended in 50 μl of Reagent B (Fix and Perm kit, Invitrogen Ltd, UK) and were stained with anti-human Perforin- FITC antibody (Biolegend, UK) or with
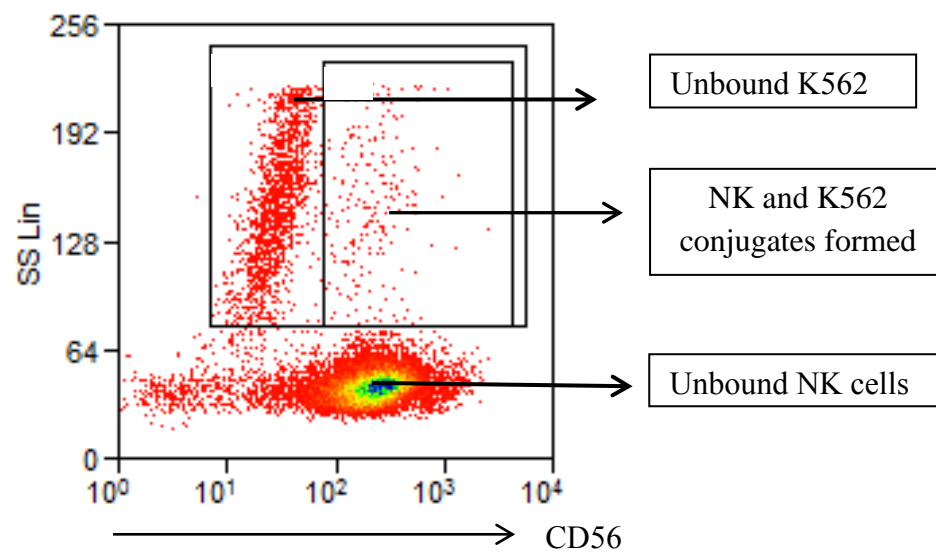
Figure 3.2 Flow cytometric analysis of NK cell – target conjugate formation

Flow cytometric plot showing immunostaining of effector and target (K562) co-cultures with CD56-PE for identification of NK cells and K562 cells 2 hr post culture.

anti-human Granzyme- FITC antibody (Biolegend, UK) for 30 minutes in the dark at room temperature. Finally, the cells were washed with 300 μl PBS and centrifuged at 250 x g for 5 minutes. Cells were then taken up in 300 μl of PBS and assessed using Cyan^{TM} ADP. (Dako Ltd, UK). The percentage of perforin^{+ve} NK cells and granzyme $^{+ve}$ NK as well as perforin (MFI) and granzyme expression levels by NK cells were recorded.

3.2.15 Measurement of serum cortisol and DHEAS levels

Serum cortisol and DHEAS levels were determined using a commercially available ELISA kit (IBL international, Germany). The ELISA was performed according to manufacturer's instructions. Firstly, frozen serum was thawed on ice and 25 μl of serum was added to a 96 well plate with wells coated with anti-cortisol or anti-DHEAS antibody. 200 μl of enzyme conjugate was added to each well and the plate was incubated at room temperature for 1hr. Post incubation, liquid was aspirated and the plate was washed three times using 300 μl of washing solution. Post washing, 100μl of Streptavidin – horseradish peroxidase (HRP) was added to each well and incubated for 15 minutes at room temperature. The enzyme substrate reaction was stopped by adding 100μl of stop reaction (H_2SO_4) in to each well. The plate was read on a spectrophotometer (BioTek® Synergy HT, UK) at 450 nm as the primary wavelength within 5 minutes of adding stop solution. The OD values for individual samples were calculated using a standard curve created by GraphPad Prism® software (GraphPad Software Limited, USA).

3.2.16 Multiple cytokine analysis – Luminex

A Bio-plex cytokine assay for IL1β, IL4, IL6, IL8, IL10, IL13, IL17, TNFα and GM-CSF (Bio-Rad Laboratories, Germany) was used for a multiple cytokine analysis using Luminex technology. The assay was performed according to manufacturer's instructions. The Bio-plex® system (Bio-Rad Laboratories, Germany) was calibrated prior to set up of the assay to

standardise the fluorescent signal. The standard was reconstituted and four fold dilutions were performed to generate a ten-point standard curve. Coupled beads were prepared by dilution of stock coupled beads with assay buffer in a falcon tube (Becton Dickinson, UK) covered with foil. 50 μl of prepared coupled beads were added to a flat bottom 96 well black plate (Corning, USA), followed by two washes with 100 μl of wash solution using a magnetic washing plate (BioTek® Synergy HT, UK). 50 μl of standards, blanks and undiluted serum was added to each well and the plate was covered with sealing tape and foil and shaken at 1100 rpm for 30 seconds then left for 30 min on the plate shaker set at 300 rpm. Post incubation, liquid was aspirated and plate was washed three times with 100 μl wash buffer. Detection antibody was diluted as per manufacturer's instructions and 25 μl of diluted detection antibody was added to each well of the plate. The plate was again covered with sealing tape and foil and left on the plate shaker at 300 rpm for 30 minutes. The liquid was aspirated and plate was washed with 100 μl wash buffer three times using a magnetic washing plate (BioTek® Synergy HT, UK). 50 μl of streptavidin-PE was added to each well and the plate was covered with sealing tape and foil and incubated on the shaker set at 300 rpm for 10 minutes. Finally, the plate was washed three times with 100 μl of wash buffer and 125μl of assay buffer was added to each well and the plate was covered with sealing tape and shaken at 1100 rpm for 30 seconds after which the plate was read on the Bio-plex® system (Bio-Rad Laboratories, Germany). Data acquisition and analysis was done using Bio-Plex Manager software version 6.0. (Bio-Rad Laboratories, Germany).

3.2.17 Dexamethasone treatment of NK cells

NK cells were isolated from healthy young individuals by negative selection using MACS technology (described in section 3.2.13). 180 μl of NK cells in complete RPMI 1640 (1 x 10^6 cells/ml) were treated with dexamethasone (Sigma Aldrich, UK) at 10^{-5}M, 10^{-7}M and 10$^-$

^{9}M in a 96-well round bottomed plate (Becton Dickinson, UK) for 18 hr at 37°C in a humidified 5% CO_2 atmosphere. The physiologically relevant concentration of dexamethasone approximates to 10^{-7}M [501, 524]. Post incubation, NK cells were centrifuged at 250 x g for 5 minute and the cells were washed twice using 300 μl of RPMI 1640. Post washing, the pellet of NK cells was resuspended in complete RPMI medium (1 x 10^6 cells/ml) for analysis of NK cell apoptosis , NK cell cytotoxicity, NK cell conjugate formation, and NK cell activation.

3.2.18 Assessment of NK cell apoptosis

NK cells were stained with Annexin V and sytox blue to enumerate apoptotic cells using flow cytometry. NK cells (1 x 10^6cells/ml) pre-treated with dexamethasone (10^{-7}M) were resuspended in 100μl of Annexin V Binding buffer [0.1 M Hepes (pH 7.4), 1.4 M NaCl, 25 mM CaCl2] and 1 μl of Annexin V- FITC (BD Biosciences, UK) was added to the cells and incubated for 10 minutes at 4°C in the dark. NK cells were then transferred into a FACS tube (Starsted, UK) containing 300 μl of Annexin V Binding buffer NK cell death was also measured by immunostaining isolated NK cells (1 x 10^6) resuspended in 100μl of PBS with 10μl of sytox blue cell stain (pre-diluted 1:800 in PBS; Invitrogen, UK). The Annexin V positive NK cells and Sytox blue positive NK cells were detected via flow cytometry using a Cyan ^{TM} ADP (Dako Ltd, UK).

3.2.19 Measuring NK cell activation status

To determine NK cell activation status immunostaining for cell surface marker CD69 expression was performed. 100μl NK cells in pre-warmed complete medium (1x 10^6/ml) were dispensed into wells of a 96-well round bottomed plate (Becton Dickinson, UK) containing 100μl K562 cells (1 x 10^5/ml) or 100μl medium only (control), after which cells

were centrifuged at 50 x g for 1 minute and the cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 2 hours. Post incubation, samples were centrifuged at 250 x g for 5 minutes, the supernatant was removed and the pellet was re-suspended in 100μl PBS. Cells were immunostained with anti- human CD56 PE antibody (Dako, UK) and anti- human CD69-FITC antibody (eBiosciences, UK) and incubated for 20 minutes in the dark at 4°C. Post incubation, the cells were washed using 300μl of PBS and centrifuged at 250 x g for 5 minutes. Finally, the cell pellet was re-suspended in 300μl of PBS and cells were transferred to FACS tubes (Starsted, UK) and the samples were analysed by flow cytometry using a Cyan ™ ADP (Dako Ltd, UK) and the percentage of CD69 positive NK cells was recorded.

3.2.20 CD107a degranulation assay

Lytic granule fusion with the NK cell membrane was assessed using a slightly modified version of a CD107a degranulation assay previously described by Alter and colleagues [525]. PBMCs were suspended in per-warmed complete medium ($1x10^6$/ ml) and were stimulated with K562 cells ($1x10^6$/ml) at an E:T ratio of 1:1 in the presence of 5μl of anti-CD107a-FITC antibody (eBiosciences , UK) for 1 hr at 37°C in a humidified 5% CO_2 atmosphere. 6μg/ml of monensin (Sigma Aldrich, UK) was added and the samples incubated for 2 hr at 37°C in a humidified 5% CO_2 atmosphere. NK cells ($1x10^6$/ ml) cultured alone served as controls. Post incubation, cells were pelleted by centrifugation at 250 x g for 5 minutes and resuspended in 100 μl PBS and stained with anti-human CD3 Pacific blue (Biolegend, UK) and anti-human CD56 PE (Dako, UK) for 20 minutes in the dark at 4°C. Cells were washed using 300μl of PBS and centrifuged at 250 x g for 5 minutes and taken up in 300 μl of PBS and staining analysed by flow cytometry on a Cyan ™ ADP (Dako Ltd, UK). CD107a expression on 5,000 NK cells was recorded. CD107a expressed by NK cells alone was

subtracted from the CD107a expression by NK cells plus K562 cells to account for spontaneous degranulation by NK cells.

3.2.21 Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics 20 software (IBM software, UK). Differences between the three groups of patients on demographic and health behaviors were tested using a chi-square test. Univariate ANOVA with least significant difference post-hoc tests was used to assess differences between the groups on immune variables. Where demographic variables differed significantly between groups, analyses were rerun adjusting for these variables using ANCOVA with the demographic variable as a covariate. Pearson's correlations were used to examine associations between depressive symptoms score and immune cell function and hormone levels. Repeated measures ANOVA was used to assess group differences in immune variables at the week 6 and month 6 time points.

3.3 Results

Hip fracture patients were classified into two groups on the basis of GDS scores: hip fracture patients with a GDS score of 5 or less were classified as non-depressed (HF; hip fracture only), those with a score of 6 or greater were categorised as depressed (HF+D; Hip fracture patients with depressive symptoms). 38 (37%) of the hip fracture patients developed depressive symptoms as a response to stressful life event of a hip fracture [526]. Recruitment and withdrawal data for this study in [Figure 3.3], The consort diagram shows that 2260 hip fracture patients were screened in this study , but only 103 hip fracture patients were tested at the 6 week time point, mainly because most of the patients screened did not fulfil the inclusion criteria or declined to participate because they were feeling too ill or thought that participation in the study was time consuming or too demanding. Drop-out post consent was also common and mostly due to a change of mind by the patient or family member. From the 103 hip fracture patients tested, later two patients who were later found to violate the inclusion criteria were excluded from the analysis. At 6 month time point, 68 hip fracture patients were tested, but only 66 hip fracture patients were included in later analysis, the two hip fracture patients that violated the inclusion criteria were excluded from analysis. The main reasons of withdrawal between the 6 week and 6 month time point included: death, being to unwell to be tested, receiving treatment for depressive symptoms, or being non-contactable.
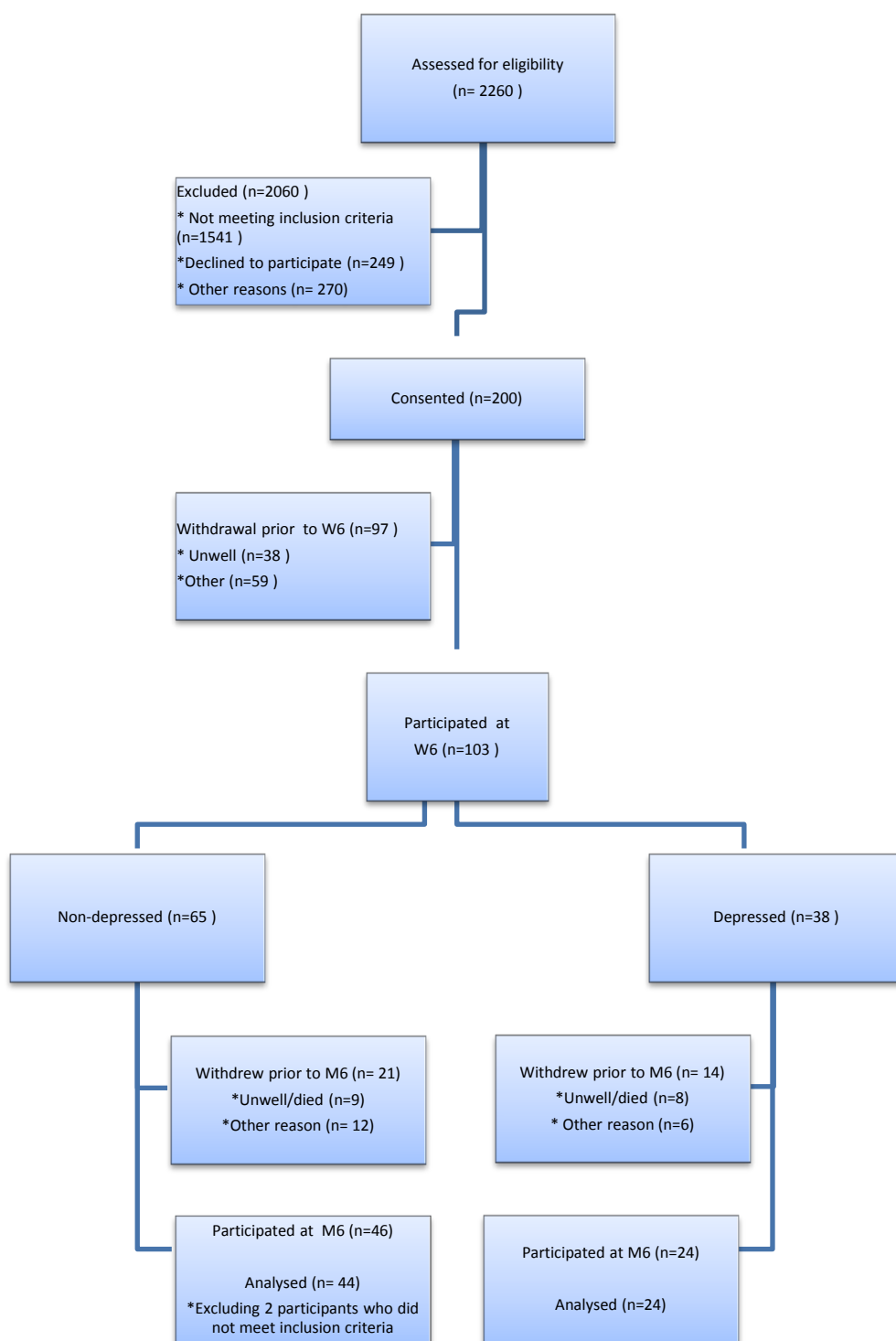
Figure 3.3 Consort diagram showing recruitment rates.

The demographic statistics for the study participants are shown in Table 3.2. The majority of hip fracture patients (80%) were females, which is not surprising as a higher incidence of hip fracture has been reported in women and women predominate in the older age group [527]. The two groups of hip fracture patients were comparable in key variables, including age, BMI, type of fracture, dietary intake and health behaviours, but the healthy controls were younger than the hip fracture patients and had a higher BMI [Table 3.2].

There were no differences in the presence of co-morbidities between the three groups, except that both the groups of hip fracture patients had higher incidence of osteoporosis compared with healthy controls, which is not surprising as osteoporosis is a major risk factor for hip fracture [528] [Table 3.3].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
Week 6		Mean (SD)	/ N(%)	
N	43	63	38	
Age	74.9 (5.64)	83.8 (7.48)	84.0 (8.62)	<.001
Sex (male)	17 (40)	13 (21)	7 (18)	.05
BMI	27.3 (4.87)	23.5 (3.81)	22.7 (4.03)	<.001
Type of fracture	x	20 (59)	37 (63)	.71
Days between surgery and interview	x	42.6 (14.06)	42.8 (12.81)	.94
Length of inpatient stay (days)	x	23.35 (16.87)	25.74 (20.27)	.53
Number of infections during hospital stay	x	.27 (.50)	.40 (.63)	.28
Patient discharged to rehabilitation centre	x	10 (16%)	13 (34%)	.04
Number of readmissions	x	.23 (.50)	.52 (.93)	.06
Occupational group	x	28 (43)	21 (58)	.21
Smokers (one or more cigarettes /day)	2 (8%)	7 (11%)	6 (19%)	.60
Alcohol consumption (one or more units per week)	13 (50%)	24 (37%)	13 (34%)	.42
Sleep (< 8hr per night)	24 (55.81%)	19 (30.15 %)	28 (73.36%)	.75

Table 3.2 Patient demographics six weeks post-surgery .

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms	p
Week 6		Mean (SD) /	N (%)	
Co-morbidities				
Osteoarthritis	3 (6.9%)	30 (47.61%)	16 (42.10%)	<.001
Hypertension	8 (18.60%)	24 (38.09%)	15 (39.47%)	.06
High Cholesterol	7 (16.27%)	11 (17.46%)	13 (34.21%)	.08
Angina	7 (16.27%)	13 (20.63%)	9 (23.68%)	.70
Other	21 (48.83)	40 (63.49%)	35 (92.10%)	.12
Diet				
Number of main meals	x	1.1 (.41)	1.2 (.40)	.40
Fat content of diet	x	11.1 (4.88)	12.4 (4.24)	.16
Vegetable content of diet	x	7.9 (2.71)	8.7 (2.89)	.17
Number of caffeinated	x	4.9 (2.1)	4.8 (2.23)	.90

Table 3.3 Participant co-morbidities and health behaviours six weeks post-surgery.

At the 6 month follow up, data were available for 66 hip fracture patients; 17 (25.7%) of these participants were depressed. The dietary intake, smoking status and alcohol intake did not change between week 6 and month 6 [Table 3.4]. Further, the medical notes of 76 hip fracture patients were accessed 6 months post hip fracture to record the number of infections and mortality rate in these patients [Table 3.5].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms	p
Month 6		Mean (SD)	/ N (%)	
Depressed	x	13	4	
Non depressed	x	11	36	
Smokers (one or more cigarettes /day)	x	3 (12.5%)	4 (9.8%)	.73
Alcohol consumption (one or more units per week)	x	9 (37.5%)	19 (46.3)	.49
Sleep (< 8hr per night)	x	16 (66.7%)	27 (67.5%)	.95
Number of patients put on antidepressants	x	X	2	
Diet				
Number of main meals	x	1.1 (.28)	1.1 (.30)	.85
Fat content of diet	x	13.5 (4.87)	12.6 (4.77)	.46
Vegetable content of diet	x	7.4 (2.27)	8.2 (2.64)	.23
Number of caffeinated drinks	x	5.3 (1.88)	4.6 (1.79)	.12

Table 3.4 Participant demographics and health behaviours 6 months post-surgery.

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
		N (%)		
N (76)	x	46	30	
Infections over 6 month period	x	21(45.6%)	15 (50%)	.21
Number of individuals with urinary tract infections	x	14 (30%)	11 (36.6%)	.71
Number of individuals with chest infections	x	2 (4.3%)	5 (16.6%)	.15
Number of individuals with wound infections	x	4 (8.6 %)	3 (10%)	.42
Number of individuals with other infections	x	10 (21.7%)	3 (10%)	.36
Mortality	x	2 (3.17%)	5 (13.15%)	.29

Table 3.5 Infection and mortality data in hip fracture patients 6 months post-surgery.

3.3.1 Psychosocial, frailty and cognitive measures in hip fracture patients

37% of older adults that had recently suffered from the physical trauma of a hip fracture had depressive symptoms. Participants classified as depressed thus had significantly higher GDS scores, $F (1, 99) = 14.76$, $p< .001$, $\eta^2 =.66$, at week 6 than those classed as non-depressed [Table 3.6]. At month 6, this difference in GDS scores remained significant, $F (1, 64) = 30.10$, $p< .001$, $\eta^2 =.32$ [Table 3.6] [526]. There was no detectable change in GDS score between week 6 and month 6, $F (1, 65) = .00$, $p = .95$, $\eta^2 =.00$. However, of those who provided data at both follow-ups, six of the non-depressed patients became depressed, as classified by the GDS at month 6, and 11 of the depressed patients were classified as non-depressed by month 6. The main analysis by group will focus on participant's depressive

symptoms classification at week 6 in order to examine the effect of emergence of depressive symptoms post-fracture on frailty, cognitive function and immune function. The difference in depressive symptoms was also confirmed by HADS depressive symptoms scale, the healthy controls recruited in this study were also asked to complete the HADS questionnaire. At week 6, significant differences were seen between the three groups, $F (1, 136) = 55.42$, $p < .001$, $\eta^2 = .45$ [Table 3.6], such that hip fracture patients with depressive symptoms had significantly higher HADS depressive symptoms scores than the hip fracture group without symptoms , $p < .001$ and the control group, $p < .001$. At month 6 , the difference in HADS score between hip fracture patients with and without depressive symptoms still remained significant , $F (1,64) = 19.46$, $p < .001$, $\eta^2 = .23$ [Table 3.6]. Similarly, HADS anxiety scores were also significantly higher among the group with depressive symptoms at week 6, $F (1, 98) = 22.83$ $p < .001$, $\eta^2 = .18$, and month 6, $F (1, 64) = 11.80$, $p = .001$, $\eta^2 = .15$ [Table 3.6] [526].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
Week 6		Mean (SD)		
GDS score	x	2.6 (1.53)	8.2 (2.47)	<.001
HADS depressive	2.4 (1.71)	3.4 (2.31)	9.1 (4.71)	<.001
HADS anxiety	x	4.0 (3.71)	8.0 (4.55)	<.001
Month 6				
GDS score	x	3.1 (1.99)	7.0 (3.75)	<.001
HADS depressive	x	4.0 (3.71)	8.0 (4.55)	<.001
HADS anxiety	x	3.5 (3.01)	7.0 (5.07)	.001

Table 3.6 Psychological measures in hip fracture patients and healthy controls.

Physical frailty was assessed in hip fracture patients using four measures. Firstly, a higher Oxford hip score (OHS) was reported in hip fracture patients without depressive symptoms compared with hip fracture patients with depressive symptoms at week 6, $F (1, 72) = 65.02$, $p< .001$, $\eta^2 =.45$, suggesting that hip fracture patients with depressive symptoms were less able to engage in activities of daily living (ADL) and the differences between OHS score of the two groups still remained significant at month 6, $F (1, 57) = 6.22$, $p =.02$, $\eta^2 =.09$ [Table 3.7]. The hand grip strength did not differ between hip fracture patients with and without depressive symptoms at week 6 or month 6, $F (1, 86) = 1.32$, $p = .25$, $\eta^2 =.01$, and $F (1, 57) = 1.34$, $p = .25$, $\eta^2 =.02$, respectively [Table 3.7]. Further, on examining the timed up and go (TUG) score it was observed that the hip fracture patients with depressive symptoms took longer to complete the TUG test at week 6, $F (1, 82) = 4.16$, $p = .05$, $\eta^2 =.04$, and at month 6 $F (1, 54) = 3.90$, $p = .05$, $\eta^2 =.06$. [Table 3.7]. Finally, the hip fracture patients with depressive symptoms scored significantly worse on the Berg balance scale (BBS) at week 6, $F (1, 78) = 8.35$, $p = .005$, $\eta^2 =.09$, but by month 6 there were no significant differences between the two groups of hip fracture patients, $F (1, 46) = .83$, $p = .37$, $\eta^2 =.16$ [526].

Cognitive function was measured in hip fracture patients using the MMSE test, no significant differences were detected between the two groups of hip fracture patients at week 6, $F (1, 98) = .08$, $p = .78$, $\eta^2 =.001$ or at month 6, $F (1, 64) = .44$, $p = .51$, $\eta^2 =.007$ time point [Table 3.7].

Variable	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
Week 6	Mean (SD)		
OHS	30.0 (8.04)	22.1 (9.33)	<.001
Hand grip score (kg)	15.9 (6.69)	14.3 (6.20)	.25
TUG (seconds)	52.4 (30.31)	69.6 (47.03)	.04
BBS	27.4 (14.16)	17.6 (14.80)	.005
MMSE score	24.7 (4.60)	24.4 (4.08)	.78
Month 6			
OHS	35.3 (8.14)	29.6 (8.97)	.02
Hand grip score (kg)	16.3 (6.56)	14.2 (6.7)	.25
TUG (seconds)	34.4 (25.81)	50.8 (36.23)	.05
BBS	36.1 (12.71)	32.7 (13.4)	.37
MMSE score	25.9 (3.59)	25.3 (3.89)	.51

Table 3.7. Physical frailty and cognitive measures in hip fracture patients.

3.3.2 Neutrophil function in older hip fracture patients

On comparing granulocyte count (mainly neutrophils) in peripheral blood, significant differences were observed between the three groups, $F_{(2, 66)} = 4.76$, $p = .01$, $\eta^2 = .12$, such that the healthy controls had significantly lower numbers of granulocytes than the hip fracture patients with, $p = .01$ and without depressive symptoms, $p = .01$ [Figure 3.4a]. As the demographic variables (sex, BMI and age) differed significantly between the groups, analyses were rerun adjusting for these variables using ANCOVA with using sex, BMI and

age as covariates. When all of the above analyses were repeated with adjustment for sex, age and BMI the results remained significant (data not shown).

Neutrophil phagocytosis in response to opsonised *E.coli* did not differ significantly between the two groups of hip fracture patients and healthy controls, $F_{(2,112)} = 2.29$, $p = .11$, $\eta^2 = .03$, suggesting that the physical stress of hip fracture and psychological stress of depressive symptoms did not have an impact on neutrophil phagocytic ability [Figure 3.4b] [522].

Neutrophil superoxide production in response to PMA, $F_{(2, 87)} = 5.53$, $p = .005$, $\eta^2 = .11$ differed between the groups [Figure 3.5a], but the significant impairment was restricted to the hip fracture patients with depressive symptoms compared to healthy controls, $p = .006$ and hip fracture patients without depressive symptoms, $p = .01$. Similarly, neutrophil superoxide production in response to opsonised *E.coli* also differed between the groups, $F_{(2,134)} = 6.53$, $p = .002$, $\eta^2 = .08$, [Figure 3.5b], such that the significant impairment was restricted to the hip fracture patients with new onset depressive symptoms compared to controls, $p = .003$ and compared to hip fracture patients without depressive symptoms, $p = .001$. When the above analyses were repeated with adjustment for age, sex and BMI, the superoxide production in response to PMA and *E.coli* remained significant (data not shown) [522].

In order to check for associations with depressive symptoms scores as a continuous variable rather than discrete groups split at GDS = 6, regression analyses were also performed in hip fracture patients. It was observed that GDS score was negatively correlated with neutrophil superoxide production in hip fracture patients in response to PMA, $\beta = -.26$, $p = .05$, $\Delta R^2 = .06$ (data not shown) and *E.coli,* $\beta = -.30$, $p = .002$, $\Delta R^2 = .09$, [Figure 3.5c], such that hip fracture patients with greater depressive symptom scores had poorer neutrophil superoxide production [522].

Further, on examining neutrophil superoxide production in response to *E.coli* in hip fracture patients six weeks and six months post-surgery, a significant main effect of time was observed for hip fracture patients, $F_{(1,56)} = 6.83$, $p = .01$, $\eta^2 = .10$, such that superoxide production improved in both groups. The significant improvement in superoxide production over time in both groups meant that by six months, neutrophil function did not differ between the groups, $p = .34$ [Figure 3.6]. Although the improvement in the depressed group was greater, there was no significant group x time interaction, $F_{(1, 56)} = 1.23$, $p = .27$, $\eta^2 = .02$ [522].
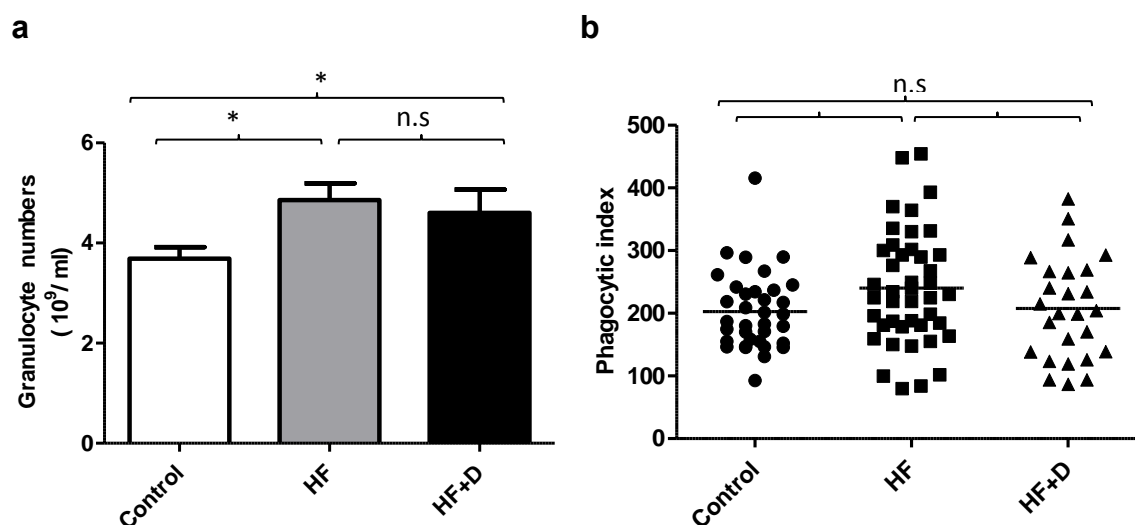
Figure 3.4 Granulocyte count and neutrophil phagocytic ability in hip fracture patients.

(a) Peripheral granulocyte count in healthy controls (n=29), hip fracture patients without depressive symptoms (HF; n=23), hip fracture patients with depressive symptoms (HF+D; n=17). Data is expressed as mean ± SEM (b) Neutrophil phagocytic ability, presented as phagocytic index, of healthy controls (n=35), hip fracture patients without depressive symptoms (HF; n = 29); hip fracture patients with depressive symptoms (HF+D; n = 23). The mean value is indicated by the bar.* p <.05.
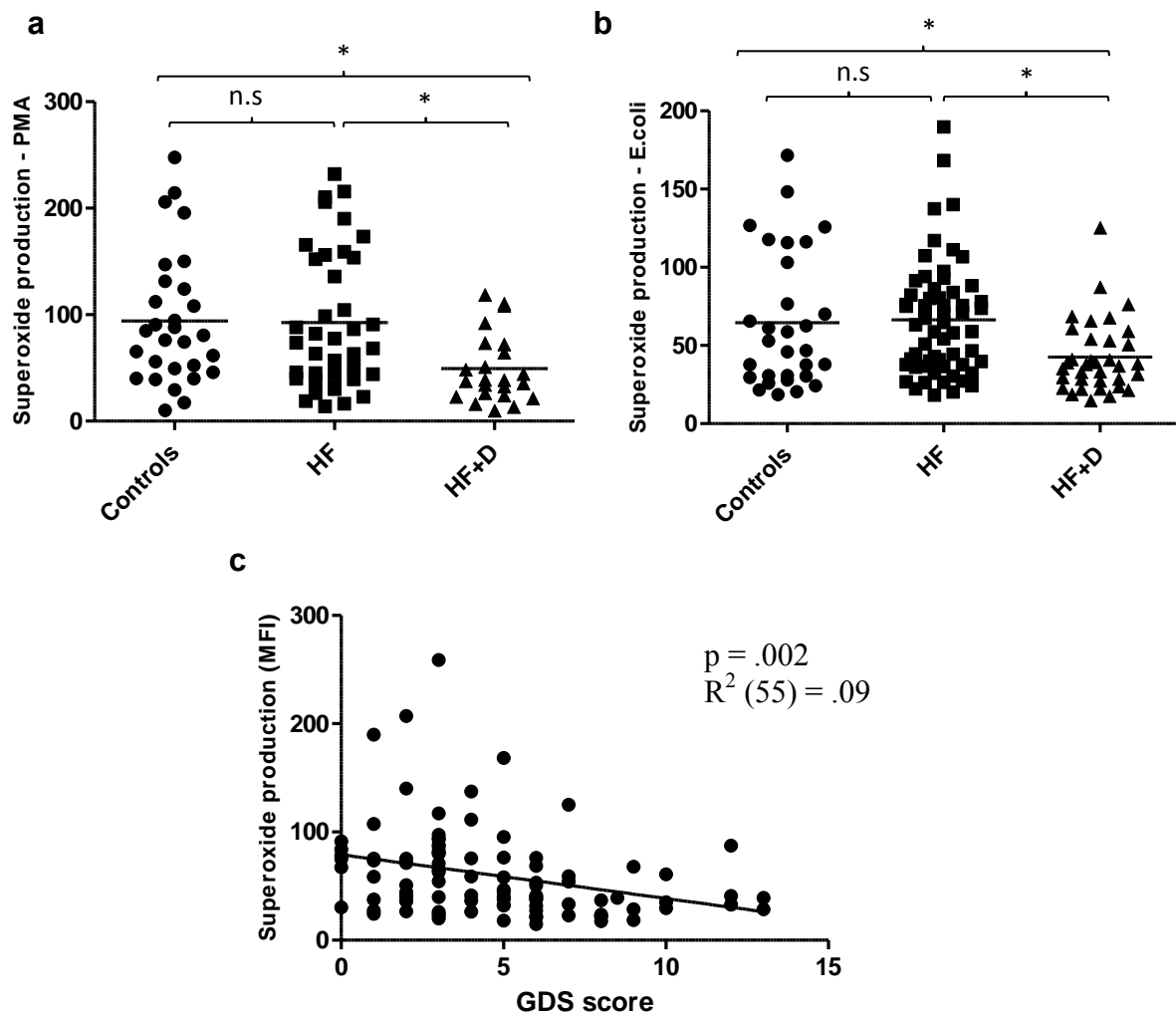
Figure 3.5 Neutrophil superoxide production in hip fracture patients.

(a) Neutrophil superoxide production (MFI) in response to PMA stimulus for healthy controls (n=29), hip fracture patients without depressive symptoms (HF; n=38), and hip fracture patients with depressive symptoms (HF+D; n=23). The mean value is indicated by the bar. (b) Neutrophil superoxide production (MFI) in response to *E.coli* stimulus for healthy controls (n=38), hip fracture patients without depressive symptoms (HF; n= 62), and hip fracture patients with depressive symptoms (HF+D; n=38). The mean value is indicated by the bar. (c) Correlation between neutrophil superoxide production in response to *E.coli* and depressive symptoms (GDS) scores in hip fracture patients (n = 55).* p <.05.
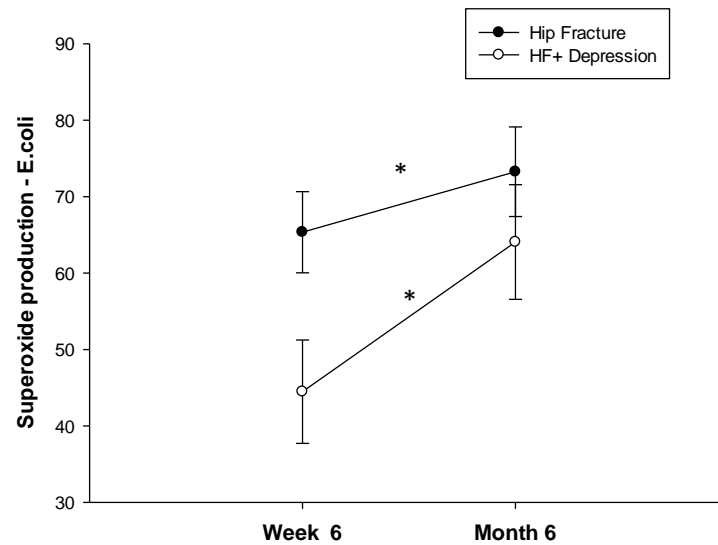
Figure 3.6 Neutrophil superoxide production in hip fracture patients six weeks and six months post-surgery.

Neutrophil superoxide production for hip fracture patients with depressive symptoms (n = 24) and without depressive symptoms (n = 39) at 6 weeks and 6 months post-surgery. Data are mean ± SEM and * indicates $p < .05$.

3.3.3 Monocyte phenotype and function in hip fracture patients

The differential expression of CD14 and CD16 have been used to define the three peripheral subsets of monocytes: classical ($CD14^{+ve}$ $CD16^{-ve}$) , intermediate ($CD14^{+ve}$ $CD16^{+ve}$) and non-classical monocytes ($CD14^{+ve}$ $CD16^{++ve}$) monocytes [64]. On comparing monocyte count in peripheral blood no significant differences were found between the three groups, $F(2, 65)$ = .37, p = .69, η^2 = .01 [Table 3.8]. On further evaluation of the distribution of monocyte subsets, no differences were seen in the percentage of 'classical'monocytes, $F(2, 67)$ = .23, p = .79, η^2 = .007 [Table 3.8]; 'intermediate' monocytes $F(2, 67)$ = .30, p = .73, η^2 = .009 or 'non-classical' monocytes $F(2, 67)$ = .43, p = .64, η^2 = .01 between the three groups [Table 3.8].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms	p
Week 6		Mean (SD)		
Monocyte count (10^9/ml)	. 53 (.04)	.50 (.04)	.55 (.05)	.69
$CD14^{+ve}CD16^{-ve}$ monocytes	89.32 (5.24)	89.29 (5.42)	88.31 (5.92)	.79
$CD14^{+ve}CD16^{+ve}$ monocytes	5.07 (2.29)	4.65 (2.94)	5.26 (2.75)	.73
$CD14^{+ve}CD16^{++ve}$ monocytes	5.37 (.78)	5.42 (.76)	6.38 (.90)	.64

Table 3.8 The distribution of monocyte subsets in hip fracture patients.

Similar to neutrophil phagocytosis, monocyte phagocytic ability in response to opsonised *E.coli* did not differ significantly between the three groups, $F(2, 81)$ = 2.15, p = .12, η^2 = .05 [Figure 3.7a]. However, on examining the ability of monocytes to produce superoxide in response to opsonised *E.coli* significant differences were observed between the three groups $F(2, 82)$ = 7.41, p = .001, η^2 = .15, but the significant impairment in monocyte superoxide

generation was restricted to the hip fracture patients with depressive symptoms compared to healthy controls, p = .008, and compared to hip fracture patients without depressive symptoms p = .002 [Figure 3.7b]. When the above analyses were repeated with adjustment for age, sex and BMI, the superoxide production in response *E.coli* still remained significantly different between the groups (data not shown). There was also a negative correlation between monocyte superoxide production and depressive symptoms (GDS score) in hip fracture patients, $\beta = -.32$, p = .01, $\Delta R^2 = .10$, [Figure 3.7c], such that hip fracture patients with a greater depressive symptom score had lower monocyte superoxide production.

On evaluating the long term effect of hip fracture and depressive symptoms on monocyte superoxide production, the main effect of time on both groups of hip fracture patients was not significant $F(1, 38) = 2.31$, p = .13, $\eta^2 = .05$ [Figure 3.8]. Further, there was no significant group x time, $F(1, 38) = .11$, p = .73, $\eta^2 = .003$, such that the two groups of hip fracture patients did not significantly differ in their recovery in the ability of monocytes to generate superoxide.

Monocyte activation via lipopolysaccharide (LPS) is known to upregulate production and secretion of TNFα [529]. PBMCs were stimulated with LPS for 1hr, 2hr, 4hr and 24 hr with LPS and TNFα production increased in a time dependent manner, being highest after 4 hrs. [Figure 3.9]. Upon prolonged stimulation (24 hr) with LPS a drop in TNFα production by monocytes was seen compared with the 4 hr time point [Figure 3.9]. Hereafter, TNFα production by monocytes was examined by stimulation of whole PBMCs with LPS for 4 hr.

On assessing TNFα cytokine production by monocytes no significant differences were observed in the percentage of monocytes producing TNFα between the three groups in the basal state, $F_{(2, 33)} = 1.24$, $p = .30$, $\eta^2 = .07$ [Figure 3.10a] or after 4hr LPS stimulations, $F_{(2, 32)} = .08$, $p = .92$, $\eta^2 = .005$ [Figure 3.9c]. Similarly, no significant differences were detected in the amount of TNFα produced by monocytes (MFI) in the basal state, $F_{(2, 33)} = 0.60$, $p = .55$, $\eta^2 = .03$ [Figure 3.10b] or after 4hr LPS stimulation, $F_{(2, 32)} = 2.13$, $p = .32$, $\eta^2 = .14$ [Figure 3.9d]. Previous findings have reported that CD14^{+ve}CD16^{+ve} monocytes produce higher levels of pro-inflammatory cytokines such as TNF compared with CD16^{-ve} monocytes [530], but in this thesis it was not possible to distinguish between the TNF production between the monocyte subsets due to loss of CD16 expression on LPS stimulation.

In addition it was not possible to isolate monocytes from our hip fracture patients due to the limited volume of blood available and as a result TNFα secretion was assessed in whole PBMCs on LPS stimulation. TNFα production by different immune cells was measured to confirm that monocytes were the main producers of TNFα post LPS stimulation [Figure 3.11a], which has also been previously reported [531]. Further, no significant differences were observed in TNFα secretion between our three groups in the basal state $F_{(2, 26)} = .04$, $p = .95$, $\eta^2 = .003$ [Figure 3.11b] or on 4hr LPS stimulations $F_{(2, 22)} = .20$, $p = .75$, $\eta^2 = .02$ [Figure 3.11c].
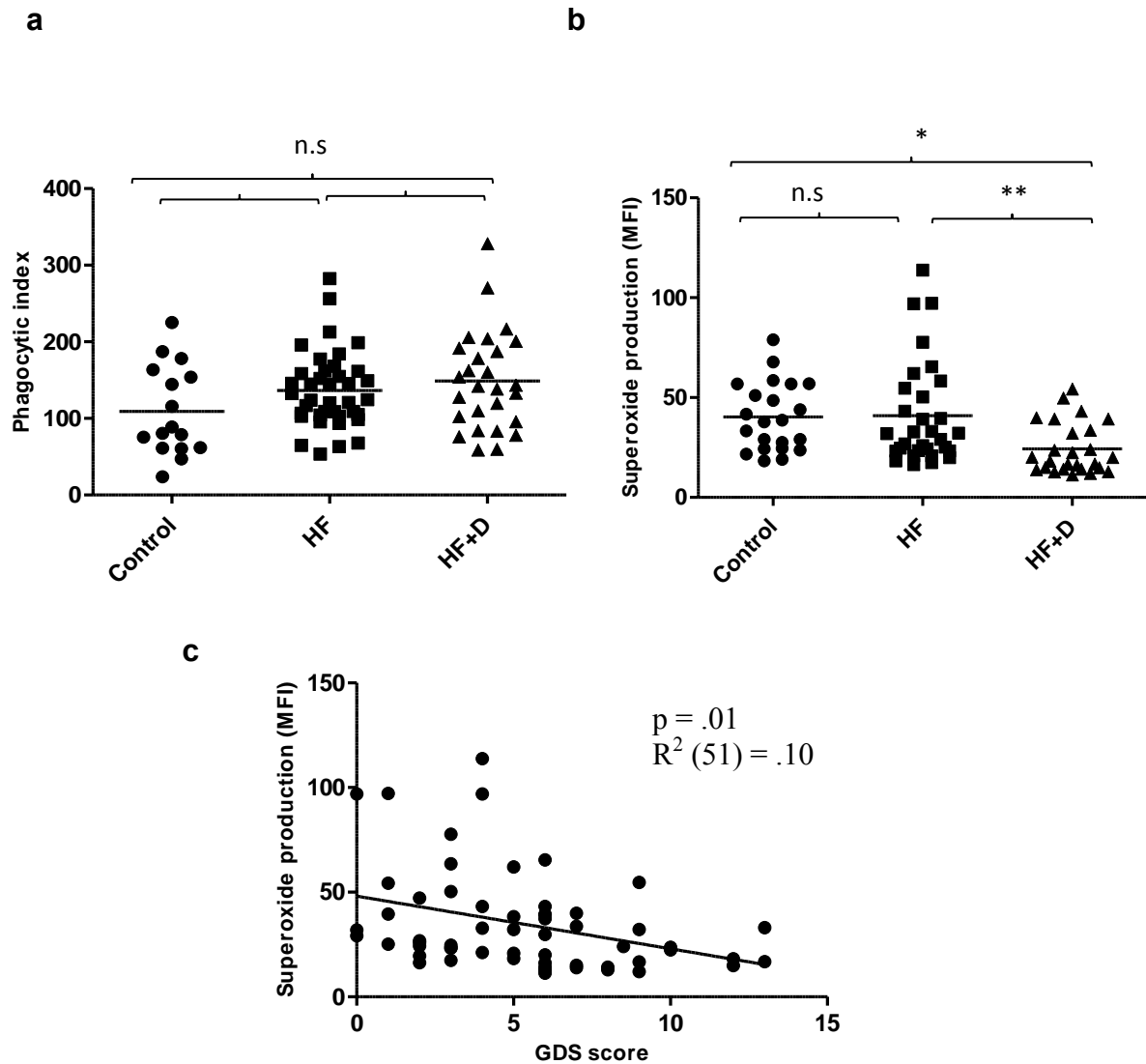
Figure 3.7 Monocyte phagocytic ability and superoxide production in hip fracture patients

(a) Monocyte phagocytic ability in healthy controls (n=18), hip fracture patients without depressive symptoms (HF; n =31), and hip fracture patients with depressive symptoms (HF+D; n = 26). The mean value is indicated by the bar. (b) Monocyte superoxide production in response to *E.coli* in healthy controls (n = 22), hip fracture patients without depressive symptoms (HF; n=41) and hip fracture patients with depressive symptoms (HF+D; n = 26). The mean value is indicated by the bar. (c) Correlation between GDS depressive symptoms scores and monocyte superoxide production in response to *E.coli* (n = 51). * indicates $p < .05$, ** $p < .005$.
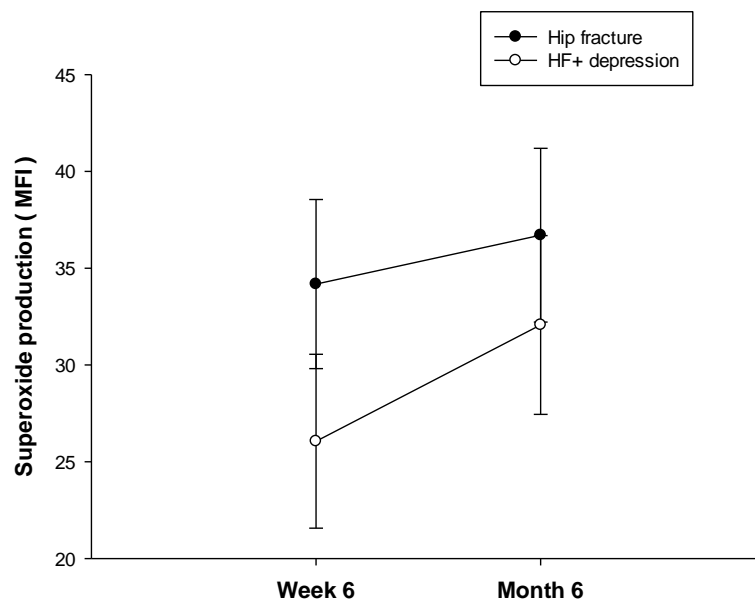
Figure 3.8 Monocyte superoxide production in hip fracture patients six months post-surgery

Monocyte superoxide production in hip fracture patients with depressive symptoms (n = 17) and without depressive symptoms (n= 18) at week 6 and 6 month post-surgery.
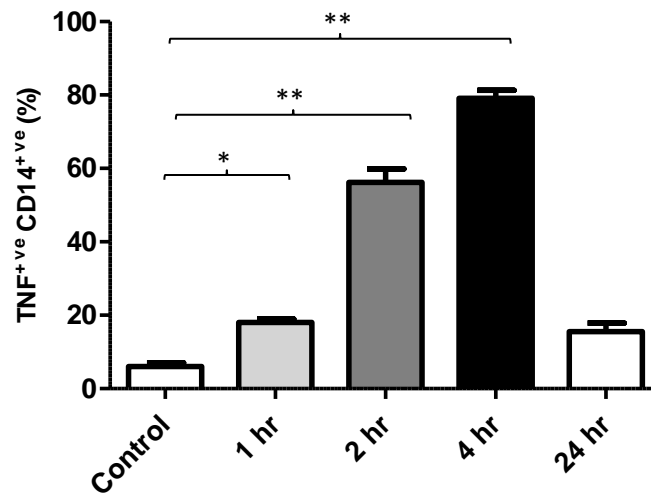
Figure 3.9 Time course of TNFα production by monocytes in response to LPS.

Percentage of TNFα producing monocytes after LPS stimulation of PBMCs from healthy young donors (n = 3). Data is expressed as mean ± SEM. * p <.05, ** p <.005.
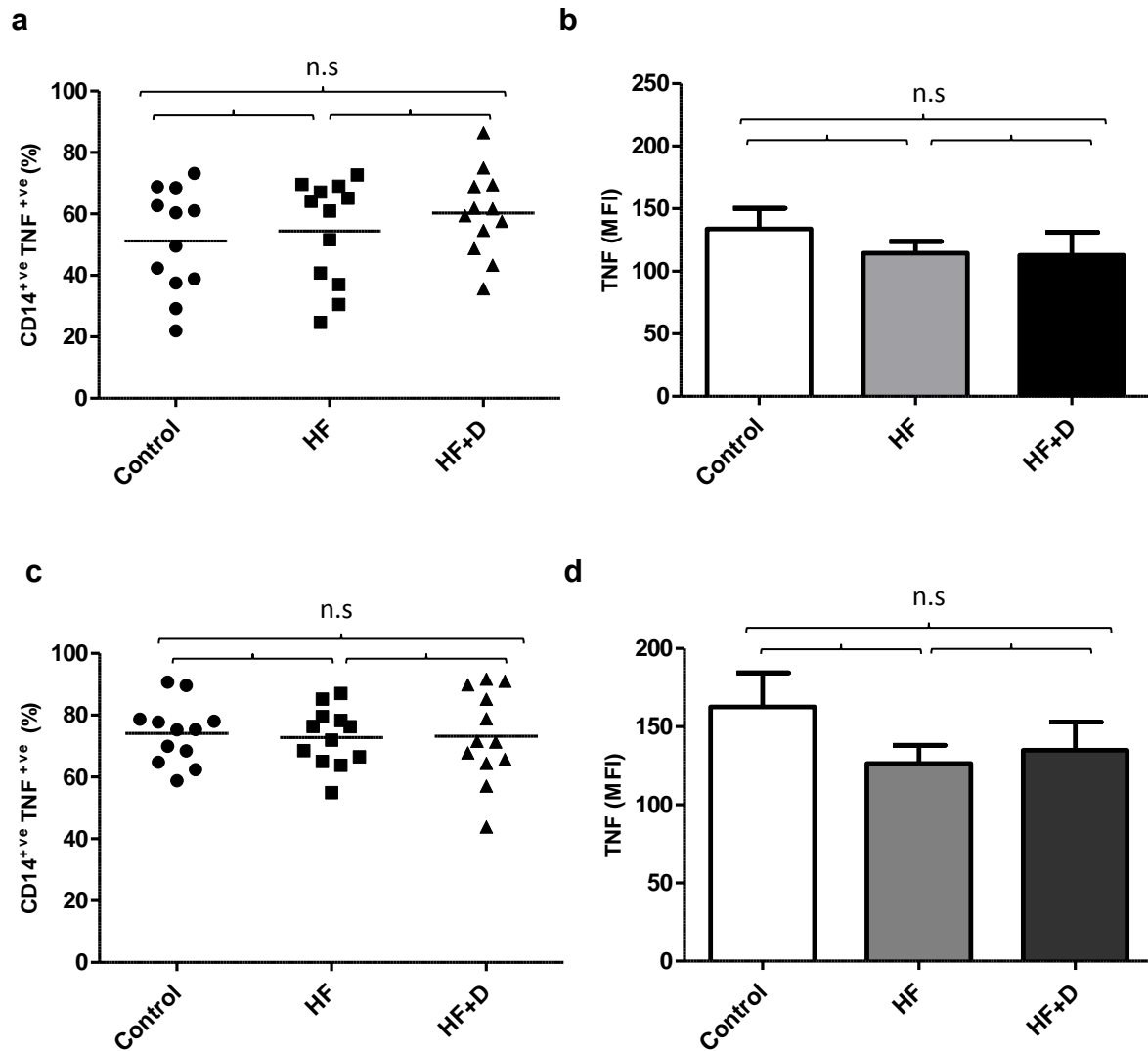
Figure 3.10 Monocyte TNFα productions in hip fracture patients.

Percentage of monocytes producing TNFα in the (a) basal state, or (c) after LPS stimulation for 4 hr in healthy controls (n = 12); hip fracture patients without depressive symptoms (HF; (n = 12) and hip fracture patients with depressive symptoms (HF+D; n = 12). The mean value is indicated by the bar. Amount of TNFα produced by monocytes (MFI) in (b) the basal state, or (d) after 4 hr LPS stimulation in healthy controls (n = 12); hip fracture patients without depressive symptoms (HF; n=12) and hip fracture patients with depressive symptoms (HF+D; n=12). Data is expressed as mean ± SEM.
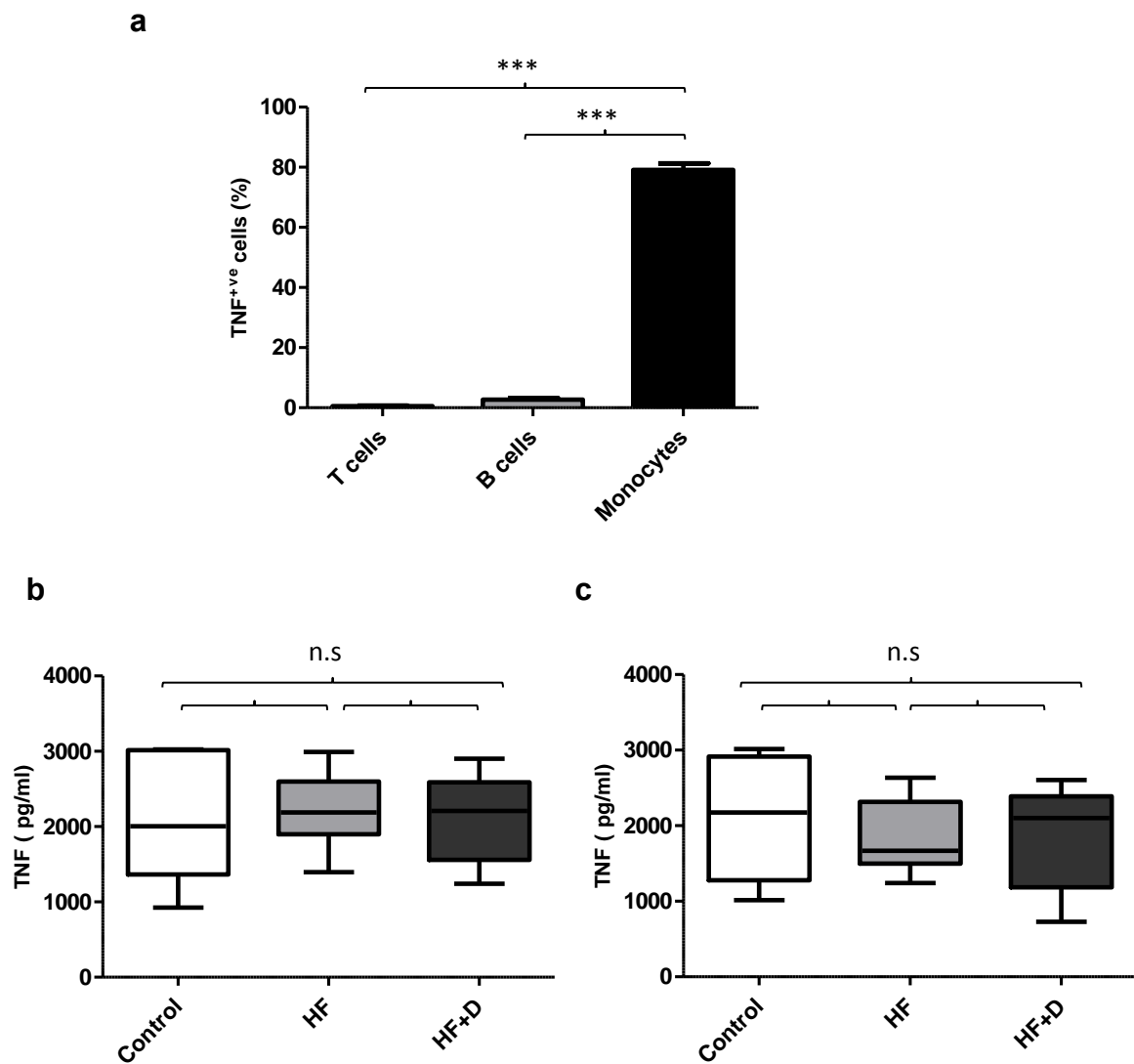
Figure 3.11 TNFα secretion post LPS stimulation in hip fracture patients.

(a) TNFα production by monocytes, T cells and B cells upon 4 hr LPS stimulation of PBMCs of healthy young donors (n = 3). Data is expressed as mean ± SEM. *** p <.001. TNFα secretion in (b) the basal condition, or (c) after 4 hr LPS stimulation in healthy controls (n = 10), hip fracture patients without depressive symptoms (HF; n = 10), and hip fracture patients without depressive symptoms (HF+D; n= 10). The solid bar represents the mean value.

To investigate possible mechanisms for the reduced monocyte superoxide generation the expression of relevant receptors was assessed. On measuring HLADR expression on monocytes, no differences were observed in the percentage of HLADR^{+ve} monocytes, $F_{(2, 50)} = 1.58$, $p = .21$, $\eta^2 = .06$, or in the cell surface expression of HLADR, $F_{(2, 50)} = .62$, $p = .53$, $\eta^2 = .02$ between our groups [Table 3.9]. HLADR expression on monocyte subsets also showed no significant differences in the percentage of HLADR^{+ve} monocytes or the surface expression of HLADR in any of the subsets between the three groups [Table 3.9].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
Week 6		Mean (SD)		
HLADR^{+ve} monocytes (%)	89.96 (5.87)	93.73 (7.46)	93.79 (8.63)	.21
HLADR expression (MFI)	79.38 (32.30)	78.24 (30.84)	68.21 (35.89)	.53
HLADR^{+ve}CD14^{+ve}CD16^{-ve}	94.41 (7.64)	93.59 (8.68)	93.64 (7.86)	.94
HLADR expression (MFI)	71.36 (36.46)	64.41 (26.48)	64.32 (29.30)	.75
HLADR^{+ve}CD14^{+ve}CD16^{+ve}	97.14 (2.24)	96.18 (4.73)	95.12 (5.77)	.44
HLADR expression (MFI)	174.70 (91.08)	179.60 (112.47)	144.10 (87.31)	.50
HLADR^{+ve}CD14^{+ve}CD16$^{+\ ve}$	86.55 (13.72)	86.98 (19.12)	85.32 (14.07)	.95
HLADR expression (MFI)	90.48 (42.92)	115.17 (88.04)	76.81 (56.44)	.22

Table 3.9 HLADR expression on monocyte subsets in hip fracture patients.

Upon evaluation of the expression of co-stimulatory molecules CD80 and CD86 on monocytes of hip fracture patients the percentage of CD80^{+ve} monocytes, $F_{(2, 41)} = 1.67$, $p = .20$, $\eta^2 = .07$ and the CD80 expression levels on monocytes $F_{(2, 41)} = 1.86$, $p = .16$, $\eta^2 = .08$ were similar between the three groups [Table 3.10]. Additionally, on examining CD80

expression on monocyte subsets no differences were observed in the percentage of CD80^{+ve} cells in the different subsets [Table 3.10] or between the three subject groups. Similarly negative data were found for CD86 cell positivity and expression on monocytes, monocyte subsets between the three subject groups [Table 3.10].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
Week 6		Mean	(SD)	
CD80^{+ve} monocytes (%)	73.98 (16.83)	79.47 (16.46)	85.00 (15.31)	.20
CD80 expression (MFI)	16.11 (5.13)	20.50 (11.56)	24.40 (14.72)	.16
CD80^{+ve}CD14^{+ve}CD16$^{-\ ve}$ (%)	71.50 (18.71)	79.94 (17.17)	83.18 (16.04)	.21
CD80 expression (MFI)	15.66 (5.36)	19.94 (10.02)	20.22 (10.48)	.37
CD80^{+}CD14^{+}CD16^{+} (%)	78.65 (15.71)	83.76 (16.42)	87.86 (15.56)	.33
CD80 expression (MFI)	17.35 (3.37)	24.79 (2.92)	24.93 (3.02)	.18
CD80^{+ve}CD14^{+ve}CD16$^{++\ ve}$ (%)	81.49 (14.88)	86.16 (14.49)	86.80 (14.98)	.61
CD80 expression (MFI)	19.84 (6.81)	28.60 (19.14)	27.35 (14.45)	.28
CD86^{+ve} monocytes (%)	96.83 (5.90)	97.60 (3.55)	96.52 (8.80)	.87
CD86 expression (MFI)	57.29 (28.83)	45.94 (16.17)	47.56 (17.57)	.25
CD86^{+ve}CD14^{+ve}CD16$^{-\ ve}$ (%)	96.50 (6.97)	97.74 (3.68)	96.51 (8.92)	.81
CD86 expression (MFI)	52.65 (28.77)	44.43 (15.33)	45.76 (18.57)	.48
CD86^{+ve}CD14^{+ve}CD16$^{+\ ve}$ (%)	99.11 (1.64)	98.82 (1.96)	97.38 (8.24)	.54
CD86 expression (MFI)	77.28 (35.52)	57.22 (19.41)	59.42 (25.46)	.09
CD86^{+ve}CD14^{+ve}CD16$^{++\ ve}$ (%)	98.59 (2.05)	96.70 (4.82)	96.14 (9.62)	.49
CD86 expression (MFI)	81.17 (32.31)	59.55 (25.32)	65.05 (26.98)	.07

Table 3.10 CD80 and CD86 expression on monocyte subsets in hip fracture patients.

3.3.4 NK cell numbers and function in hip fracture patients

On examining NK cells in peripheral blood of hip fracture patients, there were no significant differences in percentages, $F_{(2, 109)} = 1.12$, $p = .32$, $\eta^2 = .02$ or absolute numbers of NK cells, $F_{(2, 56)} = 1.98$, $p = .14$, $\eta^2 = .06$ [Table 3.11] between the three groups. Further, on examining the distribution of the two NK cell subsets – $CD56^{dim}$ and $CD56^{bright}$ no differences in the percentage of $CD56^{dim}$ NK cells, $F_{(2, 95)} = .26$, $p = .76$, $\eta^2 = .006$ or in the absolute numbers of $CD56^{dim}$ NK cells, $F_{(2, 40)} = .06$, $p = .94$, $\eta^2 = .003$ [Table 3.11] were seen between the three subject groups. Similarly, the percentage of $CD56^{bright}$ NK cells, $F_{(2, 95)} = .45$, $p = .63$, $\eta^2 = .01$ and absolute numbers of $CD56^{bright}$ NK cells, $F_{(2, 40)} = .83$, $p = .44$, $\eta^2 = .04$ were similar between the groups [Table 3.11]. Therefore, the $CD56^{dim/bright}$ ratio did not differ between hip fracture patients with and without depressive symptoms and healthy controls, $F_{(2, 95)} = .42$, $p = .65$, $\eta^2 = .009$ [Table 3.11].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
NK cells (%)	12.74 (7.58)	14.66 (6.99)	15.32 (8.70)	.32
NK cell numbers (10^9/ml)	.18 (.08)	.17 (.09)	.26 (.24)	.14
$CD56^{dim}$ NK (%)	14.32 (6.68)	14.70 (7.57)	13.41 (7.12)	.76
$CD56^{dim}$ numbers (10^9/ml)	.18 (.08)	.18 (.08)	.19 (.15)	.94
$CD56^{bright}$ NK (%)	.67 (.44)	.58 (.40)	.59 (.37)	.63
$CD56^{bright}$ numbers (10^9/ml)	.01 (.02)	.006 (.003)	.01 (.01)	.44
$CD56^{dim/bright}$ ratio	34.48 (23.44)	36.99 (29.69)	31.37 (20.17)	.65

Table 3.11 NK cell subsets in hip fracture patients.

NK cells play an essential role in defence against viral or tumour infected cells, mainly mediated via granule exocytosis [127, 532]. NK cell cytotoxicity towards K562 target cells was found to differ between the groups, $F (2, 116) = 7.55$, $p = .001$, $\eta^2 = .11$ [Figure 3.12a], but the impairment in NK cell cytotoxicity was restricted to the hip fracture patients with depressive symptoms compared to healthy controls, $p = .01$, and hip fracture patients without depressive symptoms, $p = .001$. When the above analyses were repeated with adjustment for age, sex and BMI, the difference in NK cell cytotoxicity between the three groups still remained significant (data not shown). Further, depressive symptoms (GDS scores) were correlated with reduction in NK cell cytotoxic activity towards K562 cells in hip fracture patients, $\beta = -.31$, $p = .005$, $\Delta R^2 = .09$ [Figure 3.12b], such that hip fracture patients with a greater depressive symptom score had poorer NK cell cytotoxic activity.

To investigate possible mechanisms for reduced NK cell function, various NK cell receptors were assessed as well as different stages of the cytotoxic process. CD57 is a marker for mature terminally differentiated NK cells [533]. On examining the frequency of CD57[+ve] NK cells $F (2, 54) = 1.31$, $p = .27$, $\eta^2 = .04$ and CD57 expression levels on NK cells $F (2, 54) = 0.51$, $p = .60$, $\eta^2 = .01$ no differences were observed between the three groups [Table 3.12]. Similarly, the frequency of CD16[+ve] NK cells, $F (2, 45) = 1.08$, $p = .34$, $\eta^2 = .04$ and the CD16 expression levels on NK cells, $F (2, 45) = .43$, $p = .65$, $\eta^2 = .01$ also did not differ [Table3.12].

a

b

p = .005
$R^2 (78) = .09$
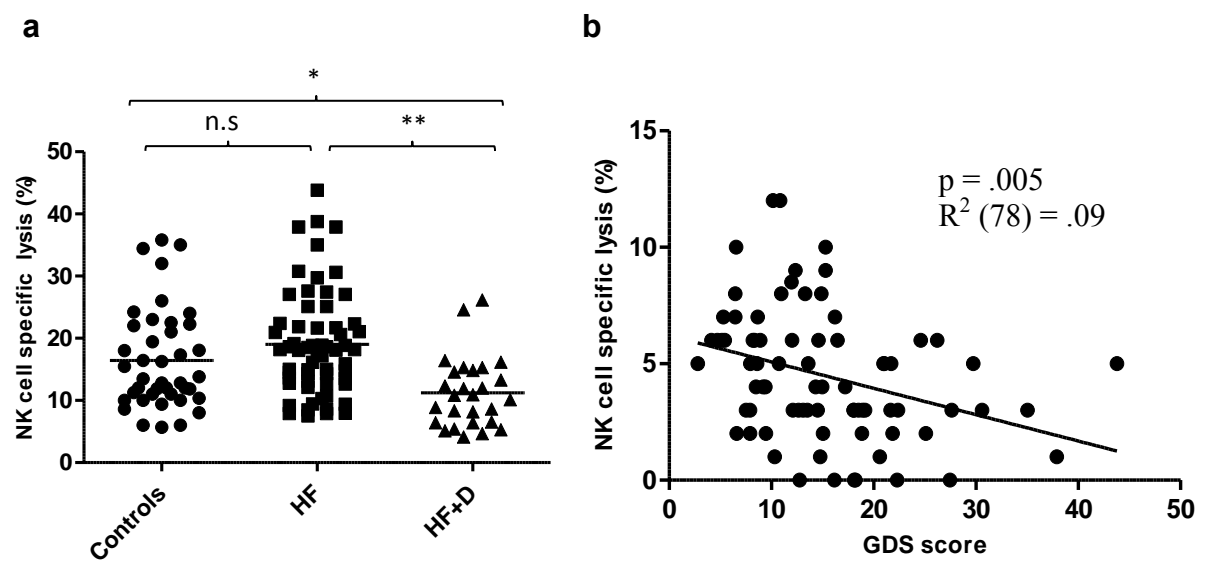
Figure 3.12 NK cell cytotoxicity in hip fracture patients

(a) NK cell cytotoxicity of healthy controls (n = 40), hip fracture patients without depressive symptoms (HF; n=50), and hip fracture patients with depressive symptoms (HF+D; n=28). The mean value is indicated by the bar. (b) Correlation between GDS depressive symptoms scores and NK cell cytotoxicity in hip fracture patients (n = 78). * indicates p < .05, ** indicates p<.005.

A range of activation receptors expressed by NK cells (NKp30, NKP46, NKG2D) are involved in recognising ligands on tumour cells and have been associated with NK cell cytotoxicity[157]. However, this study only evaluated NKG2D expression on NK cells and did not record any differences in the percentage of NK cells expressing NKG2D, $F_{(2, 42)}$ = .76, p = .47, η^2 = .03 or in the expression levels of NKG2D, $F_{(2, 42)}$ = 1.02, p = .36, η^2 = .04 between the three groups [Table3.12].

| Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
CD57[+ve] NK cells (%)	52.17 (13.46)	49.79 (16.53)	57.59 (14.90)	.27
CD57 expression (MFI)	166.56 (81.68)	163.74 (85.49)	191.91 (117.96)	.60
CD16[+ve] NK (%)	79.52 (13.97)	85.34 (9.81)	85.76 (13.91)	.34
CD16 expression (MFI)	201.56 (44.37)	207.02 (70.95)	225.70 (96.96)	.65
NKG2D[+ve] NK (%)	91.53 (4.66)	93.47 (5.78)	92.70 (4.32)	.47
NKG2D expression	25.70 (4.11)	29.32 (5.78)	37.84 (40.10)	.36

 Table 3.12 NK cell phenotypic characterisation in hip fracture patients.

The induction of NK cell cytotoxicity is dependent on contact between the NK cell and the target cell, to ensure precise targeting of the cytolytic process [129]. No significant differences were observed in the ability of NK cells to form conjugates with K562 target cells between healthy controls and hip fracture patients with and without depressive symptoms $F_{(2, 72)}$ = .41, p = .66, η^2 = .01   [Figure 3.13a]. Cell conjugate formation is a dynamic process involving adhesion molecules. The engagement of LFA-1 (CD18/CD11b) with its ligand ICAM-1 on target cells is a central step in stable adhesion of NK cells to their target cells

and is essential for NK cell cytotoxicity [534]. On examining LFA1 expression on NK cells no significant differences were found, $F_{(2, 37)} = .30$, $p = .74$, $\eta^2 = .01$ [Figure 3.13b] between the groups. Impaired NK cell cytotoxicity seen in hip fracture patients with depressive symptoms is therefore not a result of impaired ability of NK cells to form conjugates with target cells in these patients.

Perforin and granzyme are the main constituents of cytotoxic granules that are responsible for inducing target cell death [127]. On examining perforin expression by NK cells no significant differences were observed in the percentage of NK cells expressing perforin, $F_{(2, 75)} = .28$, $p = .75$, $\eta^2 = .008$ [Figure 3.14a] between the three groups. However, the perforin expression levels on NK cells differed between the three groups, $F_{(2, 75)} = 7.99$, $p = .001$, $\eta^2 = .17$, but the significant reduction in perforin expression was restricted to the hip fracture patients with new onset depressive symptoms compared to controls, $p < .001$ [Figure 3.14b]. Although perforin expression on NK cells of hip fracture patients without depressive symptoms was lower than healthy controls this difference was not statistically significant , $p = .08$. When the above analyses were repeated with adjustment for age, sex and BMI, the results remained significant (data not shown).

In contrast, no significant differences were observed in the percentage of NK cells expressing granzyme B, $F_{(2, 56)} = 0.662$, $p = .52$, $\eta^2 = .02$ [Figure 3.14c] or in the granzyme B expression levels in NK cells, $F_{(2, 56)} = .13$, $p = .87$, $\eta^2 = .005$ [Figure 3.14d] between the subject groups.
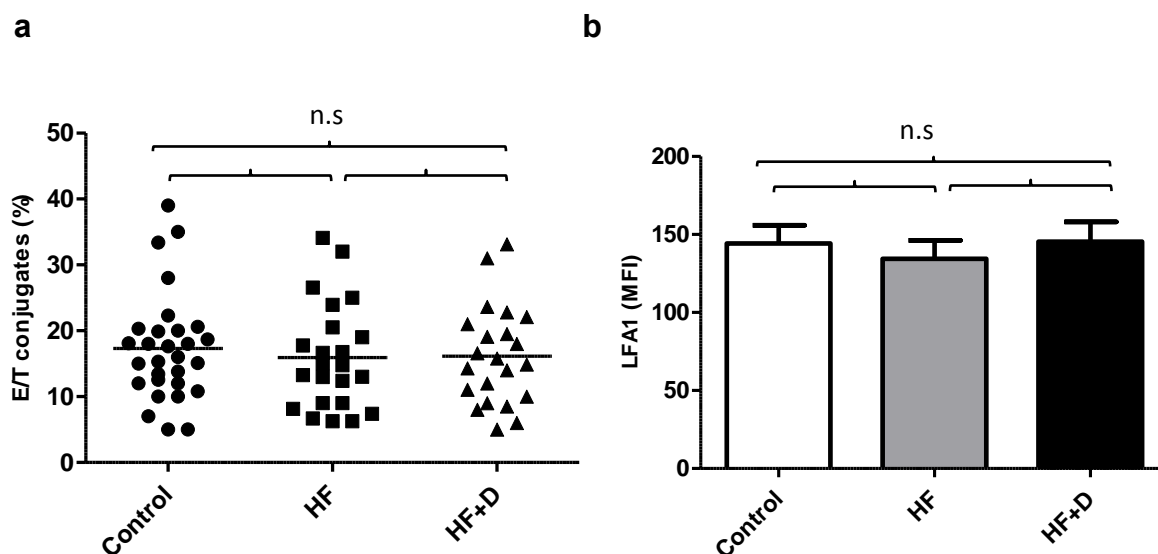
Figure 3.13 NK cell conjugate formation in hip fracture patients.

(a) NK and target cell conjugate formation in healthy controls (n=29), hip fracture patients without depressive symptoms (HF; n=22), hip fracture patients with depressive symptoms (HF+D; n=22). The mean value is indicated by the bar. (b) LFA-1 cell surface expression on NK cells of healthy controls (n=15), hip fracture patients without depressive symptoms (HF; n=15), and hip fracture patients with depressive symptoms HF+D; n=13). Data is expressed as mean ± SEM.
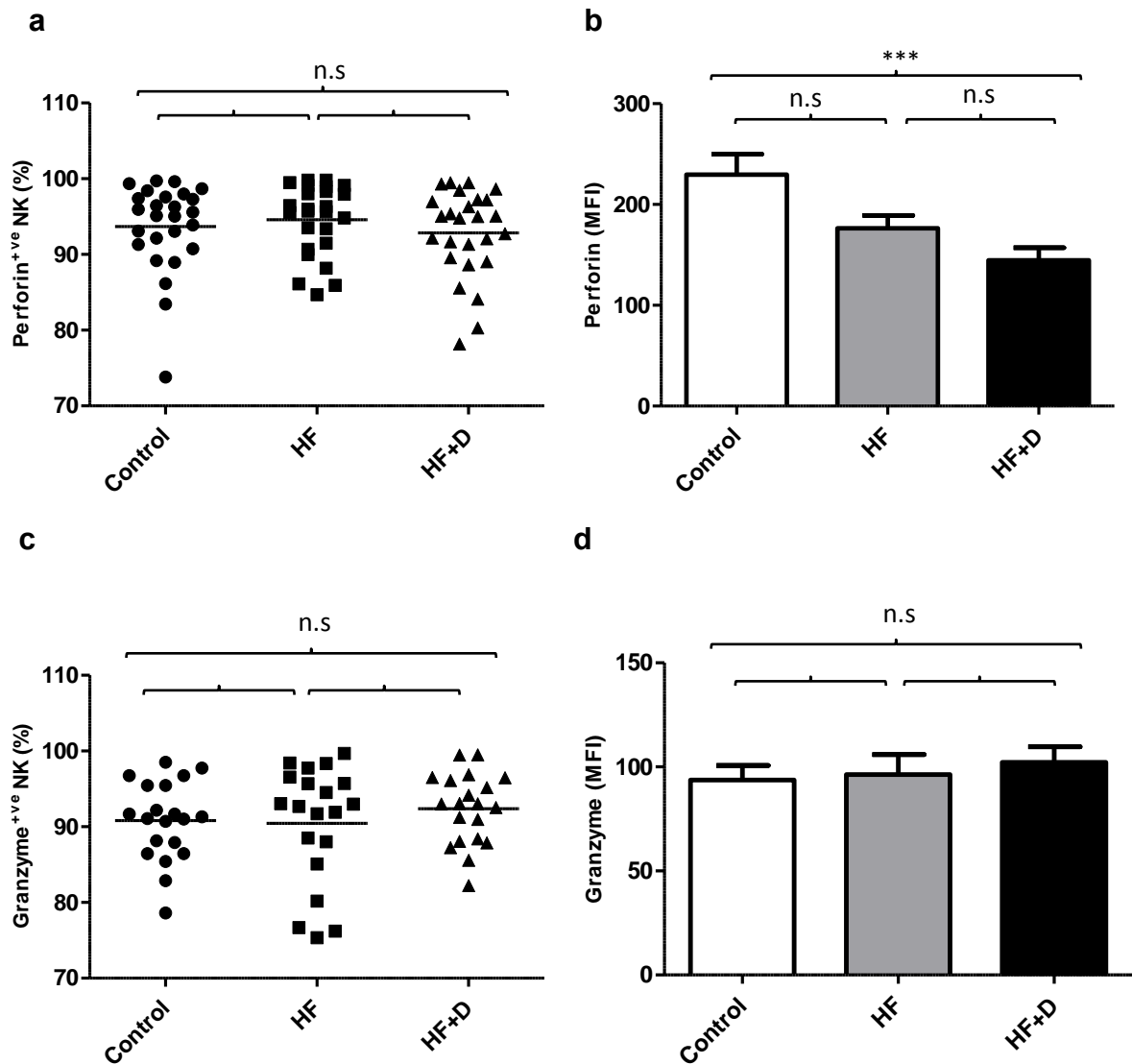
Figure 3.14 Perforin and granzyme B expression in NK cells of hip fracture patients.

(a) Percentage of perforin[+ve] NK cells, or (b) the perforin expression in healthy controls (n=26), hip fracture patients without depressive symptoms (HF; n=26), and hip fracture patients with depressive symptoms (HF+D; n=26).(c) Percentage of granzyme B[+ve] NK cells, or (d) Granzyme B expression in healthy controls (n=20), hip fracture patients without depressive symptoms (HF; n=20), and hip fracture patients with depressive symptoms (HF+D; n=20). *** indicates p<.001.

On measuring NK cell cytotoxicity in hip fracture patients with and without depressive symptoms at 6 weeks and 6 month post-surgery there was no significant effect of time, $F(1,40) = 1.36$, $p = .25$, $\eta^2 = .03$, such that the NK cell activity did not change overall with time [Figure 3.15a]. There was no significant group x time interaction, $F (1, 40) = .36$, $p = .55$, $\eta^2 = .009$, such that both the groups of hip fracture patients did not differ in their recovery of NK cell cytotoxicity six months after surgery. The NK cell activity in hip fracture with depressive symptoms was reduced in comparison to hip fracture patients without depressive symptoms even six months post-surgery, $p = .04$.

Next, the perforin expression in NK cells of hip fracture patients with and without depressive symptoms was evaluated 6 weeks and 6 months post-surgery , there was no significant effect of time, $F (1, 28) = .13$, $p = .71$, $\eta^2 = .005$ [Figure 3.15b] ,such that perforin expression in NK cells did not improve even 6 months post-surgery in hip fracture patients .
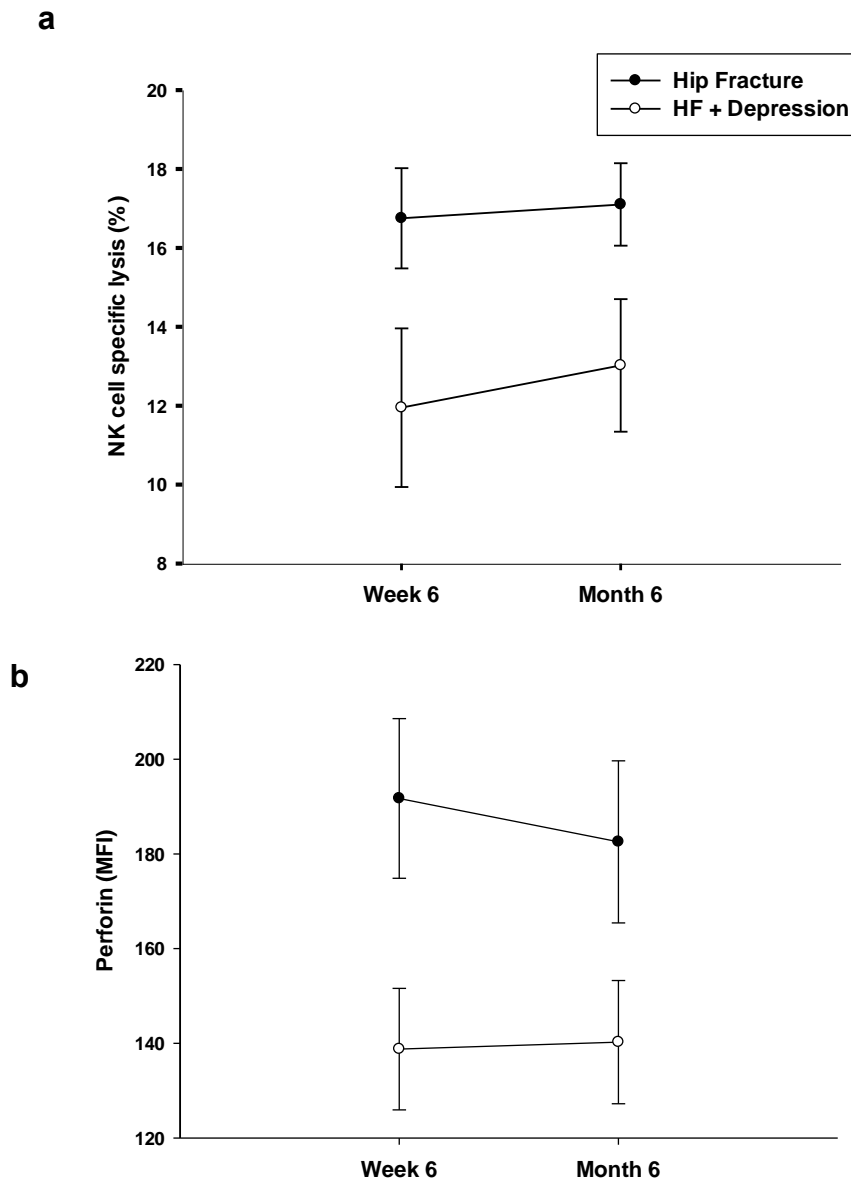
Figure 3.15 NK cell cytotoxicity and NK cell perforin expression in hip fracture patients six weeks and six months post-surgery.

(a) NK cell specific lysis for hip fracture patients with depressive symptoms (HF+D; n = 12) and without depressive symptoms (HF; n=30) at 6 weeks and 6 months post-surgery. Data are mean ± SEM. (b) Perforin expression in NK cells in hip fracture patients with depressive symptoms (HF+D; n = 19) and without depressive symptoms (HF; n = 11) at 6 weeks and 6 months post injury. Data are mean ± SEM.

3.3.5 The HPA axis in hip fracture patients

The two main adrenal hormones; DHEAS and cortisol are known to play role in maintaining immune homeostasis [535]. Analysis of HPA axis hormone levels revealed significant group differences in serum cortisol levels, $F_{(2,127)} = 10.32$, $p < .001$, $\eta^2 = .14$, [Figure 3.16a] , driven by significantly higher serum cortisol levels in hip fracture patients with depressive symptoms compared with healthy controls, $p = .006$ or with hip fracture patients without depressive symptoms, $p < .001$. DHEAS levels, $F_{(2,127)} = 10.47$, $p < .001$, $\eta^2 = .14$, were also significantly different between the subject groups [Figure 3.16b], lower DHEAS levels were observed in hip fracture with depressive symptoms compared with healthy controls, $p < .001$, and the hip fracture alone group, $p = .008$. Also, significant differences were observed between DHEAS levels of hip fracture patients without depressive symptoms and healthy controls, $p = .03$ [522].

As a consequence , the overall serum cortisol:DHEAS ratio differed significantly between the three groups, $F_{(2,127)} = 9.36$, $p < .001$, $\eta^2 = .12$, such that only those hip fracture patients with depressive symptoms had a higher ratio when compared with healthy controls, $p < .001$ and with hip fracture patients without depressive symptoms , $p = .006$, [Figure 3.16c]. When the above analyses were repeated with adjustment for age, sex and BMI, the serum cortisol:DHEAS ratio still remained significantly different between the three groups (data not shown) [522]. An elevated serum cortisol: DHEAS ratio is a known marker for depressive symptoms [536] and in this study the presence of depressive symptoms, specifically the GDS score, in hip fracture patients correlated with the cortisol: DHEAS ratio, $\beta = .28$, $p = .006$, $R^2 = .07$, such that hip fracture patients with higher depressive symptoms had an elevated cortisol: DHEAS ratio [Figure 3.16d] [522].

Further, on measuring serum adrenal hormone levels in hip fracture patients six months post- surgery there was no significant effect of time on the serum cortisol levels, $F (1, 51) = 1.90$, $p = .17$, $\eta^2 = .03$ and there remained a significant effect of group, such that serum cortisol levels were higher in hip fracture patients with depressive symptoms even 6 months post-surgery, $p = .01$ [Figure 3.17a]. Serum DHEAS levels in hip fracture patients six months after surgery showed a trend towards a decline in patients without depressive symptoms, but there was no significant effect of time on the serum DHEAS levels, $F (1, 51) = 1.23$, $p = .27$, $\eta^2 = .02$. However, the effect of group did not remain significant, such that serum DHEAS levels did not significantly differ between hip fracture patients with and without depressive symptoms 6 months post-surgery , $p =.14$ [Figure 3.17b].

Overall, the serum cortisol: DHEAS ratio did not significantly alter with time, $F (1, 51) = .94$, $p = .34$, $\eta^2 = .01$, and there remained a significant effect of group, such that the cortisol: DHEAS ratio was higher in the hip fracture patients with depressive symptoms even six months post-surgery, $p = .02$ [Figure 3.17c] [526].
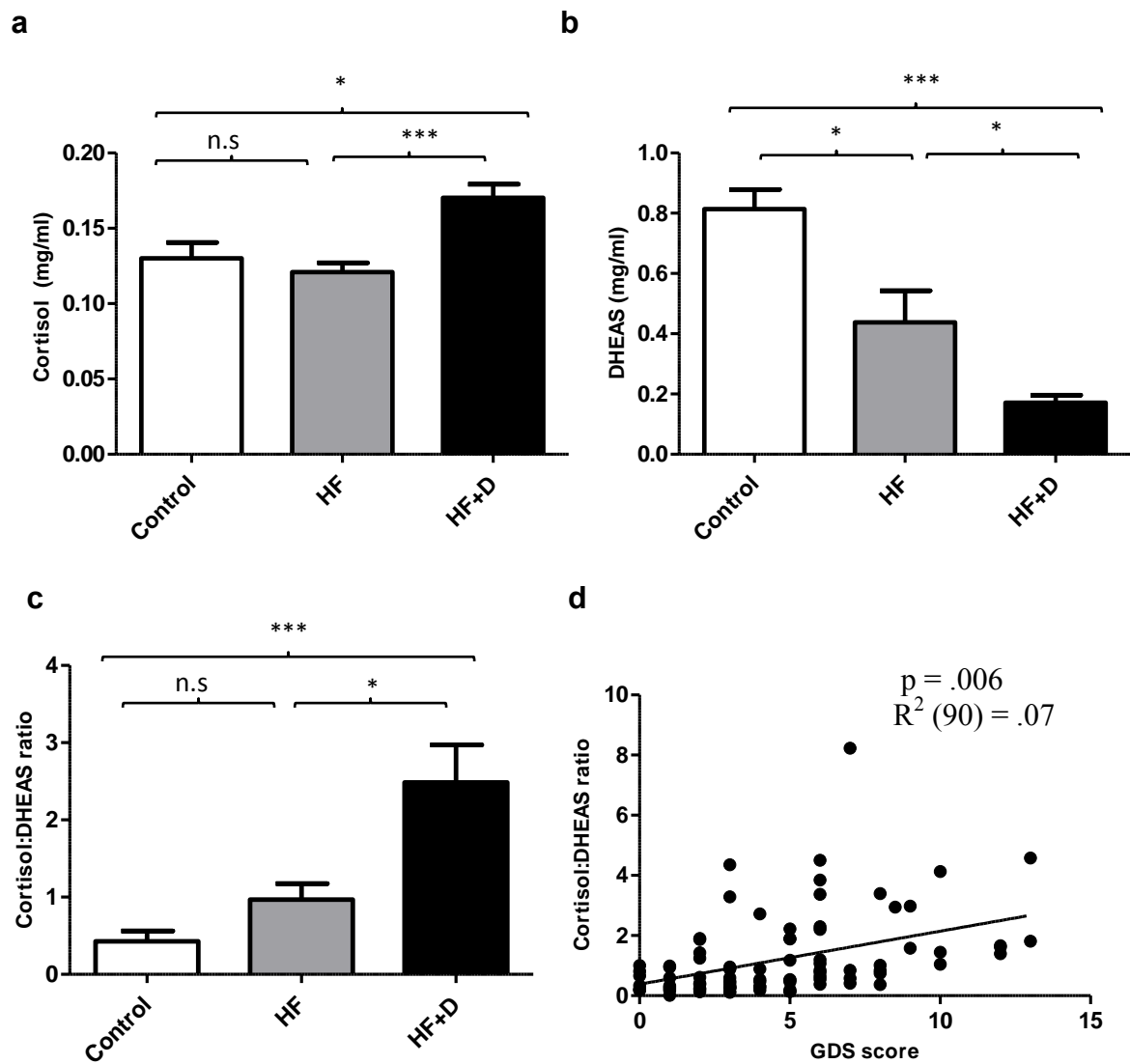
Figure 3.16 Adrenal hormone levels in hip fracture patients.

Serum (a) cortisol, (b) DHEAS, or (c) the cortisol: DHEAS ratio in healthy controls (n=32); hip fracture patients without depressive symptoms (HF; n= 62), and hip fracture patients with depressive symptoms (HF+D; n=38). (d) Correlation between GDS depressive symptoms scores and serum cortisol: DHEAS ratio in hip fracture patients (n = 90). Data are mean ± SEM. * p <.05 **p <.005 and ***p <.001.
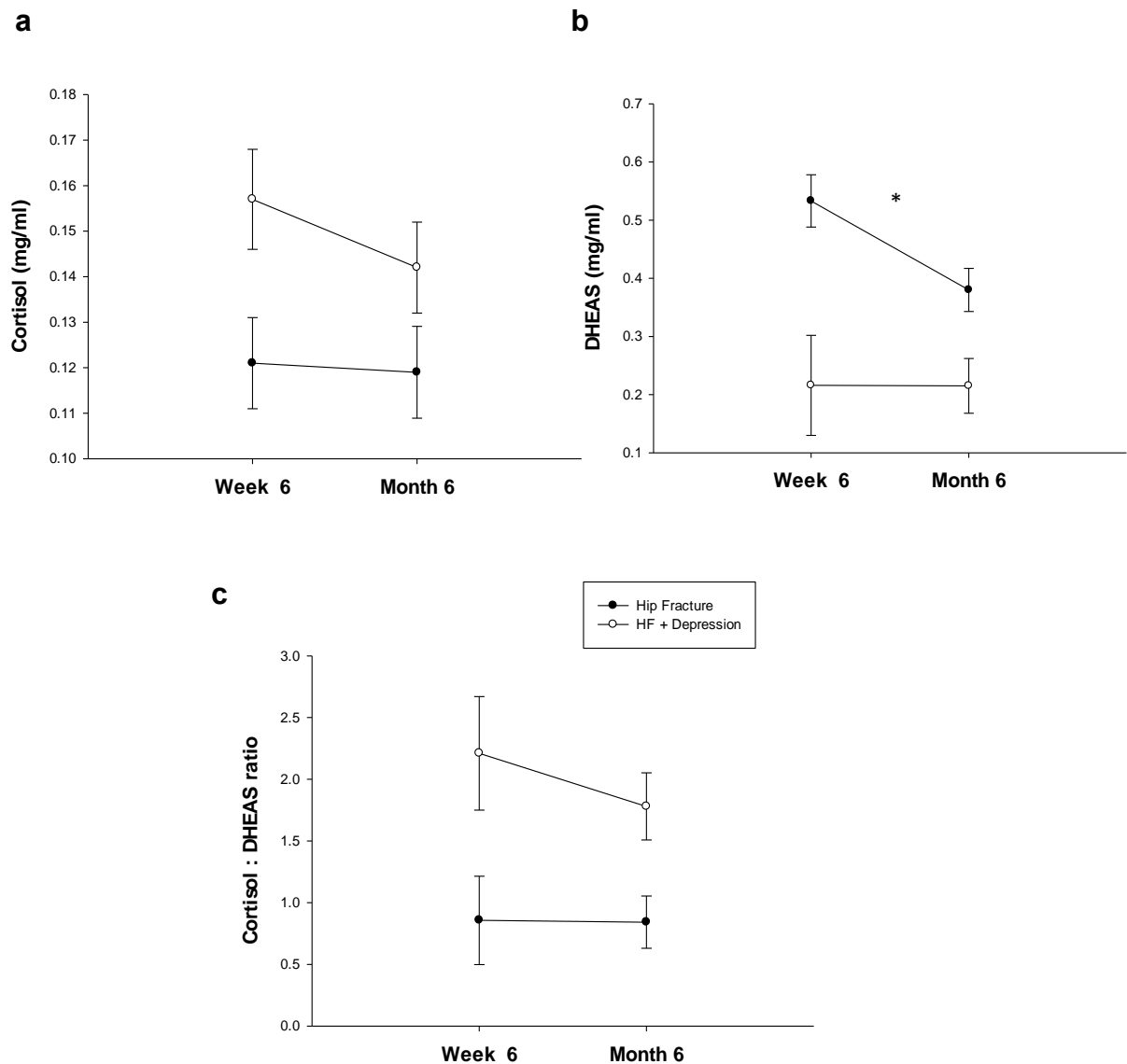
Figure 3.17 Adrenal hormones in hip fracture patients six months post-surgery.

Serum (a) cortisol, (b) DHEAS or (c) the cortisol:DHEAS ratio in hip fracture patients without depressive symptoms (HF; n = 33) and hip fracture patients with depressive symptoms (HF+D; n = 20) at 6 weeks and 6 months post-surgery. Data are mean ± SEM. * p <.05.

3.3.6 *In-vitro* treatment of NK cells with dexamethasone

Glucocorticoids are known to exert a suppressive effect on NK cell killing [501, 503]. As elevated serum cortisol level in hip fracture patients with depressive symptoms was found here [Figure 3.15a] this might explain the reduced NK cell activity seen in the patients with depressive symptoms. In order to test this hypothesis, NK cells isolated from healthy young individuals were treated with dexamethasone for 18 hr prior to testing their cytotoxic potential. Overnight incubation of NK cells with dexamethasone resulted in a dose dependent reduction in NK cell mediated lysis of target cells [Figure 3.18]. 10^{-7}M dexamethasone is close to the equivalent physiological serum cortisol levels observed in hip fracture patients with depressive symptoms and has been referred to as a physiologically relevant concentration in other studies [501, 524]. This data supports the hypothesis that the elevated cortisol levels seen in the hip fracture patients with new onset depressive symptoms might be one of the factors contributing towards suppressed NK cell cytotoxicity.

To exclude the possibility that suppressed NK cell cytotoxicity reported upon treatment with dexamethasone was due to an increase in NK cell apoptosis, NK cells treated overnight with dexamethasone (10^{-7}M) were immunostained with Annexin V to detect apoptosis and sytox green for necrosis. No significant differences were observed in the percentage of Annexin V positive [p = .77; Figure 3.19a] or Sytox positive [p = .57; Figure 3.19b] NK cells between untreated and 10^{-7}M dexamethasone treated NK cells. Thus, dexamethasone treatment does not affect NK cell viability.

The first step of NK cell mediated target cell lysis involves conjugate formation between NK cells and target cells [129]. No significant differences were observed in the ability of NK cells to form conjugates in response to treatment with a range of dexamethasone concentrations [Figure 3.20], which contradicts previous findings in an NK cell line [501].
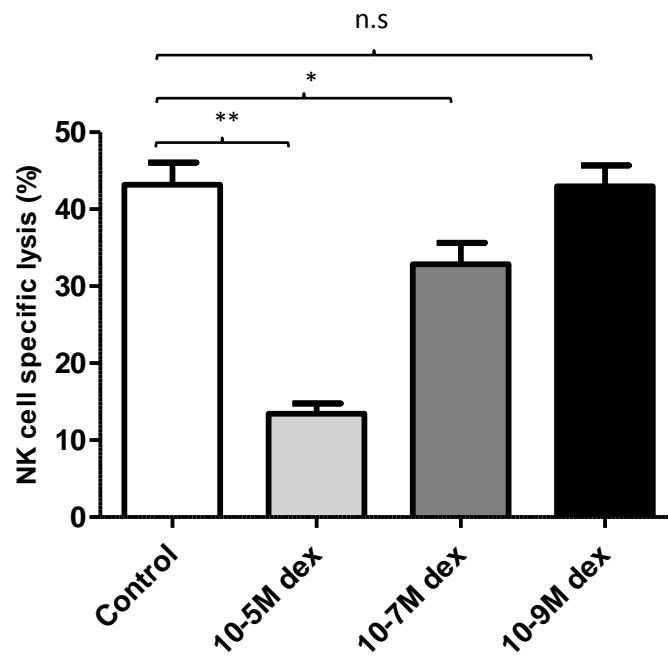
Figure 3.18 NK cell cytotoxicity with dexamethasone treatment.

NK cell cytotoxicity in cells from healthy young donors (n = 9) treated with dexamethasone (10^{-5}M, 10^{-7}M and 10^{-9}M) for 18 hr. Data are mean ± SEM. * indicates p < .05, ** p < .005.
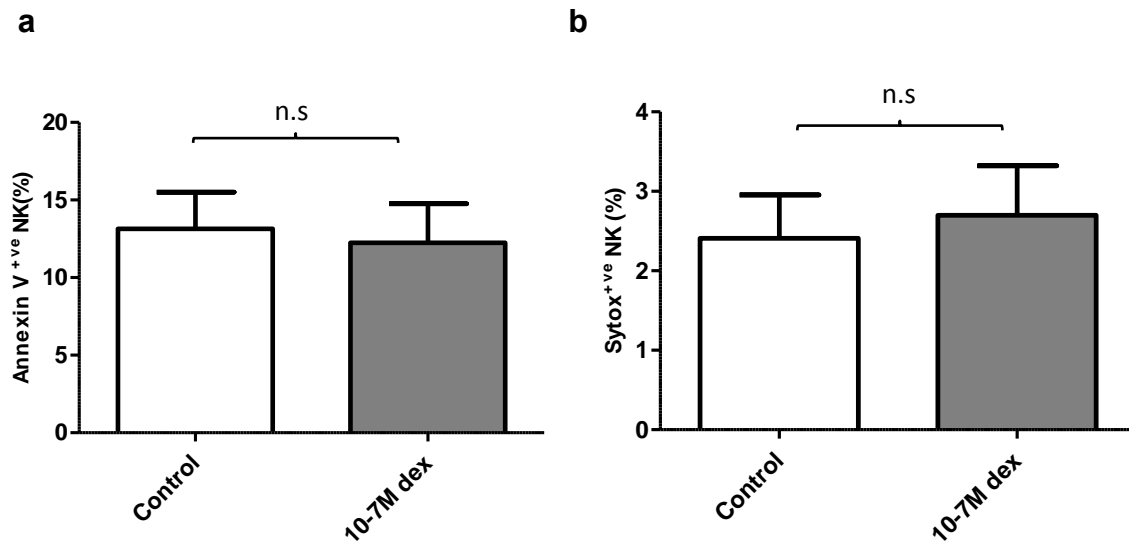
Figure 3.19 NK cell apoptosis and necrosis after dexamethasone treatment.

Frequency of (a) Annexin V[+ve] NK cells or (b) Sytox[+ve] NK cells after treatment with buffer alone (control) or 10^{-7}M dexamethasone (dex) in NK cells isolated from healthy young donors (n = 5). Data is expressed as mean ± SEM.
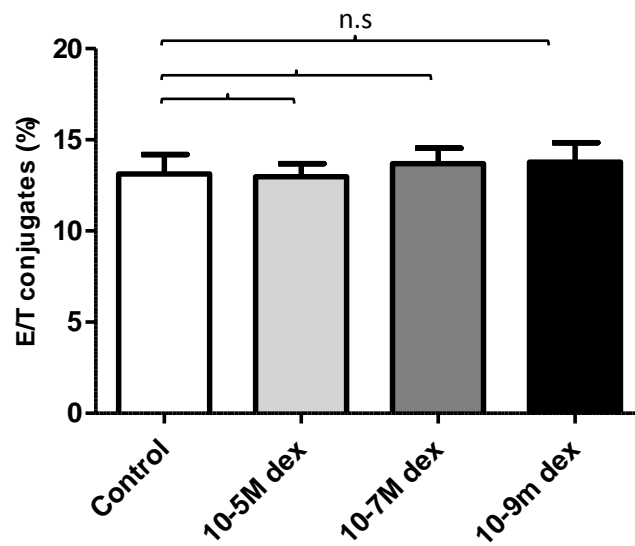
Figure 3.20 NK-target cell conjugate formation with dexamethasone treatment.

Percentage of effector-target cell conjugates formed by NK cells from healthy young donors (n = 9) upon treatment with dexamethasone (10^{-5}M, 10^{-7}M and 10^{-9}M) for 18 hr. Data are mean ± SEM.

Examination of NK cell activation after co-culture with K562 cells by staining NK cells for CD69 expression [537, 538] showed no significant effect of treatment with 10^{-7}M dexamethasone, p = .72 [Figure 3.21].

NK-target cell conjugate formation is followed by migration of cytolytic granules towards the immunological synapse to induce target cell death[131]. Cytolytic granule membranes are lined by highly glycosylated membrane proteins, such as lysosomal associated membrane protein1 (LAMP1 or CD107a). NK cell conjugation with tumour cells results in degranulation and CD107a appearance on the cell surface of NK cells followed by exocytosis of cytotoxic proteins (perforin and granzyme) [539]. On measuring the percentage of NK cells expressing CD107a post-dexamethasone treatment, no significant differences were observed CD107a surface expression [Figure 3.22] in agreement with a previous report [501].

Finally, the effect of dexamethasone on perforin and granzyme B expression by NK cells was examined. On measuring the frequency of perforin^{+ve} NK cells post dexamethasone treatment, no significant differences were observed in response to treatment with dexamethasone [Figure 3.23a], though perforin expression levels on a per cell basis were reduced with 10^{-5}M , p = .001 and 10^{-7}M dexamethasone , p = .02 compared to untreated NK cells (control) [Figure 3.23b]. In contrast, no differences were found in the frequency of granzyme B $^{+ve}$ NK cells [Figure 3.23c] or granzyme B expression levels per NK cell after dexamethasone treatment [Figure 3.23d].
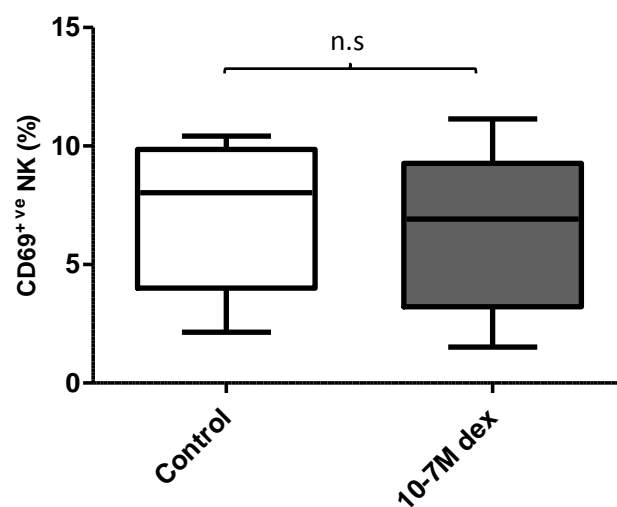
Figure 3.21 CD69 expression upon dexamethasone treatment of NK cells

Frequency of CD69^{+ve} untreated (control) and dexamethasone treated (10^{-7}M dex) NK cells post-stimulation with K562 target cells for 2 hr using NK cells isolated from healthy young donors (n = 5). Data is expressed as mean ± SEM.
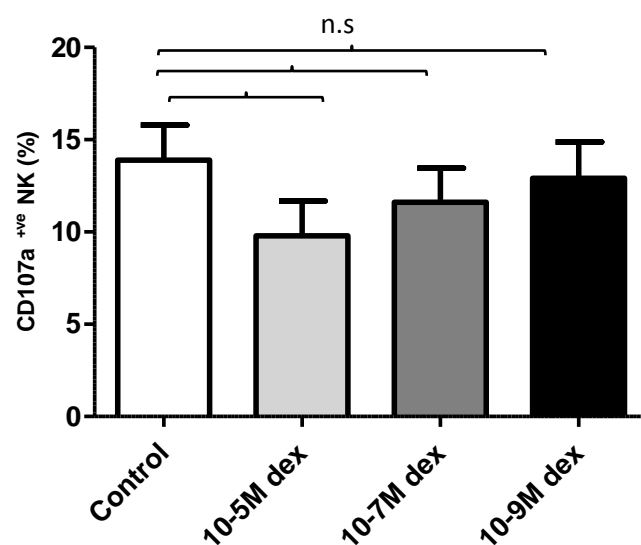
Figure 3.22 CD107a expressions by NK cells post dexamethasone treatment.

Frequency of CD107a^{+ve} untreated (control) and dexamethasone treated NK cells (10^{-5}M dex, 10^{-7}M dex and 10^{-9}M dex) post-stimulation with K562 target cells for 2 hr in NK cells from healthy young donors (n = 7). Data is expressed as mean ± SEM.
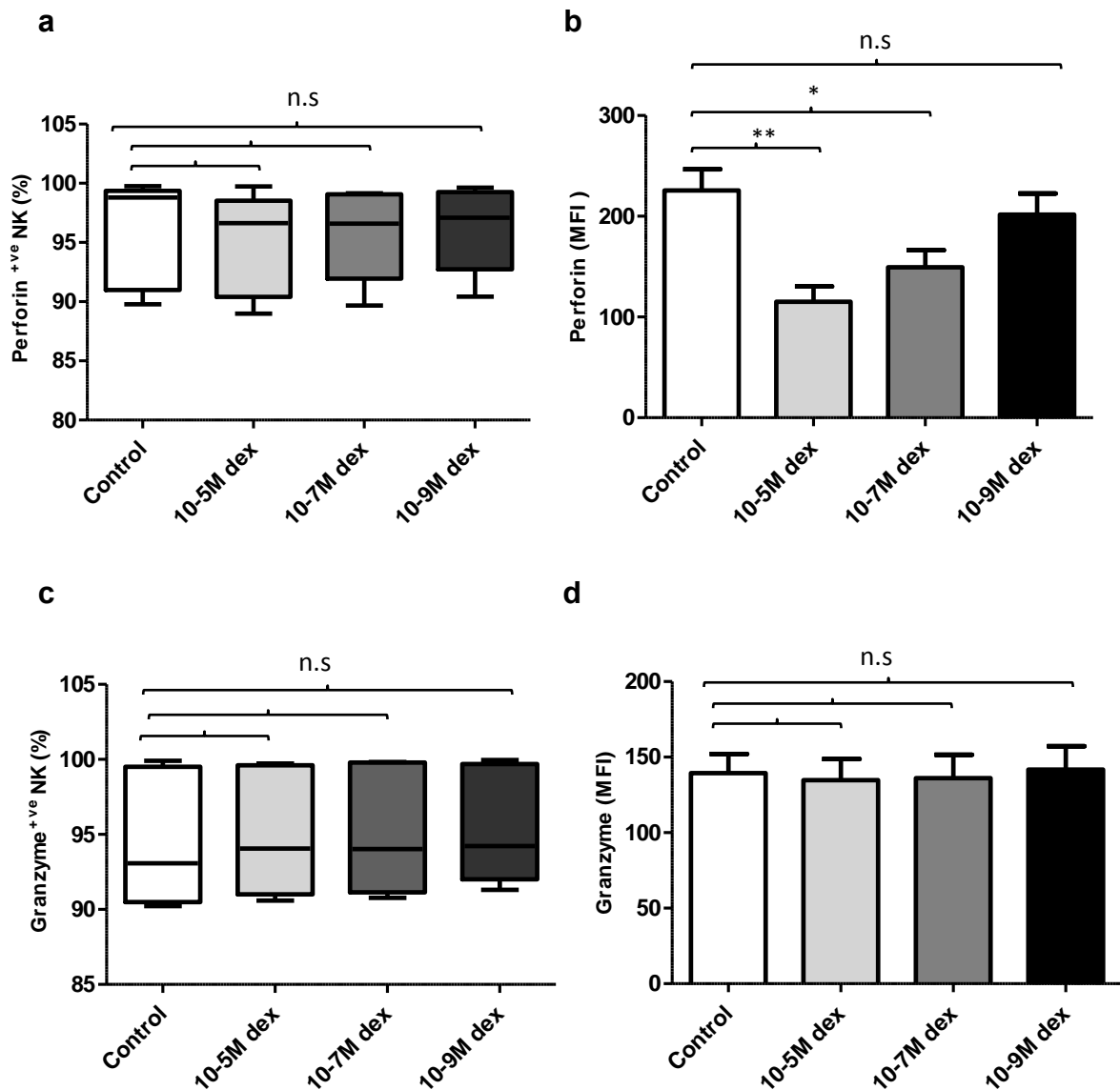
Figure 3.23 Perforin and granzyme B expression by NK cells with dexamethasone treatment.

Percentage of (a) Perforin[+ve] or (b) granzyme B[+ve] and (c) perforin or (d) granzyme B expression levels in untreated (control) and dexamethasone treated NK cells (10^{-5}M dex, 10^{-7}M dex and 10^{-9}M dex) for NK cells from healthy young donors .Data is expressed as mean ± SEM. * p<.05 ** p < .005.

3.3.7 Serum cytokine levels in hip fracture patients

In this chapter, serum levels of nine different cytokines were also measured in our hip fracture patients. Significant differences were seen in serum IL6 levels between the three groups, $F (2, 101) = 10.25$, $p < .001$, $\eta^2 = .16$, such that a significant increase in serum IL6 levels were detected in depressed hip fracture patients compared with healthy controls, $p < .001$ as well as non-depressed hip fracture patients , $p = .002$ [Table 3.13]. In addition, a significant association was found between GDS scores and serum IL6 levels in hip fracture patients, $\beta = .29$, $p = .02$, $\Delta R^2 = .08$, such that those with greater depressive symptoms had higher circulating IL6 levels (data not shown).

Serum TNFα levels also showed significant differences between our three groups, $F (2, 62) = 9.52$, $p < .001$, $\eta^2 = .23$, such that a significant increase was observed in serum TNFα levels in hip fracture patients with depressive symptoms compared with healthy controls, $p < .001$ and non-depressed hip fracture patients, $p = .02$. [Table 3.13]. However, we did not observe a significant association between GDS scores and TNFα levels in hip fracture patients, $\beta = .18$, $p = .23$, $\Delta R^2 = .01$ (data not shown).

Similarly, significant differences were also found in the serum levels of the anti-inflammatory cytokine IL10 between our three groups, $F (2, 66) = 7.25$, $p = .001$, $\eta^2 = .18$, such that a significant increase in serum IL10 levels was seen in hip fracture patients with depressive symptoms compared with healthy controls, $p = .001$ [Table 3.13]. No significant differences were observed in serum IL10 levels between hip fracture patients with and without depressive symptoms, $p = .21$. There was no association between GDS scores and circulating IL10 levels in hip fracture patients, $\beta = .23$, $p = .21$, $\Delta R^2 = .02$ (data not shown).

When the above analyses were repeated with adjustment for age, sex and BMI, the differences in IL6, TNFα and IL10 levels between the three groups still remained significant (data not shown).

Finally , no significant differences were found in serum IL8 levels, $F_{(2, 93)} = 2.57$, $p = .08$, $\eta^2 = .05$; IL1β levels, $F_{(2, 58)} = 2.04$, $p = .13$, $\eta^2 = .06$; IL17 levels, $F_{(2, 63)} = .36$, $p = .69$, $\eta^2 = .01$; GM-CSF levels, $F_{(2, 60)} = 2.38$, $p = .10$, $\eta^2 = .07$; IL4 levels, $F_{(2, 89)} = .821$, $p = .44$, $\eta^2 = .01$, or in serum IL13 levels $F_{(2, 63)} = 1.85$, $p = .16$, $\eta^2 = .05$ between our three subject groups [Table 3.13].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
		Mean (SD)		
IL-6 (pg/ml)	6.25 (6.40)	7.31 (5.15)	13.79 (9.75)	**<.00**
TNFα (pg/ml)	4.28 (4.58)	9.96 (6.06)	19.25 (19.52)	**<.00**
IL-10(pg/ml)	5.27 (5.34)	10.37 (10.15)	22.41 (28.91)	**.001**
IL-1β (pg/ml)	18.41 (13.77)	12.84 (7.92)	26.29 (29.62)	.13
IL-8 (pg/ml)	25.95 (17.20)	19.09 (14.47)	18.82 (10.91)	.08
GM-CSF(pg/ml)	86.67 (79.27)	39.13 (19.48)	64.85 (79.37)	.10
IL-17(pg/ml)	.82 (.57)	1.48 (4.71)	.79 (.41)	.69
IL-4(pg/ml)	2.37 (2.21)	1.45 (3.60)	3.37 (9.64)	.44
IL-13 (pg/ml)	16.48 (2.67)	7.47 (4.02)	15.84 (3.77)	.16

Table 3.13. Serum cytokine levels in hip fracture patients.

3.3.8 Cortisol: DHEAS ratio and innate immune parameters

An elevated serum cortisol: DHEAS ratio acts as a marker for depressive symptoms [536]. In this chapter, a significant association was found between depressive symptoms (GDS scores) and the serum cortisol: DHEAS ratio. However, there was no association between serum cortisol: DHEAS ratio and neutrophil superoxide production, $\beta = -.05$, $p = .39$, $\Delta R^2 = .003$, or monocyte superoxide production, $\beta = -.12$, $p = .29$, $\Delta R^2 = .01$ in the hip fracture patients (data not shown).

Similarly, no significant association was found between serum cortisol: DHEAS ratio and NK cell cytotoxicity in the hip fracture patients, $\beta = -.11$, $p = .24$, $\Delta R^2 = .01$ (data not shown). Further, no significant association was observed between serum cortisol: DHEAS ratio and serum cytokines levels; including IL6 $\beta = .10$, $p = .41$, $\Delta R^2 = .01$ or TNFα levels $\beta = .16$, $p = .32$, $\Delta R^2 = .02$ or IL10 levels $\beta = -.19$, $p = .33$, $\Delta R^2 = .03$ (data not shown).

3.4 Discussion

Mood disorders, especially depression, have been well studied in older patients with hip fracture [433, 434]. About one third of our hip fracture patients had significant depressive symptoms post-surgery. The data shown here reveal for the first time that what we presume to be new onset depressive symptoms post hip fracture (participants were exluded if they had previous depression or treatment for it) are associated with physical frailty, innate immune dysregulation, a higher serum cortisol: DHEAS ratio and elevated serum levels of pro-inflammatory cytokines.

Firstly, the data indicated that depressive symptoms post hip fracture were associated with poor physical functioning including; impaired ability to perform activities of daily living and reduced walking speed [526]. These findings are in line with previous studies showing a considerable negative impact of depressive symptoms on quality of life [540] and physical functioning post hip fracture [437, 541]. Also, this impairment in functional ability persisted up to six months post fracture, suggesting that the presence of depressive symptoms post hip fracture surgery is a major contribution to disability in this population. However, the development of depressive symptoms post fracture did not have an effect on cognitive impairment. These findings might be due to exclusion of individuals with dementia in our study which was not an exclusion criterion for the other study.

Next, on examining the additive effect of physical and psychological distress on neutrophil functioning, it was found that neutrophil phagocytosis remained unaltered. These findings are consistent with a previous study from our group showing that hip fracture did not have an effect on neutrophil phagocytic ability [359]. However, we did find that neutrophil superoxide production was impaired in hip fracture patients that developed depressive

symptoms[522]. In the previous study from our group ,we reported suppression in neutrophil superoxide production in hip fracture patients compared to healthy controls [359], however these patients were not screened for depressive symptoms and it is possible that only patients that developed depressive symptoms had supressed neutrophil function in the previous study as well. Our findings are supported by another study which showed that depression has a suppressive effect on neutrophil superoxide production [542]. Emotional stressors, such as bereavement in older adults have also been associated with impaired neutrophil superoxide generation, and again neutrophil phagocytic ability remained unaltered [360]. Interestingly, it has also been shown that older adults with fewer stress coping factors, such as hobbies and close links with friends and family, have lower neutrophil superoxide production compared to those with greater numbers of these factors[543].

Assembly and activation of the NADPH oxidase complex involving association of cytosolic components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ with membrane bound $gp91^{phox}$ and $p22^{phox}$, catalyses electron transfer from NADPH to oxygen resulting in superoxide generation [544]. A study examining the effect of academic psychological stress on neutrophil functioning reported that decreased superoxide production was accompanied by diminished expression of subunit $p47^{phox}$ of NADPH complex [545]. Although not examined in this thesis, there is a possibility that diminished expression of $p47^{phox}$ on neutrophils of hip fracture patients with depressive symptoms might be a contributing factor towards their reduced ability of superoxide generation.

On examining the effect of physical and psychological distress on monocytes ,no significant differences were observed in peripheral monocyte count between hip fracture patients with or without depressive symptoms, which is in line with previous findings of unaltered numbers of circulating monocytes in depressed individuals [487]. Further, the presence of

depressive symptoms in hip fracture patients did not have an effect on the peripheral distribution of monocyte subsets, which is also in line with a previous study in depressed patients [487]. Similar to our findings in neutrophils, a suppression of superoxide production by monocytes was seen in hip fracture patients with depressive symptoms. However, another study reported an increase in superoxide generation by monocytes in depressed individuals, but in that study only ten individuals with major depression were assessed and these were all young individuals [487]. Interestingly, the effect of depressive symptoms on monocyte function in hip fracture patients was not universal and there was no effect on phagocytic ability, TNFα secretion or expression of co-stimulatory and antigen presenting molecules. These findings are consistent with a previous study that reported intact monocyte cytokine production upon stimulation with bacterial components and expression of MHC-II molecule human leukocyte antigen DR (HLA-DR) in young patients with depressive symptoms [546].

Neutrophils and monocytes play a prime role in defence against bacterial infections, thus it is tempting to hypothesise that this reduction in neutrophil and monocyte bactericidal properties might be of clinical significance and increase susceptibility of infections in hip fracture patients with depressive symptoms, resulting in ill-health and increased risk of mortality. A high incidence of urinary tract infection (33%) and chest infections (9.2 %) were found in hip fracture patients over the 6 month follow up period, which is in line with previous reports [359, 547]. Although, hip fracture patients with and without depressive symptoms did not differ in the number of infections they developed during their hospital stay or during the six month follow up period, the study may not have had sufficient power to detect such changes. Further, excess mortality has been reported in depressed individuals [548], especially in depressed patients post hip fracture [435]. In this study, a trend towards a

higher death rate in hip fracture patients with depressive symptoms was seen, but this did not reach statistical significance.

Depressive symptoms post hip fracture were also associated with suppressed NK cell activity, in line with numerous studies reporting a suppressive effect of severe life stressors such as caregiving [549] , bereavement [550-552] , occupational stress [491, 553] and depression [492, 554-556] on NK cell function. Interestingly, an association between depressive symptoms scores and NKCC in hip fracture patients was also seen, which has been previously reported in people undergoing severe life stressors, such as bereavement [557]. Even though a number of studies have reported an impaired NK cell activity on chronic exposure to psychological distress, none of these studies have attempted to explain the reason behind this impaired killing by NK cells. In this chapter, it was shown that the suppression in NKCC of hip fracture patients with depressive symptoms was not a result of altered numbers of circulating NK cells [554, 558] or changes in the CD56^{dim} /CD56^{bright} NK cell distribution. Instead, a significant decline in perforin content on a per cell basis was detected in hip fracture patients with depressive symptoms. This reduction in perforin expression might result in reduced granzyme delivery to target cells which in turn reduces target cell killing, suggesting one possible mechanism contributing towards impaired NK cell activity Studies in perforin-deficient mice have reported an increased susceptibility towards tumours [559] and inefficient control of viral challenges [560] in these mice due to impairments in target cell lysis by NK cells [561]. Interestingly, another study examining the effect of psychotherapy, reported a reduction in depression scores and also an improvement in NK cell activity by increasing NK cell perforin expression [562].

Hyperactivity of the HPA axis resulting in elevated basal cortisol levels is one of the most consistent findings in depressed patients [563, 564] . In this chapter, it was shown that hip

fracture patients with depressive symptoms had elevated serum cortisol levels compared to hip fracture patients without depressive symptoms and healthy controls [522]. Reduced feedback inhibition by glucocorticoids due to impaired functioning of the glucocorticoid receptor has been suggested to be responsible for the HPA axis hyperactivity in depressed individuals [565, 566]. Although not assessed in this thesis, previous studies have shown a reduction in the GR expression in depressed patients [567, 568]; it is possible that reduced GR expression on immune cells in hip fracture patients with depressive symptoms might contribute towards elevated cortisol levels in these patients. Additionally, a decline in DHEAS levels also occurred in hip fracture patients with depressive symptoms, which is consistent with previous findings of reduced DHEAS levels in depressed individuals [364, 569, 570]. However, there has been a contradictory report showing an increase in DHEA levels in depressed patients [571]. Overall, an elevated serum cortisol: DHEAS ratio occurred in hip fracture patients with depressive symptoms [522], which is also in line with previous findings for patients with depression [536].

The elevated cortisol: DHEAS ratio might be one of the mechanisms responsible for reduced superoxide production by innate immune cells in hip fracture patients with depressive symptoms. *In-vitro* studies have reported a suppressive effect of glucocorticoids on bactericidal properties, especially superoxide generation [498, 572] in neutrophils and monocytes [498, 573]. Interestingly, a study examining the effect of dexamethasone on the human monocytic THP-1 cell line reported a down regulation of NADPH oxidase activity due to inhibition of expression of p47^{phox} genes [574]. In contrast, DHEAS has been shown to enhance superoxide generation in *in-vitro* assays [575]. Although it is possible that an elevated serum cortisol:DHEAS ratio might have a direct inhibitory effect on expression of p47^{phox} genes in neutrophils and monocytes, statistical analysis using the Sobel test (kindly performed by Dr Anna Phillips) suggested that the elevated serum cortisol:DHEAS was

related to depressive symptoms but did not mediate the decrease in superoxide production by innate immune cells [522]. Further research is required to confirm this conclusion and to determine how depressive symptoms might mediate suppression of superoxide generation.

Similarly, a suppressive effect of dexamethasone (a synthetic glucocorticoid) has been reported on the cytotoxic activity of an NK cell line [501, 524, 576] and peripheral blood leukocytes enriched for NK cells [503]. In this chapter, these findings were confirmed using isolated NK cells from young donors. However, in future studies it might be interesting to examine whether a more pronounced suppressive effect of dexamethasone occurs on NK cell activity in healthy older adults. *In-vitro* incubation of NK cells with dexamethasone resulted in a reduction in perforin content per NK cell, which is in line with previous findings [524], suggesting that reduced perforin expression on NK cells on exposure to glucocorticoids might contribute towards reduced NK cell cytotoxicity seen in stressed adults. Mifepristone (a glucocorticoid antagonist) is capable of augmenting NK cell cytotoxicity via ERK-mediated increase in perforin expression, which can be blocked by cortisol [577]. Taken together with our data, it is reasonable to propose that prolonged NK cell exposure to cortisol, as occurred in hip fracture patients with depressive symptoms, reduces their expression of perforin and compromises NKCC. Although not explored in this thesis, glucocorticoids are capable of inducing epigenetic modifications and regulate gene transcription of immune responsive genes [578]. Glucocorticoids induce reduced acetylation at histone 4 lysine 8 (H4-K8), resulting in reduced promoter accessibility which has a suppressive effect on gene transcription on NK cell activity regulating genes, such as perforin, resulting in reduced NK cell functional activity [501]. Further, *in-vitro* studies have also shown that DHEA increases NK cell cytotoxicity [579], raising the possibility that elevated cortisol: DHEAS might be one of the mechanisms responsible for reduced NK cell

cytotoxicity in hip fracture patients with depressive symptoms. However, once again Sobel analysis suggested that the elevated serum cortisol: DHEAS was related to depressive symptoms, but did not mediate the decrease in NK cell activity in hip fracture patients with depressive symptoms.

In addition to the HPA axis, depression has also been associated with sympathetic nervous system activation, resulting in release of neuropeptides such as norepinephrine [580-582]. Norepinephrine (noradrenalin) mediates its effects via adrenergic receptors, which are expressed in high levels on NK cells and has a suppressive effect on NK cell activity [554, 583, 584] by reducing levels of perforin and granzyme B [585]. Future research could investigate this possibility.

Depression has been characterised by some as an inflammatory disorder accompanied by elevated levels of pro-inflammatory cytokines, including IL6, TNFα and IL1β [509, 586, 587]. In this chapter, elevated levels of TNFα and IL6 were seen in hip fracture patients with depressive symptoms compared with patients without depressive symptoms and healthy controls [522]. This is consistent with the findings of a previous study in which older hip fracture patients with high levels of pro-inflammatory cytokines such as IL6 also had higher GDS scores [588]. Epidemiological studies have also reported an association between IL6 and depressive symptoms in older people [589, 590]. Further, studies in animal models have shown that administration of pro-inflammatory cytokines is known to induce 'sickness behaviour' resulting in behavioural changes including ; sleep disturbance, loss of appetite, memory impairment and loss of interest in social activities that overlap with many of the symptoms of depression in humans [591, 592]. This 'sickness behaviour can be reversed on withdrawal of cytokines [593]. A few studies conducted in older adults have suggested that inflammation might play a potential causal role in the development of depressive symptoms [594, 595].

Interestingly, IL6 knockout mice are resistant to development of stress-associated depression-like behaviour [596], suggesting that elevated levels of pro-inflammatory cytokines in certain hip fracture patients might increase the risk of development of depressive symptoms.

As discussed previously, depressive symptoms is associated with HPA axis hyperactivity, resulting in elevated cortisol levels. Interestingly, there is evidence to suggest that pro-inflammatory cytokines (IL6, TNFα, and IL1β) have neuromodulatory properties and can result in activation of the HPA axis [597-599]. Glucocorticoid resistance has been suggested as a potential mechanism relevant to HPA axis activation induced by pro-inflammatory cytokines [510, 600]. In the present study, depressive symptom scores significantly correlated with IL6 levels, but there was no association between serum cortisol levels and IL6.

Anti-inflammatory cytokines, such as IL10 are responsible for reducing production of pro-inflammatory cytokines and dampening immune responses [312]. In this thesis, in addition to an elevation in levels of pro-inflammatory cytokines, an elevated serum IL10 was seen in hip fracture patients with depressive symptoms [522]. A similar elevation of IL10 levels has been previously reported in depressed individuals [601, 602] and in animal models of depressive behaviour [603] which might be a counter regulatory mechanism to reduce inflammatory responses and restore homeostasis [601].

Chronic stress induced adverse health consequences and immune dysregulation may persist even after chronic stress has abated [357, 604, 605]. This lasting, detrimental effect of stress might reflect a premature ageing of the immune system associated with chronic stress [606]. Interestingly, studies done on long term follow up have reported that the mortality disadvantage in hip fracture patients can persist for up to five years after the fracture [607,

[608]. Recovery after hip fracture is a long process and only one-third hip fracture patients return to their pre-fracture functional status one year post-surgery [422]. In this chapter, it was observed that neutrophil superoxide generation in hip fracture patients with depressive symptoms had improved after 6 months, but monocyte superoxide generation and NK cell cytotoxicity remained impaired in the depressed hip fracture patients 6 months post-surgery. Additionally, perforin expression on NK cells in hip fracture patients with depressive symptoms did not improve even 6 months post-surgery and might contribute towards suppressed NK cell function in these patients 6 month post-surgery. Elevated serum cortisol: DHEAS ratio was also observed in the hip fracture patients with depressive symptoms at the six month time point.

In summary, this chapter shows for the first time that depressive symptoms in hip fracture patients is characterised by suppression of neutrophil and monocyte superoxide generation, reduced NK cell cytotoxicity, elevated pro-inflammatory cytokine levels and an increased serum cortisol:DHEAS ratio. These effects were not seen in patients with hip fracture alone. Further, this suppressed monocyte and NK cell functioning observed in hip fracture patients with depressive symptoms persisted six months after surgery and did not improve with time. Taken together the data suggest that development of depressive symptoms after a hip fracture is the main driver of immune dysregulation in these patients post-surgery.

Chapter 4

The synergistic effects of physical and psychological distress on adaptive immune functioning

4.1 Introduction

4.1.1 Background

Ageing is accompanied by a range of alterations in the adaptive immune system resulting in reduced adaptive function such as vaccination responses (reviewed in chapter 1). Similarly, chronic stress has also been associated with remodelling of the immune system and reduced immunity similar to that seen in ageing including; reduced levels of lymphocytes [609, 610], altered ratio of helper to suppressor T cells [611], accumulation of senescent T cells [612, 613], reduced telomere length and greater telomerase activity [614-616], impairment in proliferation responses to mitogens [494, 617] and a shift in CD4 helper response from a Th1 to Th2 phenotype [618, 619]. Although data regarding the effect of chronic stress on Th17 cells is limited a recent study reported an increase in inflammatory Th17 cells in mice with depression like behaviour [620]. Further, the decrement in functioning of the thymus that occurs with normal ageing has also been reported to occur during periods of psychological stress [621, 622].

Exposure to chronic stress is accompanied by HPA axis hyperactivity [363, 623]. In the previous chapter, it was reported that an elevated serum cortisol: DHEAS ratio occurs in hip fracture patients with depressive symptoms. Importantly, a range of immune modulating effects of cortisol on T lymphocytes have been previously reported including; increased T cell apoptosis [624], reduced telomerase activity [625], a shift towards Th2 lineage commitment [624, 626] and dysregulated cytokine production [627, 628]. The literature regarding the effect of chronic stress on B cells is limited. A few studies have reported that circulating numbers of B cells remain unaltered on exposure to chronic stress [629]. Moreover, no study has examined the effect of glucocorticoids on B cell function or phenotype.

Regulatory immune cells play an essential role in maintaining immune homeostasis. An age associated increase in T_{reg} frequency and a decline in suppressive capacity of regulatory T cells have been previously discussed (reviewed in chapter 1). Additionally, chronic stressors have been associated with numerical and functional alterations in $CD4^{+ve}CD25^{+ve}Foxp3^{+ve}$ T_{regs} [630, 631]. Further, a novel subset of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells with immunosuppressive properties has been recently identified in humans [301]. In this thesis, it was shown for the first time that the frequency of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells and their ability to produce IL10 post-stimulation declines with age (described in chapter 2) and that this correlated with an age-related increase in serum autoantibody (RF) in healthy older adults [441]. However, the effect of chronic stress on this novel subset of immunoregulatory B cells remains unexplored.

4.1.2 Aims

In the previous chapter the effects of development of depressive symptoms post hip fracture on phenotype and functional capacity of innate immune cells was determined. Additionally, HPA axis abnormalities and elevated pro-inflammatory cytokine levels were reported in hip fracture patients with depressive symptoms. In this chapter, the aim was to examine if the psychological distress of depressive symptoms could act additively with the physical stress of hip fracture to amplify the effect of ageing upon adaptive immunity and determine possible mediators of the additive effects.

(a) To examine the effect of physical and psychological distress on total peripheral T cell count, CD4:CD8 ratio, naïve: memory T cell ratio and frequency of senescent and activated T cells.

(b) Determine if emergence of depressive symptoms in hip fracture patients exerts an effect on frequency of regulatory immune cells, especially CD4^{+ve}CD25^{+ve} Foxp3^{+ve} (T$_{regs}$) and CD19^{+ve}CD24^{hi}CD38^{hi} (B$_{regs}$) and their IL10 production capacity.

(c) Test for role of cortisol: DHEAS ratio and the balance of pro- and anti-inflammatory cytokines as potential mediators of any additive effects observed.

(d) To test the longevity of alterations in immune status in hip fracture patients by repeating tests in which differences were observed at the six month time point.

4.2. Materials and Methods

4.2.1 Solutions and Antibodies

Freezing Medium: 10% DMSO in HiFCS

Antibody	Dilution	Company
Anti-human CD3 PEcy7 (clone : UCHT1)	1/100	eBiosciences
Anti-human CD3 APCcy7 (clone : HIT3a)	1/100	Biolegend
Anti-human CD4 Alexa fluor 450 (clone : RPA-T4)	1/100	eBiosciences
Anti-human CD8 PE (clone : UCHT4)	1/100	Immunotools
Anti-human CCR7 FITC (clone : 150503)	1/20	R and D systems
Anti-human CD45RA APC (clone : HI-100)	1/500	Biolegend
Anti-human CD25 APC (clone : BC-96)	1/20	Biolegend
Anti-human CD28 APC (clone : CD28.2)	1/200	BD biosciences
Anti-human CD57 FITC (clone : HCD57)	1/50	eBiosciences
Anti-human foxp3 PE	1/10	Biolegend

Table 4.1 List of primary antibodies used for flow cytometry.

4.2.2 Flow cytometric staining of T cells

Frozen PBMCs were thawed and washed in RPMI (described in section 2.2.4) and the

pelleted cells were re-suspended in PBS (1 x 10^6 cells/ml) and 100 μl of cells in PBS were

incubated for 20 minutes in the dark at 4°C with combinations of antibodies including; anti-

human CD3-PEcy7 (eBiosciences, UK), anti-human CD4 Alexa fluor 450 (eBiosciences,

UK), anti-human CD8 PE (Immunotools, UK), anti-human CCR7 FITC (R and D systems,

UK) and anti-human CD45RA APC (Biolegend, UK). Post incubation, cells were washed

twice with 300 μl of PBS and centrifuged at 250 x g for 5 minutes. Post washing, pelleted

cells were resuspended in 300 μl of PBS for flow cytometric analysis using a Cyan ^{TM} ADP

(Dako Ltd, UK). T cells can be classified into four distinct subsets on the basis of expression

of cell surface markers, CCR7 and CD45RA; naïve (CD45RA^{+ve} CCR7^{+ve}), central memory (CD45RA^{-ve} CCR7^{+ve}), effector memory (CD45RA^{-ve} CCR7^{-ve}) and effector memory RA (CD45RA^{-ve} CCR7^{-ve}) [Figure 4.1] [183, 184].

100 μl of isolated PBMCs resuspended in PBS (1 x 10^6/ml) in FACS tubes (Becton Dickinson, UK) were immunostained with a combination of fluorochrome conjugated antibodies including; anti-human CD3-PEcy7 (eBiosciences, UK), anti-human CD4 Alexa fluor 450 (eBiosciences, UK), anti-human CD8-PE (Immunotools, UK), anti-human CD28 APC (BD Biosciences, UK), anti-human CD57 FITC (eBiosciences, UK) ,anti-human NKG2D PEcy7 (eBiosciences , UK) , anti-human CD69 FITC (eBiosciences, UK), anti-human CD25 APC (Biolegend, UK) and anti-human HLADR PE (eBiosciences, UK) for 20 minutes in dark at 4°C. Post incubation, cells were washed twice with 300 μl of PBS and centrifuged at 250 x g for 5 minutes. Post washing, pelleted cells were resuspended in 300 μl of PBS for flow cytometric analysis using a Cyan ^{TM} ADP (Dako Ltd, UK). Results have been expressed both as the percentage of T cells that were CD28^{+ve}, CD28^{-ve}, CD57^{+ve} and NKG2D^{+ve} to identify senescent T cells. Additionally, the percentage of T cells that were CD69^{+ve}, CD25^{+ve} and HLADR^{+ve} were identified as activated T cells.
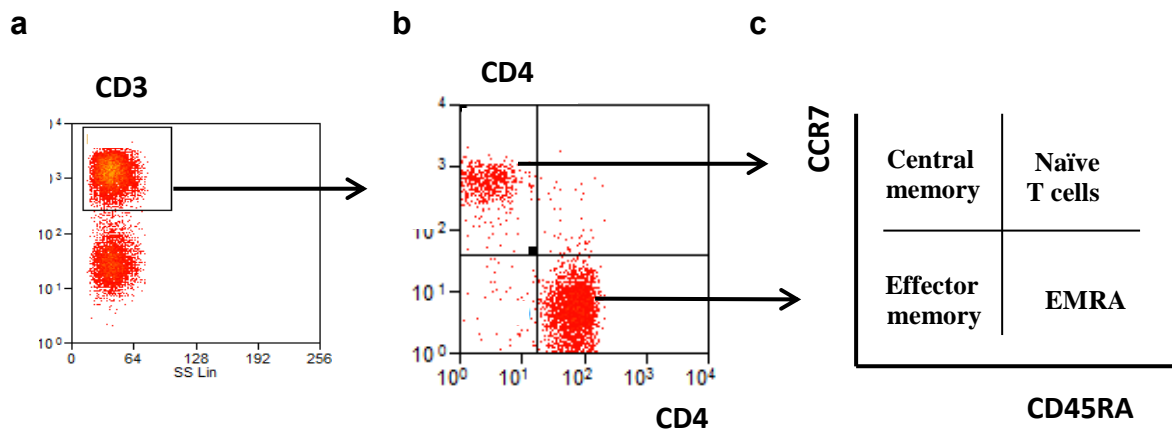
Figure 4.1 Identification of T cell subsets by immunostaining using flow cytometry.

PBMCs from a young individual were stained with anti-CD3, CD4, CD8, CD45RA and CCR7 antibodies. T cells (CD3$^+$) were gated (a) to identify CD4 and CD8 T cells (b). (c) Schematic representation of T cells subsets identified on basis of CD45RA and CCR7 expression.

4.2.3 Foxp3 staining of Regulatory T cells

To detect regulatory T cells, 100 µl of isolated PBMCs resuspended in PBS (1 x 10^6/ml) were transferred into FACS tubes (Becton Dickinson, UK) and cells were stained with anti-human CD3-PEcy7 (eBiosciences, UK), anti-human CD4 Alexa fluor 450 (eBiosciences, UK) and anti-human CD25 APC (Biolegend, UK) antibodies as described above (section 4.2.2) for 20 minutes in dark at 4°C. Post incubation, cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes. Post washing, the cells were re-suspended in 1 ml of foxp3 Fix Perm Working solution prepared by doing a 1:3 dilution of the foxp3 fix perm concentrate (eBiosciences , UK) with foxp3 diluent (eBiosciences , UK) and then left for 30 minutes in dark at room temp. Post incubation, cells were washed with 2 ml of 1x foxp3 permeabilization buffer (eBiosciences, UK) prepared by doing a 1:10 dilution with deionized water and centrifuged at 250 x g for 5 minutes. Post centrifugation, 100 µl of 1 x permeabilization buffer and anti-human foxp3 PE antibody (eBiosciences, UK) was added to the cells and left for incubation for 30 minutes in dark at room temp. Finally, the cells were washed with 2 ml of 1 x permeabilization buffer and were centrifuged at 250 x g for 5 minutes. Pelleted cells were resuspended in 300 µl of PBS for flow cytometric analysis on Cyan ^{TM} ADP (Dako Ltd , UK) and the percentage of CD3^{+ve} CD4^{+ve} CD25^{+ve} Foxp3^{+ve} T cells were recorded [Figure 4.2].

4.2.4 Stimulation of PBMCs to induce IL10 production

Human cell cultures were performed in complete RPMI1640 (Sigma Aldrich, UK) containing 10% HiFCS (Biosera, UK) supplemented with glutamine/penicillin/streptomycin (Life Technologies, UK). PBMCs were stimulated for 4 hr with PMA (50 ng/ml; Sigma Aldrich, UK) and Ionomycin (500ng/ml; Sigma Aldrich, UK) in presence of Brefeldin A (10µg/ml; Sigma Aldrich, UK). Post stimulation, cells were transferred into FACS tubes

(Becton Dickinson, UK) and centrifuged at 250 x g for 5 minutes and washed twice with 300 µl PBS. Pelleted cells were re-suspended in 100 µl PBS and stained using anti-human CD3 APCcy7 (eBiosciences, UK) and anti-human CD4 Alexa fluor 450 (eBiosciences, UK) for 20 min in the dark at 4°C. Post immunostaining, cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes. The cells were re-suspended in 50 µl Reagent A (Fix and Perm kit, Invitrogen, UK) and incubated for 30 minutes in the dark at room temperature. Post incubation, cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes. The pelleted cells were re-suspended in 50 µl Reagent B (Fix and Perm kit, Invitrogen, UK) and anti-human IL10 Alexa fluor 647 (eBiosciences , UK) was added and samples were incubated in the dark at room temperature for 30 minutes. Post incubation, the cells were washed with 300 µl PBS and centrifuged at 250 x g for 5 minutes and resuspended in 300 µl PBS for flow cytometric analysis on a Cyan ^{TM} ADP (Dako Ltd , UK). The percentage of IL10^{+ve} CD4 T cells and the IL10 expression levels (MFI) was recorded.
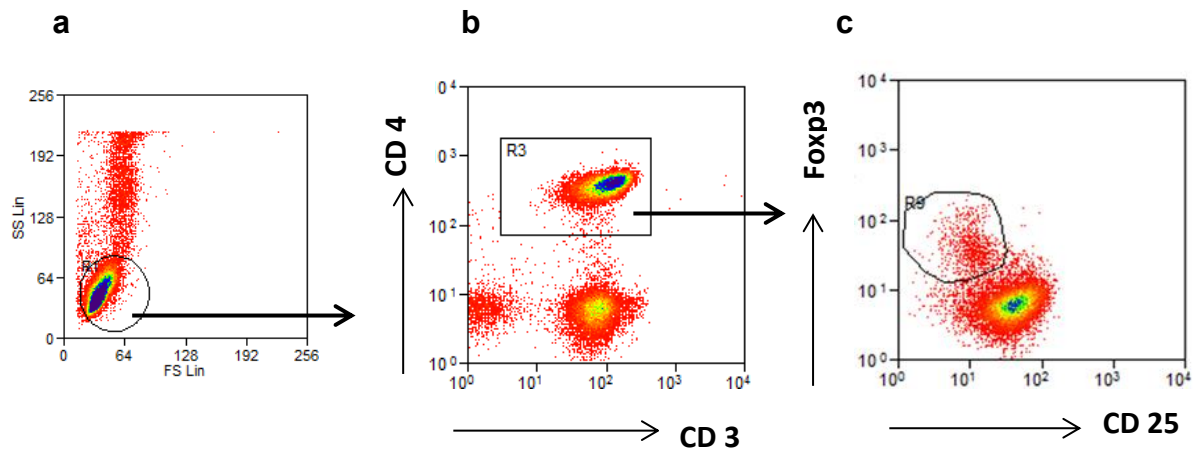
Figure 4.2 Gating strategy used to identify Regulatory T cells

PBMCs were stained for surface expression of CD3, CD4 and CD25, after which cells were fixed, followed by intracellular staining for Foxp3. (a) Firstly lymphocytes were gated on the basis of forward and side light scatter properties; (b) after gating on lymphocytes CD3^{+ve}CD4^{+ve} T cells and (c) CD25^{+ve} Foxp3^{+ve} CD4 T cells were gated, indicating the frequency of regulatory T cells in CD4 T cells.

4.3 Results

4.3.1 T cell subsets in hip fracture patients

Firstly, significant differences were observed in the percentages of CD3^{+ve} T cells in the PBMC pool between our three subject groups, $F_{(2, 90)} = 3.58$, $p = .03$, $\eta^2 = .07$, [Figure 4.3a], which was driven by a significant decline in the percentage of T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .03$, but not with hip fracture patients without depressive symptoms, $p = .75$. Further, on examining the absolute numbers of T cells significant differences were seen between our three subject groups, $F_{(2, 50)} = 6.91$, $p = .002$, $\eta^2 = .21$ [Figure 4.3b], driven by a significant decline in T cell numbers in hip fracture patients with depressive symptoms compared with hip fracture patients without depressive symptoms, $p = .03$ and a further drop was observed on comparison with healthy controls, $p = .003$. When the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown).

On further analysis, no significant association was observed between GDS scores and frequency of peripheral T cells, $\beta = -.15$, $p = .24$, $\Delta R^2 = .02$ or absolute T cell numbers, $\beta = -.15$, $p = .40$, $\Delta R^2 = .02$ in hip fracture patients (data not shown).
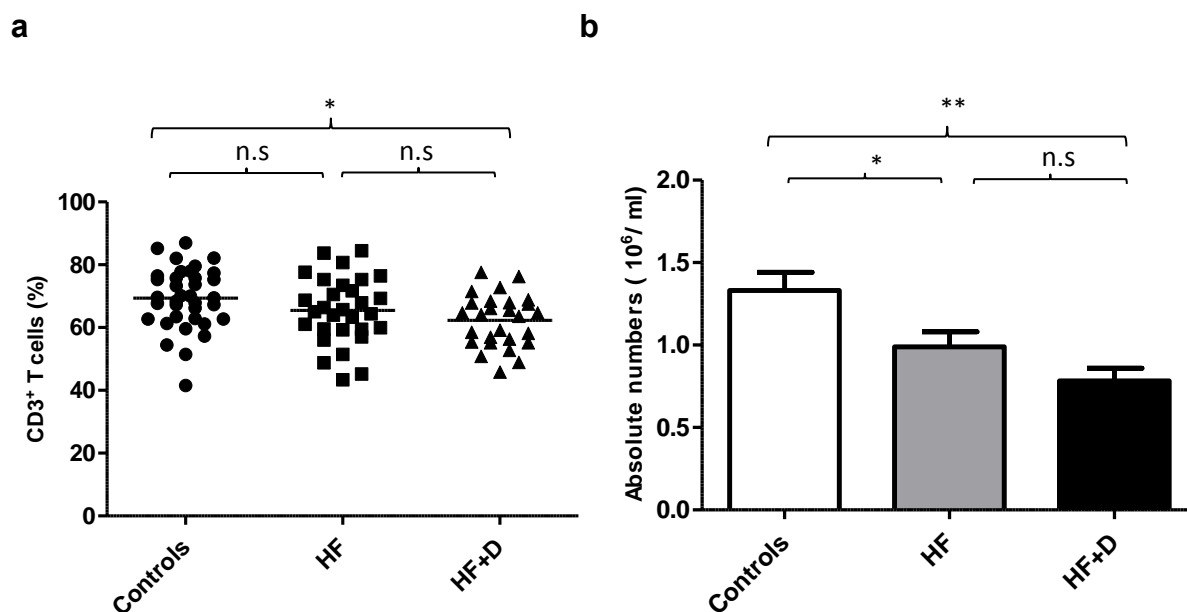
Figure 4.3 Frequency of peripheral T lymphocytes in hip fracture patients.

(a) Percentage CD3[+ve] T cells in healthy controls (n=36), hip fracture patients without depressive symptoms (HF; n=31) and hip fracture patients with depressive symptoms (HF+D; n=26). The solid bar represents the mean value. (b) Absolute numbers of T cells in healthy controls (n=20), hip fracture patients without depressive symptoms (HF; n=18) and hip fracture patients with depressive symptoms (HF+D; n=15). Data are mean ± SEM. * p <.05, ** p <.005.

Next , on comparing peripheral T cell frequency in hip fracture patients six weeks and six months post-surgery, there was no significant effect of time , $F_{(1, 28)} = .69$, $p = .41$, $\eta^2 = .02$ [Figure 4.4] and no significant group x time interaction, $F_{(1, 28)} = .10$, $p = .75$, $\eta^2 = .004$. Both groups of hip fracture patients did not differ in the T cell frequency six months after surgery, $p = .77$. These findings are not surprising; as there was no significant difference in T cell frequency at the 6 week time point between the two groups of hip fracture patients. The absolute T cell numbers in hip fracture patients at 6 month time point was not measured as the whole blood count data at 6 month time point for hip fracture patients was not collected.

T lymphocytes are composed of two main subsets, CD4 T cells (helper T cells) and CD8 T cells (cytotoxic T cells). No differences were found in the percentage of CD4^{+ve} T cells between our three groups, $F_{(2, 85)} = 2.40$, $p = .09$, $\eta^2 = .05$ [Table 4.2]. However, a significant difference occurred in absolute number of CD4^{+ve} T cells, $F_{(2, 51)} = 5.87$, $p = .005$, $\eta^2 = .19$, [Table 4.2], driven by a significant decline in absolute numbers of CD4 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .005$. No significant differences were observed in the percentage of CD8 T cells, $F_{(2, 85)} = 1.52$, $p = .22$, $\eta^2 = .03$ [Table 4.2] or in absolute number of CD8 T cells between the three groups, $F_{(2, 51)} = .27$, $p = .27$, $\eta^2 = .04$ [Table 4.2]. Thus, no significant differences occurred in the CD4:CD8 ratio between the three groups, $F_{(2, 85)} = 2.16$, $p = .12$, $\eta^2 = .04$ [Table 4.2].
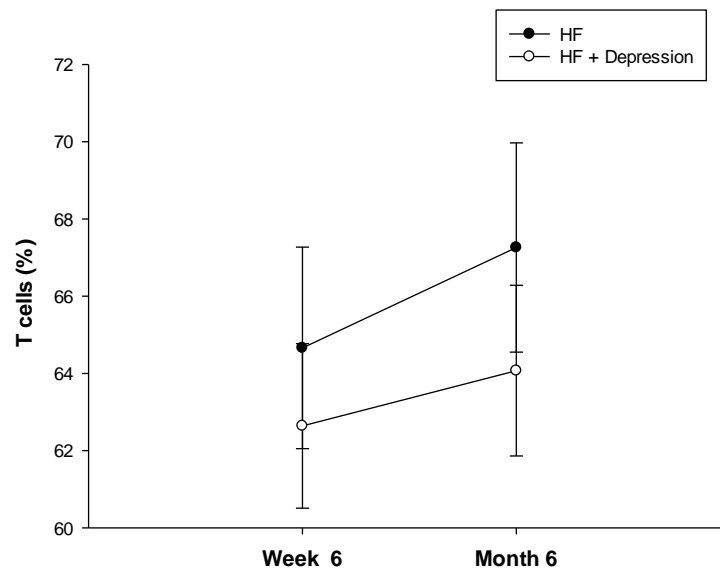
Figure 4.4 Frequency of circulating T cells in hip fracture patients six weeks and six months post-surgery.

Frequency of T cells in hip fracture patients without depressive symptoms (HF; n=12) and hip fracture patients with depressive symptoms (HF+D; n=18) at 6 weeks and 6 months post-surgery. Data are mean ± SEM.

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
CD4^{+ve} T cells (%)	65.55 (10.57)	70.90 (10.92)	70.62 (11.15)	.09
Absolute numbers (10^6/ml)	.84 (.26)	.65 (.28)	.55 (.22)	.005
CD8^{+ve} T cells (%)	28.31 (10.01)	24.89 (10.26)	23.83 (11.53)	.22
Absolute numbers (10^6/ml)	.32 (.22)	.25 (.16)	.21 (.18)	.27
CD4:CD8 ratio	2.80 (1.76)	3.84 (3.24)	4.02 (2.68)	.12

Table 4.2 The distribution of CD4 and CD8 T cells in hip fracture patients.

As previously discussed, CD4 and CD8 T cells can be divided into different subsets, on the basis of expression of CD45RA and the chemokine receptor CCR7, namely naïve, central memory (CM), effector memory (EM) and terminally differentiated effector memory cells (EMRA)[183]. On examining the percentage, $F_{(2, 85)} = .13$, $p = .87$, $\eta^2 = .003$, and absolute numbers of naïve CD4 T cells, $F_{(2, 51)} = 2.07$, $p = .13$, $\eta^2 = .07$ no significant differences were found in these cells in peripheral blood of our three subject groups [Table 4.3]. For memory CD4 T cell subsets, no significant differences occurred in percentages, $F_{(2,86)} = .90$, $p = .40$, $\eta^2 = .02$, or absolute numbers $F_{(2, 51)} = 1.37$, $p = .26$, $\eta^2 = .05$, of central memory CD4 T cells or in the percentage, $F_{(2, 86)} = .20$, $p = .81$, $\eta^2 = .005$, or in absolute numbers, $F_{(2, 51)} = .77$, $p = .46$, $\eta^2 = .03$, of effector memory CD4 T cells between our three groups [Table 4.3]. Finally, no significant differences were seen in the percentage, $F_{(2, 86)} = .23$, $p = .79$, $\eta^2 = .005$, or in absolute numbers of EMRA CD4 T cells, $F_{(2, 51)} = 1.83$, $p = .16$, $\eta^2 = .06$, between our three groups [Table 4.3].

Finally, the naïve: memory ratio in CD4 T cells also did not significantly differ between hip fracture patients with and without depressive symptoms and healthy controls, $F_{(2, 89)} = .21$, $p = .80$, $\eta^2 = .005$ [Table 4.3].

Next, the distribution of naïve and memory cell subsets amongst CD8 T cells was examined. No differences were observed in the percentage, $F_{(2, 85)} = .05$, $p = .94$, $\eta^2 = .001$, or absolute numbers of naïve CD8 T cells $F_{(2, 51)} = 1.77$, $p = .18$, $\eta^2 = .06$, between the three groups [Table 4.3]. On examining memory CD8 T cell subsets, no significant differences were observed in: percentages , $F_{(2,85)} = 1.34$, $p = .26$, $\eta^2 = .03$, or absolute numbers $F_{(2, 51)} = .80$, $p = .45$, $\eta^2 = .03$ of central memory CD8 T cells; percentage $F_{(2, 85)} = 2.77$, $p = .07$, $\eta^2 = .06$, or absolute numbers of effector memory CD8 T cells $F_{(2, 51)} = 1.21$, $p = .30$, $\eta^2 = .04$; or the percentage $F_{(2, 85)} = .55$, $p = .57$, $\eta^2 = .01$, or absolute numbers of EMRA CD8 T cells, $F_{(2, 51)} = .50$, $p = .60$, $\eta^2 = .01$, between the subject groups [Table 4.3].

 Similarly, the naïve: memory ratio in CD8 T cells did not significantly differ between hip fracture patients with and without depressive symptoms and healthy controls, $F_{(2, 86)} = .12$, $p = .89$, $\eta^2 = .003$, [Table 4.3].

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
CD4 T cells				
Naïve CD4 T cells (%)	38.92 (19.65)	36.87 (20.55)	36.55 (18.11)	.87
Absolute numbers (10^6/ml)	.35 (.27)	.22 (.22)	.21 (.15)	.13
CM CD4 T cells (%)	3.49 (6.99)	3.49 (3.37)	5.38 (6.98)	.40
Absolute numbers (10^6/ml)	.01 (.01)	.03 (.02)	.02 (.02)	.26
EM CD4 T cells (%)	19.38 (10.62)	20.33 (10.93)	21.39 (15.31)	.81
Absolute numbers (10^6/ml)	.14 (.07)	.14 (.11)	.10 (.08)	.46
EMRA CD4 T cells (%)	38.40 (17.15)	40.10 (17.36)	36.96 (16.42)	.79
Absolute numbers (10^6/ml)	.31 (.14)	.26 (.17)	.22 (.10)	.16
Total memory CD4 T cells	61.07 (19.64)	63.16 (20.54)	63.44 (18.04)	.87
Absolute numbers (10^6/ml)	.46 (.14)	.39 (.21)	.34 (.15)	.12
Naïve:memeory ratio	.82 (.87)	.87 (.84)	.73 (.63)	.80
CD8 T cells				
Naïve CD8 T cells (%)	16.98 (16.12)	15.73 (13.11)	16.61 (13.89)	.94
Absolute numbers (10^6/ml)	.05 (.07)	.02 (.04)	.02 (.01)	.18
CM CD8 T cells (%)	26.55 (14.61)	33.17 (20.09)	32.03 (16.01)	.26
Absolute numbers (10^6/ml)	.06 (.06)	.10 (.11)	.09 (.09)	.45
EM CD8 T cells (%)	41.04 (13.27)	33.40 (16.16)	33.56 (14.44)	.07
Absolute numbers (10^6/ml)	.13 (.09)	.09 (.10)	.09 (.08)	.30
EMRA CD8 T cells (%)	14.44 (11.09)	17.02 (13.50)	17.63 (13.64)	.57
Absolute numbers (10^6/ml)	.04 (.04)	.04 (.09)	.02 (.02)	.60
Total memory CD8 T cells	83.01 (16.12)	83.99 (12.89)	82.43 (14.65)	.92
Absolute numbers (10^6/ml)	.27 (.20)	.21 (.16)	.19 (.19)	.39
Naïve:memeory ratio	.26 (.41)	.23 (.26)	.28 (.45)	.89

Table 4.3 Naive and memory T lymphocytes in hip fracture patients.

4.3.2 Accumulation of senescent T cells in hip fracture patients

CD28 is a co-stimulatory molecule involved in T cell activation, proliferation, cytokine production and survival [192]. Ageing has been associated with an increase in CD28^{-ve} cells which have reduced function and have been considered as senescent. On examining the frequency of circulating CD28^{-ve}T cells, there were significant differences, $F_{(2, 56)} = 8.16$, $p = .001$, $\eta^2 = .22$ between our three groups of subjects, driven by a significant increase in the percentage of CD28^{-ve} T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .001$ and hip fracture patients without depressive symptoms, $p = .02$ [Table 4.4]. The frequency of CD28^{-ve} CD4T cells also significantly differed between groups, $F_{(2, 56)} = 6.11$, $p = .004$, $\eta^2 = .17$ [Table 4.4], but was limited to an increase in frequency of CD28^{-ve} CD4 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .001$ and hip fracture patients without depressive symptoms, $p = .02$. Similarly, the frequency of CD28^{-ve} CD8T cells was also significantly different, $F_{(2, 56)} = 6.69$, $p = .002$, $\eta^2 = .19$ between our three groups [Table 4.4], and a significant increase in the frequency of CD28^{-ve} CD8 T cells occurred only in hip fracture patients with depressive symptoms compared with healthy controls, $p = .003$ and hip fracture patients without depressive symptoms, $p = .02$. When the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant. As a consequence of the above, significant differences were seen in CD28^{+ve} T cells [Table 4.4]. However, CD28 expression levels (MFI) on T cells did not significantly differ between our subjects groups (data not shown).

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
CD28^{-ve} T cells (%)	17.75 (7.47)	20.50 (6.14)	29.14 (12.98)	.001
CD28^{+ve} T cells (%)	82.24 (7.47)	78.20 (8.18)	70.85 (12.98)	.002
CD28^{-ve} CD4 T cells (%)	6.41 (5.34)	8.20 (4.48)	13.96 (10.31)	.004
CD28^{+ve} CD4 T cells (%)	93.58 (5.33)	91.19 (5.66)	85.50 (11.35)	.006
CD28^{-ve} CD8 T cells (%)	36.32 (16.25)	38.82 (11.43)	52.29 (15.48)	.002
CD28^{+ve} CD8 T cells (%)	63.36 (16.14)	59.62 (14.54)	47.67 (15.49)	.006

Table 4.4 CD28 expression on T lymphocytes in hip fracture patients.

The expression of CD57 on T cells has also been identified as a marker for cell senescence [632]. Significant differences in CD57^{+ve} T cells, $F_{(2, 56)} = 4.05$, $p = .02$, $\eta^2 = .12$ were observed between our three groups [Table 4.5], driven by an increase in the percentage of CD57^{+ve} T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .02$ but not in comparison with hip fracture patients without depressive symptoms, $p = .68$. Further, on examining frequency of CD57^{+ve} CD4 T cells significant differences, $F_{(2, 56)} = 6.82$, $p = .002$, $\eta^2 = .19$ were found between our three groups [Table 4.5], due to accumulation of CD57^{+ve} CD4 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .002$ only and but not in comparison with hip fracture patients without depressive symptoms, $p = .10$. Similarly, frequency of CD57^{+ve} CD8 T cells was also significantly different, $F_{(2, 56)} = 5.36$, $p = .007$, $\eta^2 = .16$ between our three groups [Table 4.5], due to an increase in CD57^{+ve} CD8 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .005$ only and not in comparison with hip fracture patients without depressive symptoms, $p = .33$.

When the above analyses were repeated with adjustment for age, sex and BMI, the results remained significant. However, no significant differences were reported in CD57 expression levels (MFI value) on T lymphocytes between our three groups (data not shown).

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
CD57^{+ve} T cells (%)	12.56 (9.04)	17.57 (7.31)	21.89 (14.23)	.02
CD57^{+ve} CD4 T cells (%)	4.29 (3.36)	6.86 (4.26)	11.24 (9.25)	.002
CD57^{+ve} CD8 T cells (%)	25.31 (17.89)	33.37 (13.43)	42.55 (18.60)	.007

Table 4.5 CD57 expression on T lymphocytes in hip fracture patients.

Finally, on examining frequency of CD28^{-ve} CD57^{+ve} T cells, significant differences were seen $F_{(2, 56)} = 5.25$, $p = .008$, $\eta^2 = .15$ between our three groups [Figure 4.5a], driven by accumulation of CD28^{-ve}CD57^{+ve} T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .008$ only and not in comparison with hip fracture patients without depressive symptoms, $p = .33$. Similarly, on examining frequency of CD28^{-ve}CD57^{+ve} CD4 T cells significant differences, $F_{(2, 56)} = 6.28$, $p = .003$, $\eta^2 = .18$ occurred [Figure 4.5b], due to increased frequency of CD28^{-ve} CD57^{+ve} CD4 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .002$, but not on comparison with hip fracture patients without depressive symptoms, $p = .13$. The frequency of CD28^{-ve} CD57^{+ve} CD8 T cells were also significantly different, $F_{(2, 56)} = 4.12$, $p = .02$, $\eta^2 = .12$ between our three groups [Figure 4.5c], due to a significant increase in frequency of CD28^{-ve} CD57^{+ve} CD8 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .02$ but not in comparison with hip fracture patients without

depressive symptoms, p = .49. When the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown).On further analysis, no significant association was found between GDS scores and frequency of peripheral CD28[-ve] T cells, β = .23, p = .16, ΔR^2 = .05; peripheral CD57[+ve] T cells, β = .09, p = .59, ΔR^2 = .009 or CD28[-ve] CD57[+ve] T cells in hip fracture patients, β = .11, p = .51, ΔR^2 = .01 (data not shown).

NKG2D is a known NK cell receptor that is also expressed on T cells and acts as a marker of senescence [202]. On examining NKG2D expression on T cells, no significant differences were observed in the frequency of NKG2D[+ve] T cells, $F_{(2, 28)}$ = .13, p = .87, η^2 = .009, or the frequency of NKG2D[+ve] CD4 T cells $F_{(2, 28)}$ = .07, p = .93, η^2 = .005, or NKG2D[+ve] CD8 T cells $F_{(2, 28)}$ = .30, p = .73, η^2 = .02 [Table 4.6], between the three groups. Further, no significant differences were reported in NKG2D expression levels (MFI) on T lymphocytes between our three groups (data not shown).

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
	Mean	(SD)		
NKG2D[+ve] T cells (%)	9.28 (5.25)	10.03 (4.38)	10.50 (5.57)	.87
NKG2D[+ve] CD4 T cells (%)	5.50 (4.05)	5.55 (2.30)	5.11 (2.96)	.93
NKG2D[+ve] CD8 T cells (%)	16.15 (7.16)	13.56 (5.63)	15.12 (7.59)	.73

Table 4.6 NKG2D expression on T lymphocytes in hip fracture patients.
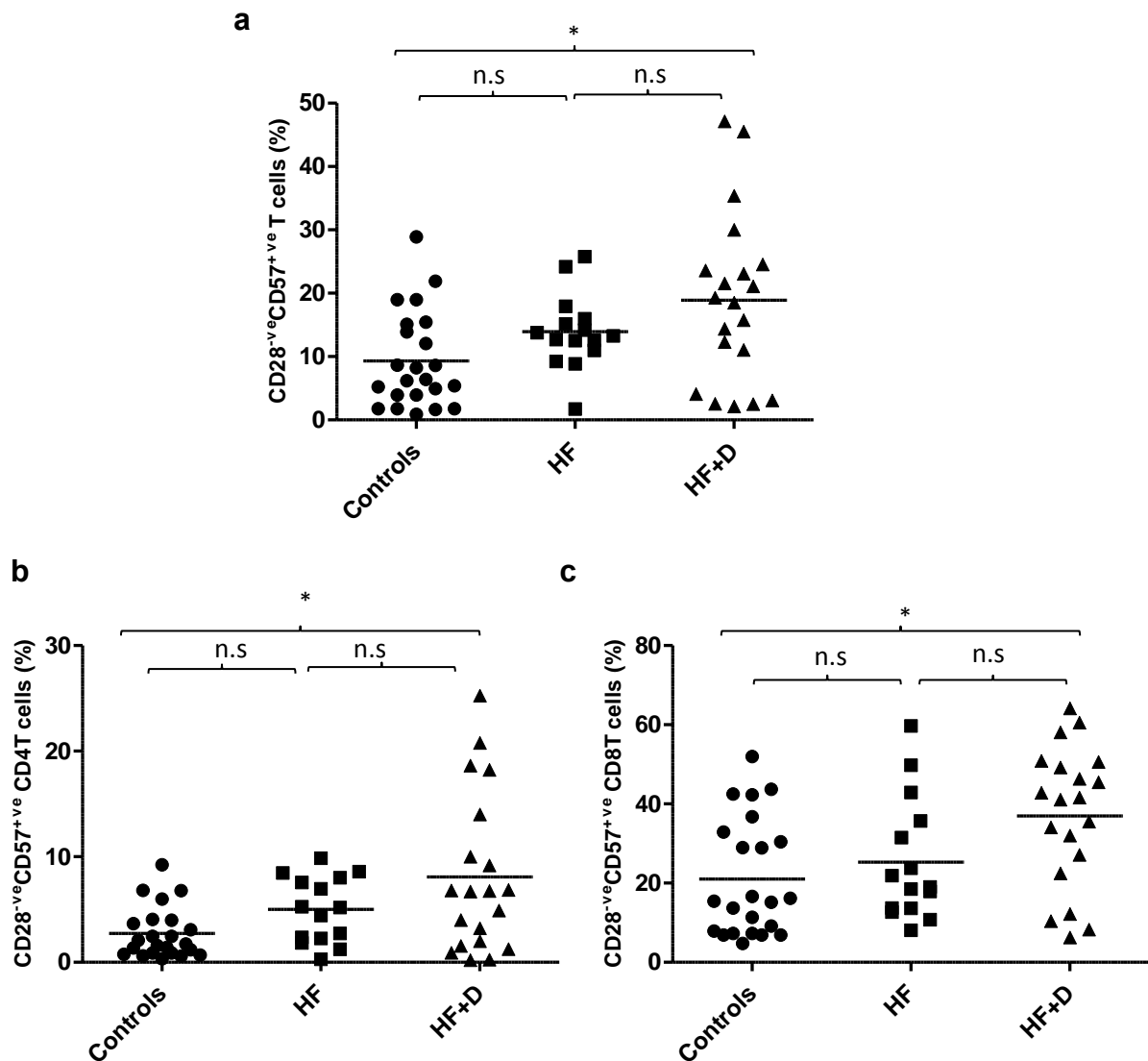
Figure 4.5 CD28^{-ve} CD57^{+ve} T lymphocytes in hip fracture patients.

Percentage of (a) CD28[-ve]CD57[+ve] T cells, (b) CD28[-ve]CD57[+ve] CD4T cells, or (c) CD28[-ve]CD57[+ve] CD8 T cells in healthy controls (n=23), hip fracture patients without depressive symptoms (HF; n=19) and hip fracture patients without depressive symptoms (HF + D; n=17). The solid bar represents the mean value. * $p < .05$.

4.3.3 Activated T cells in hip fracture patients

CD69 (early), CD25 (middle) and HLA-DR (late) are markers for T cell activation [633, 634]. In an attempt to examine activation status of circulating T cells in hip fracture patients the expression of these activation markers on T lymphocytes was compared. The percentage of CD69^{+ve} T cells, showed significant differences, $F_{(2, 53)} = 4.65$, $p = .01$, $\eta^2 = .15$, driven by a significant increase in the percentage of CD69^{+ve} T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .01$ only and not in comparison with hip fracture patients without depressive symptoms, $p = .12$ (data not shown). When the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown). Next, on comparing the frequency of CD69^{+ve} CD4 T cells $F_{(2, 53)} = 2.59$, $p = .08$, $\eta^2 = .08$ [Figure 4.6a] no significant differences were observed between our three groups. However, significant differences occurred in the percentage of CD69^{+ve} CD8 T cells, $F_{(2, 53)} = 3.50$, $p = .03$, $\eta^2 = .11$, driven by a significant increase in percentage of CD69^{+ve} CD8 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .04$ but not in comparison with hip fracture patients without depressive symptoms, $p = .17$ [Figure 4.6b]. Suggesting, an accumulation of activated CD69^{+ve} CD8 T cells in the hip fracture patients with depressive symptoms.

No significant association was found between GDS scores and frequency of peripheral CD69^{+ve} T cells, $\beta = .24$, $p = .14$, $\Delta R^2 = .05$ (data not shown). Finally, no significant differences were reported in CD69 expression levels (MFI value) on T lymphocytes between our three groups (data not shown).
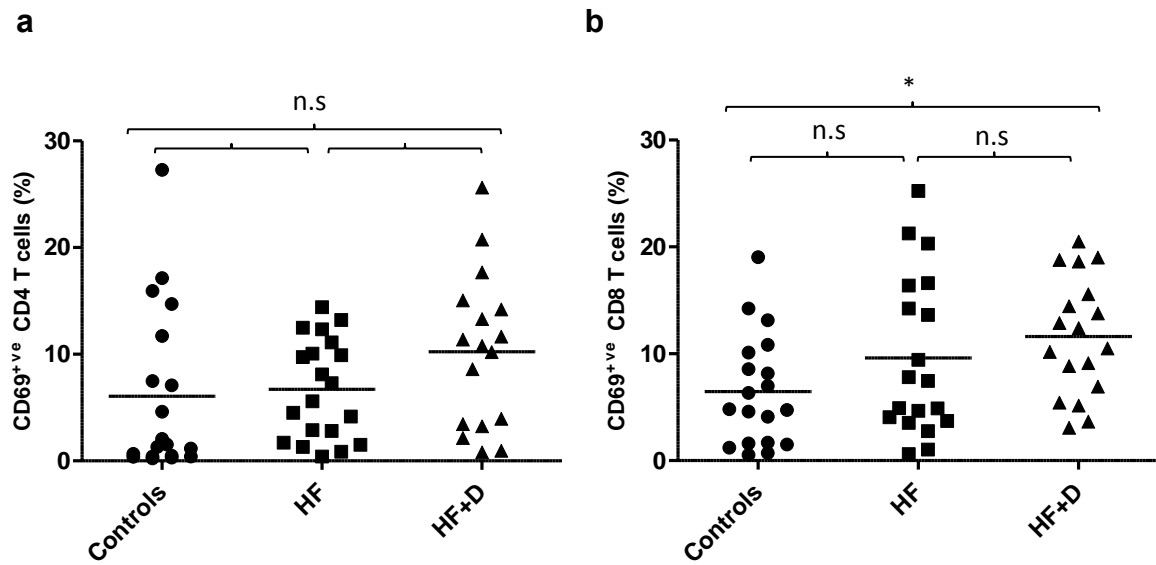
Figure 4.6. CD69 expression on CD4 and CD8 T lymphocytes in hip fracture patients

Frequency of (a) CD69^{+ve} CD4 T cells or (b) CD69^{+ve} CD8 T cells in healthy controls (n=19), hip fracture patients without depressive symptoms (HF; n=19) and hip fracture patients with depressive symptoms (HF+D; n=19). The solid bar represents the mean value. * p <.05.

Next, on examining peripheral distribution of CD25^{+ve} T cells no significant differences were found, $F_{(2, 40)} = 1.41$, $p = .25$, $\eta^2 = .06$ between our three groups [Table 4.8]. Similarly, no differences were reported between distribution of CD25^{+ve} T cells in the CD4 T cell subset $F_{(2, 40)} = .63$, $p = .53$, $\eta^2 = .03$, or the CD25^{+ve} T cells in CD8 T cell subset $F_{(2, 40)} = .57$, $p = .56$, $\eta^2 = .02$ [Table 4.8], between the three groups. However, no significant differences were reported in CD25 expression levels (MFI value) on T lymphocytes between our three groups (data not shown).

Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
		Mean	(SD)	
CD25^{+ve} T cells (%)	3.48 (1.73)	3.98 (1.08)	4.37 (1.35)	.25
CD25^{+ve} CD4 T cells (%)	5.85 (1.73)	6.14 (1.60)	6.28 (1.32)	.76
CD25^{+ve} CD8 T cells (%)	4.42 (2.05)	5.02 (1.31)	4.66 (.96)	.56

Table 4.8. CD25 expression on T lymphocytes in hip fracture patients.

Finally, on examining peripheral distribution of HLADR^{+ve} T cells, significant differences were observed, F (2, 48) = 3.43, p = .04, η^2 = .13 between our three groups (data not shown), driven by an increase in percentage of HLADR^{+ve} T cells in hip fracture patients with depressive symptoms compared with healthy controls, p = .03 but not on comparison with hip fracture patients without depressive symptoms, p = .55. When the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown). Further, on comparing frequencies of HLADR^{+ve} CD4 T cells between our three groups, F (2, 48) = 1.76, p = .18, η^2 = .06 [Figure 4.7a] no significant differences were found. In contrast, significant differences occurred in HLADR^{+ve} CD8 T cells, F (2, 48) = 7.72, p = .001, η^2 = .24, driven by an increase in percentage of HLADR^{+ve} CD8 T cells in hip fracture patients with depressive symptoms compared with healthy controls, p = .001 but not in comparison with hip fracture patients without depressive symptoms, p = .06 [Figure 4.7b]. Suggesting, an accumulation of activated HLADR^{+ve} T cells only in the CD8 T cell subset of hip fracture patients with depressive symptoms. No significant association was reported between GDS scores and frequency of peripheral HLADR^{+ve} T cells, β = .26, p = .12, ΔR^2 = .07. Also, no significant differences were reported in HLADR expression levels (MFI value) on T lymphocytes between our subject groups (data not shown).
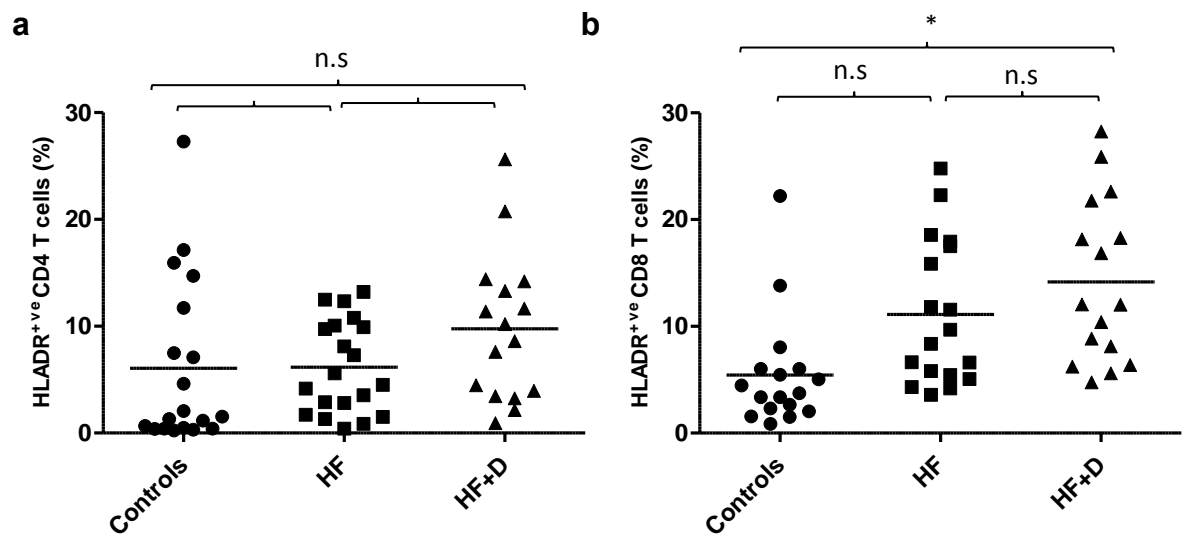
Figure 4.7. HLADR expression on CD4 and CD8 T lymphocytes in hip fracture patients.

Percentage of (a) HLADR^{+ve} CD4 T cells or (b) HLADR^{+ve} CD8 T cells in healthy controls (n=17), hip fracture patients without depressive symptoms (HF; n=18) and hip fracture patients with depressive symptoms (HF+D; n=16). The solid bar represents the mean value. * p <.05.

4.3.5 Regulatory T cells in hip fracture patients

T_{regs} have been identified as a subpopulation of T cells expressing CD4, CD25 and the transcription factor foxp3 [277, 635] that plays an essential role in T_{reg} cell differentiation and development[636]. On examining circulating frequency of T_{regs}, no significant differences were observed between hip fracture patients with and without depressive symptoms and healthy older adults, $F (2, 57) = 1.24$, $p = .29$, $\eta^2 = .04$ [Figure 4.8a]. Similarly, no significant differences were found between absolute numbers of regulatory T cells, $F (2, 31) = .98$, $p = .38$, $\eta^2 = 06$ [Figure 4.8b] between our three subject groups.

4.3.6 IL10 production by CD4 T cells in hip fracture patients

Next, on examining IL10 production by CD4 T cells on stimulation with PMA and Ionomycin (4hr) significant differences were seen in the percentage of IL10 producing CD4 T cells between our three groups, $F (2, 50) = 4.93$, $p =.01$, $\eta^2 = .16$ [Figure 4.9a], driven by a significant increase in percentage of IL10^{+ve} CD4 T cells in hip fracture patients with depressive symptoms compared with healthy controls, $p = .02$ and hip fracture patients without depressive symptoms, $p = .04$. However, no significant differences were observed in the amount of IL10 produced (MFI value) by CD4 T cells between our groups, $F (2, 42) = .46$, $p =.63$, $\eta^2 = .02$ [Figure 4.9b]. Interestingly, there was a significant association between GDS scores and frequency of peripheral IL10^{+ve} CD4 T cells in hip fracture patients, $\beta = .34$, $p = .04$, $\Delta R^2 = .11$, such that hip fracture patients with greater depressive symptoms (GDS score) had higher frequency of IL10^{+ve} CD4 T cells [Figure 4.9c].

Further, on comparing frequency of IL10^{+ve} CD4 T cells in hip fracture patients six weeks and six months post-surgery, there was a significant effect of time , $F (1, 19) = .09$, $p = .04$, $\eta^2 = .01$, [Figure 4.10] but no significant group x time interaction, $F (1, 19) = 2.62$, $p = .17$, $\eta^2 = .32$. Overall, the two groups of hip fracture patients did not differ in IL10 production by CD4 T cells on stimulation with PMA and Ionomycin (4hr) six months after surgery, $p = .76$.
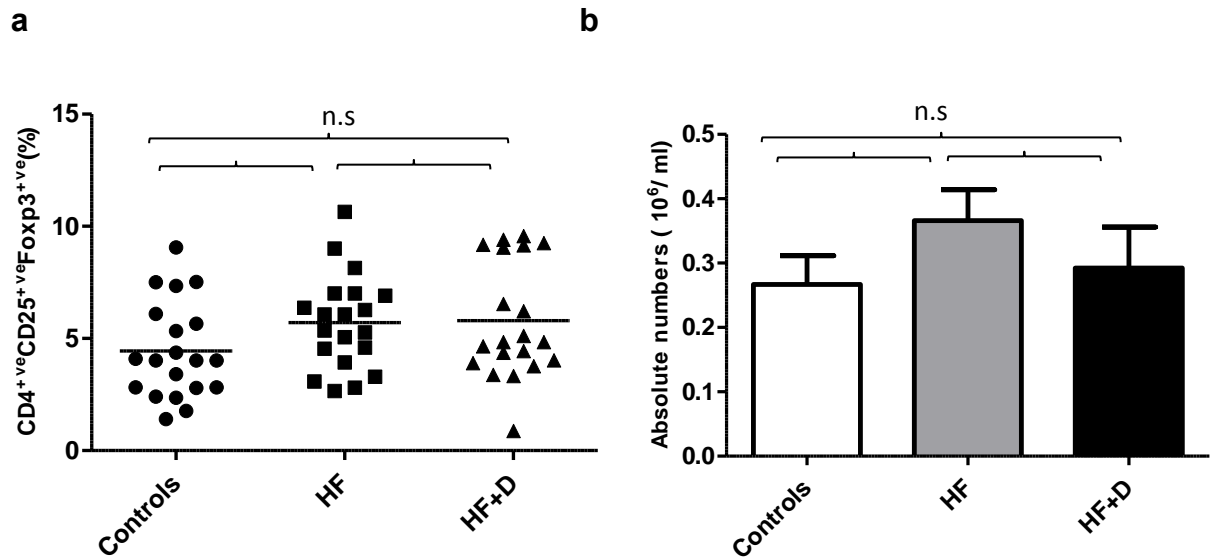
Figure 4.8 CD4^{+ve} CD25^{+ve} Foxp3^{+ve} regulatory T lymphocyte in hip fracture patients

(a) Percentage of CD4^{+ve} CD25^{+ve} Foxp3^{+ve} T cells in healthy controls (n=20), hip fracture patients without depressive symptoms (HF; n=20) and hip fracture patients with depressive symptoms (HF+D; n=20). The solid bar represents the mean value. (b) Absolute number of CD4^{+ve} CD25^{+ve} Foxp3^{+ve} regulatory T cells in healthy controls (n=12), hip fracture patients without depressive symptoms (HF; n=13) and hip fracture patients with depressive symptoms (HF+D; n= 10). Data are mean ± SEM.

Figure 4.10 IL10 production by CD4 T lymphocytes in hip fracture patients.

(a) Percentage IL10[+ve] CD4 T cells and (b) mean IL10 production (MFI value) by CD4 T cells in healthy controls (n=17), hip fracture patients without depressive symptoms (HF; n=20) or hip fracture patients with depressive symptoms (HF+D; n=20). The solid bar represents the mean value in (a) and data are mean ± SEM in (b). (c) Correlation between GDS depressive symptoms scores and IL10 production by CD4 T cells in hip fracture patients (n = 36). * p <.05.
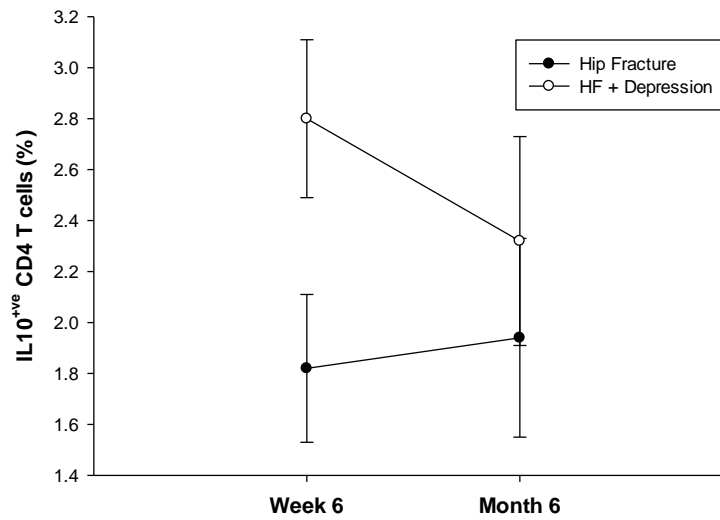
Figure 4.10 Frequency of IL10 producing CD4 T cells in hip fracture patients six months post-surgery.

Frequency of IL10^{+ve} CD4 T cells in hip fracture patients without depressive symptoms (n = 9) and hip fracture patients with depressive symptoms (n = 10) at 6 weeks and 6 months post-surgery. Data are mean ± SEM.

4.3.7 B cells in hip fracture patients

On examining B cells in peripheral blood of hip fracture patients , no significant differences were observed in B cell frequency, $F_{(2,67)} = .16$, $p = .15$, $\eta^2 = .04$, [Figure 4.11a] or absolute numbers of B cells, $F_{(2,69)} = .30$, $p = .73$, $\eta^2 = .01$ [Figure 4.11b] between the three groups.

When peripheral frequency of regulatory B cells were assessed significant differences were found in the percentages of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells, $F_{(2,61)} = 11.16$, $p < .001$, $\eta^2 = .26$ between our three groups [Figure 4.12a], due to a decline in this subset of B cells in hip fracture patients with depressive symptoms compared with healthy controls, $p < .001$ as well as hip fracture patients without depressive symptoms, $p = .001$. When all of the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown). Similarly, significant differences were seen in absolute numbers of peripheral $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells, $F_{(2,38)} = 4.85$, $p = .01$, $\eta^2 = .20$ [Figure 4.12b] between our three groups, driven by a significant decline in absolute numbers in hip fracture patients with depressive symptoms compared with healthy controls, $p = .04$ as well as hip fracture patients without depressive symptoms, $p = .02$.

Interestingly, a significant association was observed between GDS scores and frequency of peripheral $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells in hip fracture patients, $\beta = -.38$, $p = .01$, $\Delta R^2 = .15$, such that hip fracture patients with greater depressive symptoms (GDS score) had lower frequency of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells [Figure 4.12c].
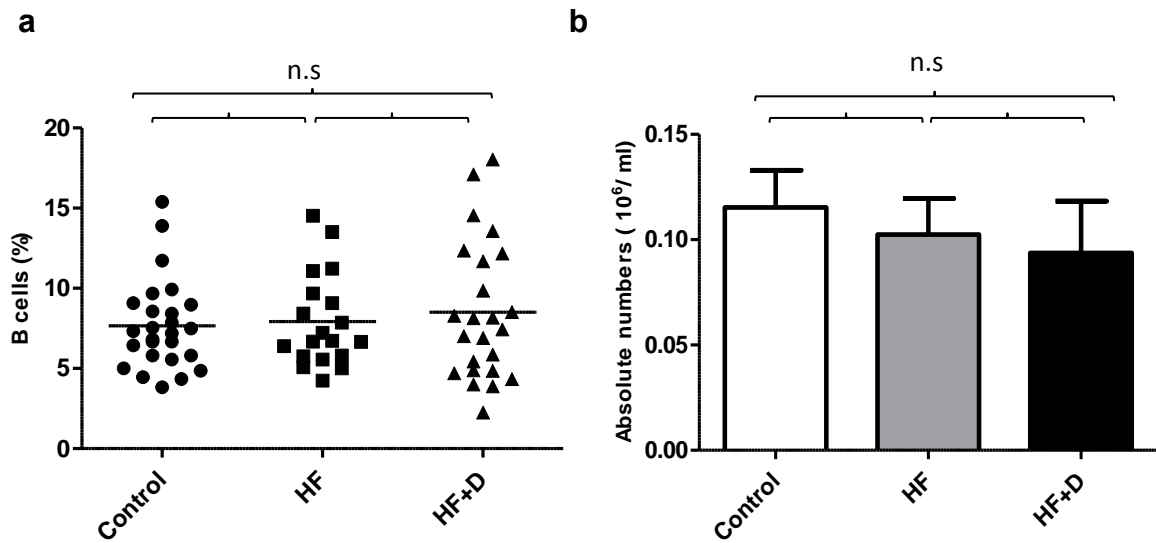
Figure 4.11 B lymphocytes in hip fracture patients.

(a) Percentage and (b) absolute numbers of CD19^{+ve} B cells in peripheral blood of healthy controls (n = 26), hip fracture patients without depressive symptoms (HF; n = 19) and hip fracture patients with depressive symptoms (HF+D; n= 25). The solid bar represents the mean value in (a) and data are mean ± SEM in (b).
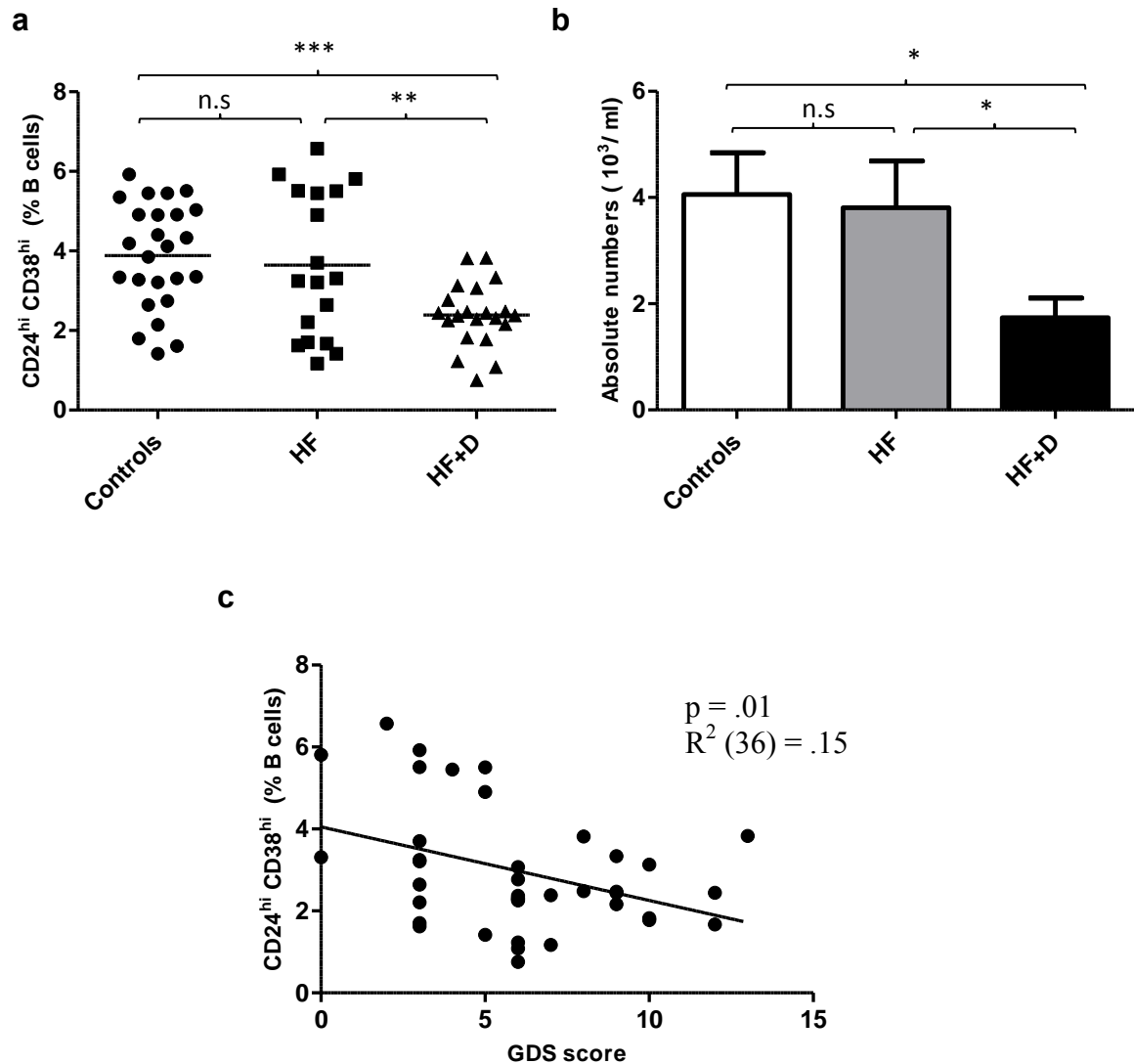
Figure 4.12 Frequency of CD19^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients.

(a) Percentage and (b) absolute numbers of CD19^{+ve}CD24^{hi}CD38^{hi} cells in healthy controls (n = 21), hip fracture patients without depressive symptoms (HF; n=15) and hip fracture patients with depressive symptoms (HF+D; n=22). The solid bar represents the mean value in (a) and the data are mean ± SEM in (b). (c) Correlation between percentage of CD19^{+ve}CD24^{hi}CD38^{hi} and GDS scores in hip fracture patients (n = 36). * p <.05, ** p <.005 and *** p <.001.

Further, on examining frequency of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells in hip fracture patients six weeks and six months post-surgery, there was a significant effect of time on hip fracture patients, F (1,25) = 13.63, p = .001, η^2 = .35, but no effect of group x time interaction F (1, 25) = 2.72, p = .11, η^2 = .09. However, the effect of group remained significant between hip fracture patients with and without depressive symptoms, p = .002 [Figure 4.14].

Next, the frequency of IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells post CD3 stimulation (72 hr) was determined. The data revealed significant differences between our groups, F (2, 55) = 4.88, p = .01, η^2 = .15, but the reduction in IL10 induction was restricted to hip fracture patients with depressive symptoms compared with healthy controls, p =.04 and hip fracture patients without depressive symptoms, p = .01 [Figure 4.15a]. When all of the above analyses were repeated with adjustment for age, sex and BMI, the results still remained significant (data not shown). Further, mean IL10 production (MFI) by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells post stimulation did not differ between the subject groups, F (2, 55) = .85, p = .43, η^2 = .03 [Figure 4.15b].

There was a significant association between GDS scores and the frequency of IL10 producing $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells post stimulation in hip fracture patients, β = -.34, p = .03, ΔR^2 = .34, such that hip fracture patients with greater depressive symptoms (GDS score) had a lower frequency of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells [Figure 4.15c].

The percentage of IL10 producing cells in the $CD24^{int}CD38^{int}$ B cell subset F (2, 55) = .04, p = .95, η^2 = .002 and their IL10 expression levels F (2, 55) = 2.90, p = .07, η^2 = .09 did not differ between our three groups [Table 4.9]. Also, the percentage of IL10 producing $CD24^{-ve}CD38^{+ve}$B cells F (2, 55) = 1.59, p = .21, η^2 = .05 and their IL10 expression levels F (2, 55) = 2.47, p =.09, η^2 = .08 did not differ significantly [Table 4.9].
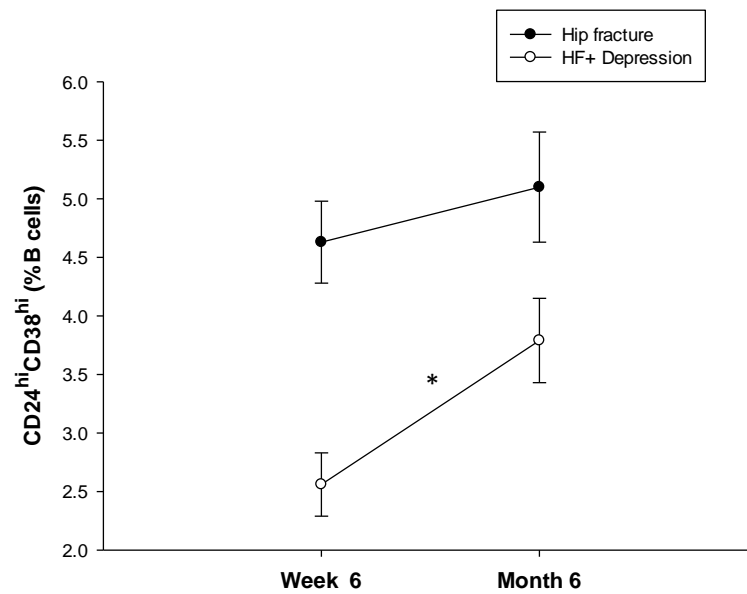
Figure 4.13 CD19^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients 6 months post-surgery.

Percentage of peripheral CD19^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients without depressive symptoms (n = 10) and hip fracture patients with depressive symptoms (n = 17) 6 weeks and 6 months post-surgery. Data are mean ± SEM.
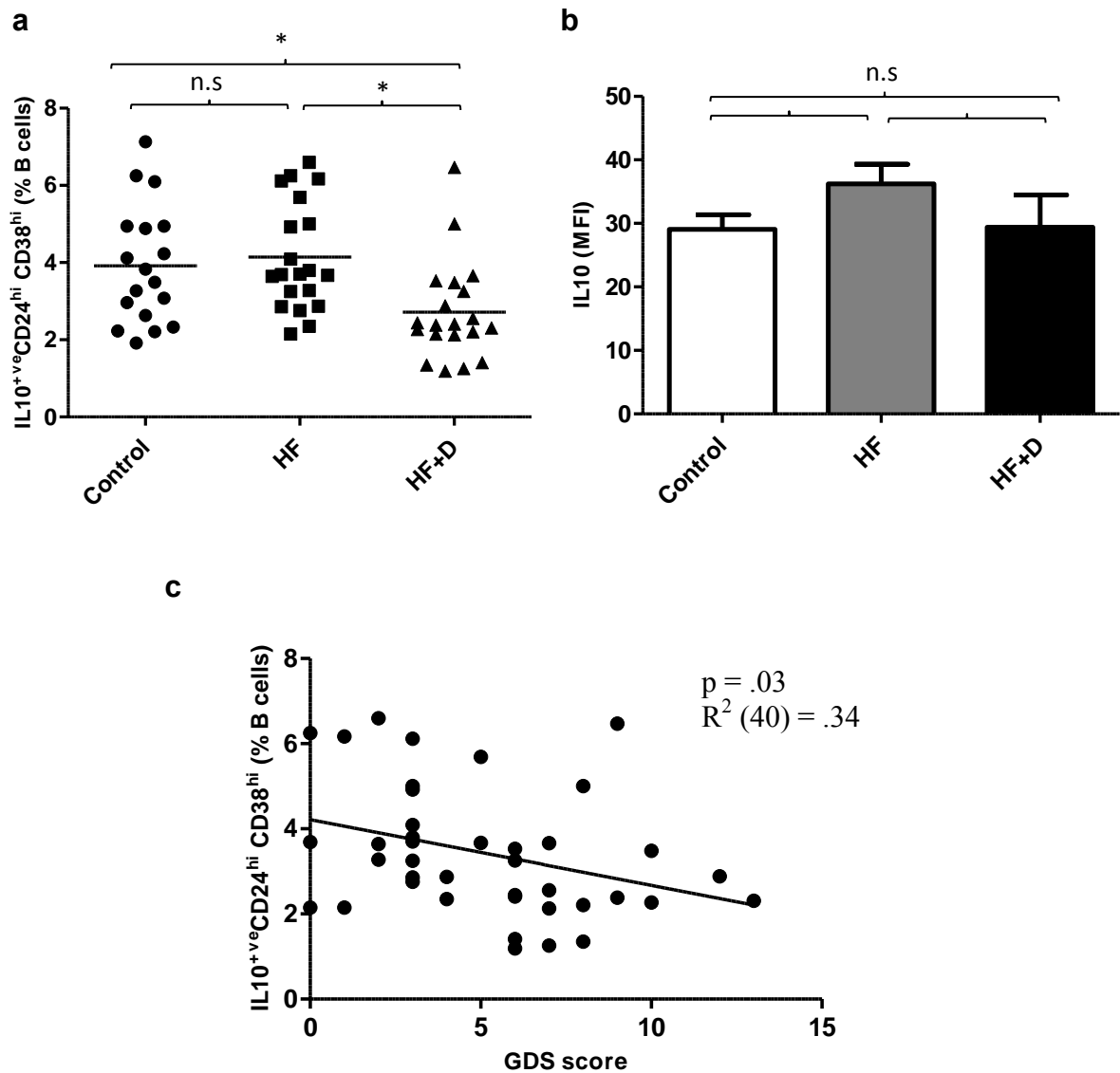
Figure 4.14 IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients.

(a) Frequency of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} cells in healthy controls (n=18), hip fracture patients without depressive symptoms (HF; n=21) or hip fracture patients with depressive symptoms (HF+D; n=19). The solid bar represents the mean value. (b) IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} cells in healthy controls (n=18), hip fracture patients without depressive symptoms (HF; n=21) or hip fracture patients with depressive symptoms (HF+D; n=19). Data are mean ± SEM. (c) Correlation between percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells post CD3 stimulation (72hr) and GDS scores in hip fracture patients (n = 40). * p <.05.

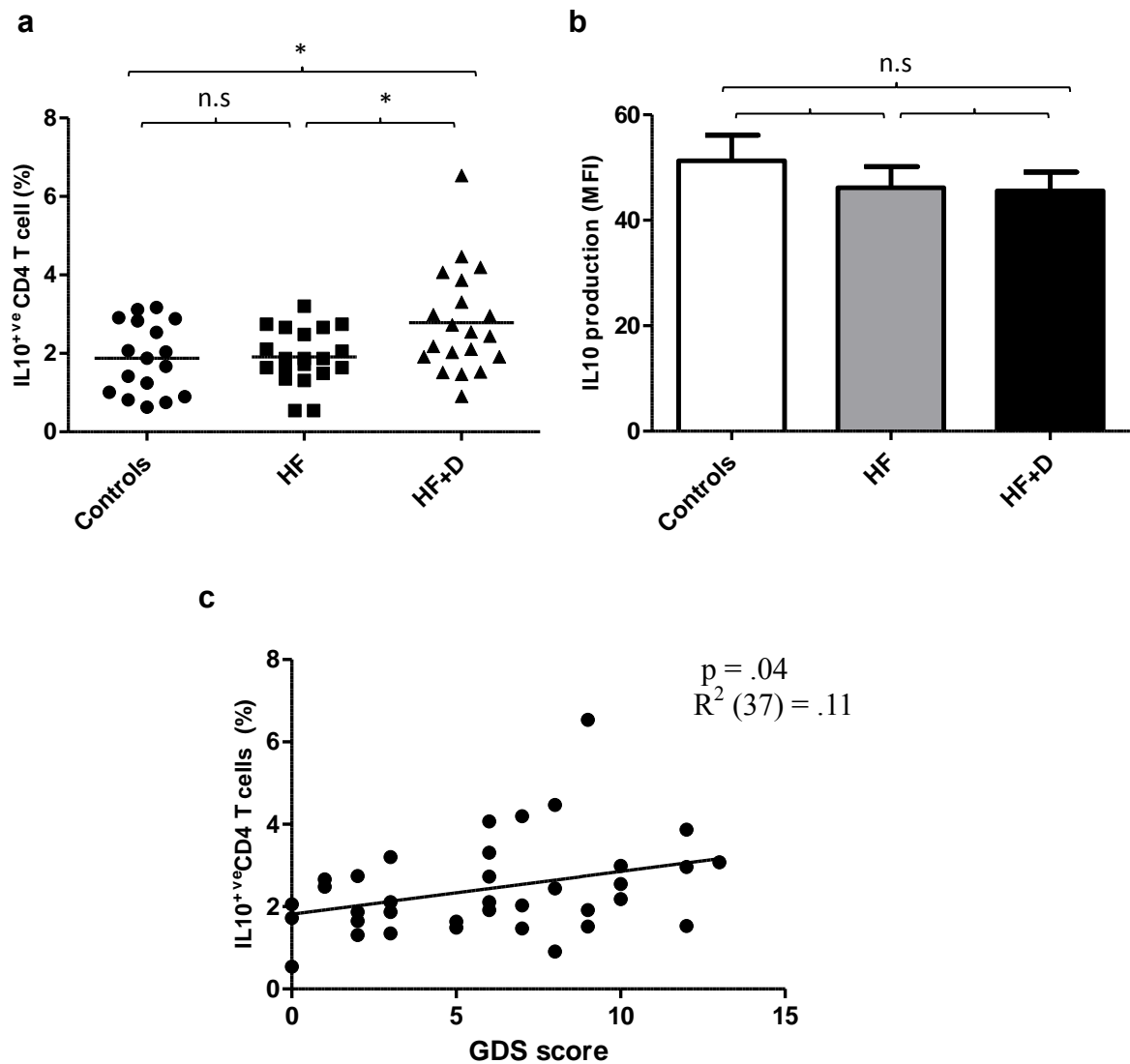| Variable	Healthy control	Hip fracture patients (HF)	Hip fracture patients with depressive symptoms (HF + D)	p
		Mean	(SD)	
IL10^{+ve} CD24^{int} CD38^{int}	.84 (.52)	.84 (.83)	.82 (.50)	.95
IL10 expression (MFI)	21.18 (6.81)	27.26 (13.10)	20.28 (8.37)	.21
IL10^{+ve} CD24^{+ve} CD38^{-ve}	1.36 (1.13)	1.93 (1.33)	2.33 (2.29)	.21
IL10 expression (MFI)	24.90 (6.57)	25.63 (7.65)	20.57 (8.57)	.09

Table 4.9 IL10 producing B cell subsets in hip fracture patients.

Further, on examining the frequency of IL10^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients six weeks and six months post-surgery post CD3 stimulation, there was no significant effect of time on hip fracture patients, $F_{(1,21)} = 2.50$, $p = .12$, $\eta^2 = .10$. Further, no significant effect of group x time interaction $F_{(1, 21)} = 2.72$, $p = .11$, $\eta^2 = .09$ was observed and the effect of group did not remain significant between hip fracture patients with and without depressive symptoms, $p = .36$, six month post-surgery [Figure 4.15].
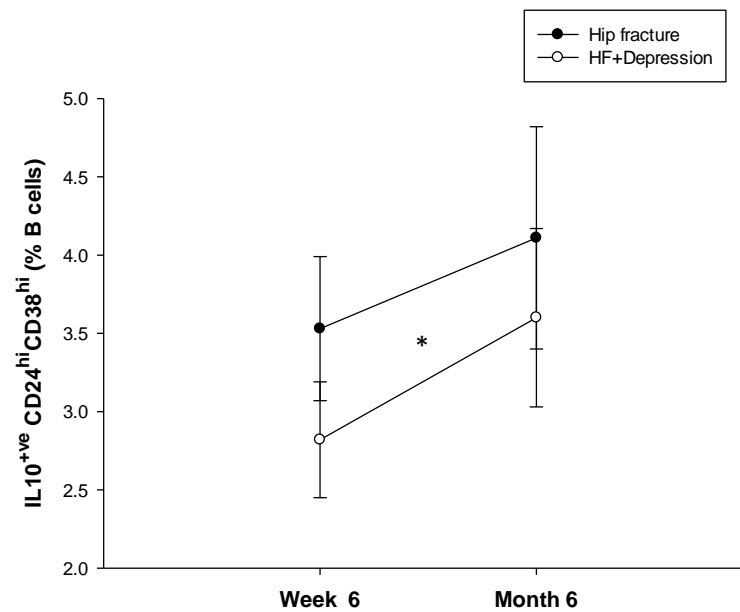
Figure 4.15 IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} B cells in hip fracture patients 6 months post-surgery.

Percentage of IL10^{+ve}CD19^{+ve}CD24^{hi}CD38^{hi} B cells post CD3 stimulation in hip fracture patients without depressive symptoms (n = 9) and hip fracture patients with depressive symptoms (n = 14) 6 weeks and 6 months post-surgery. Data are mean ± SEM and * indicates p <.05.

4.3.8 Cortisol:DHEAS ratio and adaptive immune parameters

An elevated serum cortisol:DHEAS ratio acts as a marker for depressive symptoms [536] and in this study GDS scores were also significantly associated with the cortisol:DHEAS ratio (described in chapter 3) [522]. However, there was no association between serum cortisol: DHEAS ratio and T cell frequency in hip fracture patients, β = -.09, p = .38, ΔR^2 = .009 or absolute T cell numbers β = -.21, p = .24, ΔR^2 = .04. Similarly, no association was observed between serum cortisol levels and frequency of circulating T cells β = -.17, p = .18, ΔR^2 = .03 or absolute T cell numbers β = -.20, p = .27, ΔR^2 = .04 in the hip fracture patients (data not shown).

There was also no association between serum cortisol: DHEAS ratio and the frequency of senescent CD28^{-ve} CD57^{+ve} T cells, β = .16, p = .35, ΔR^2 = .02, CD69^{+ve} T cells, β = .12, p = .47, ΔR^2 = .01 (data not shown) or HLADR^{+ve} T cells in hip fracture patients, β = .04, p = .81, ΔR^2 = .002. Further, no significant association was found between the serum cortisol: DHEAS ratio and IL10 producing CD4 T cells in hip fracture patients, β = .24, p = .10, ΔR^2 = .24, CD24^{hi}CD38^{hi} B cell frequency in the resting state, β = -.15, p = .09, ΔR^2 = .08 or in IL10 producing CD24^{hi}CD38^{hi} B cells post CD3 stimulation in hip fracture patients, β = -.15, p = .35, ΔR^2 = .02.

4.3.9 Proinflammatory cytokines and T cells

On examining associations between pro-inflammatory cytokines and circulating T cells we have reported a significant association between circulating TNFα levels and frequency of circulating T cells, β = -.34, p = .04, ΔR^2 = .12 in whole sample, such that patients with elevated serum levels of TNFα had fewer circulating T cells [Figure 4.16]. Similarly, a significant association between circulating TNFα levels and absolute numbers of circulating T cells, β = -.52, p = .03, ΔR^2 = .27 was observed in whole sample, such that patients with

elevated serum levels of TNFα had fewer circulating absolute numbers of T cells (data not shown).

However, no significant associations was reported between serum IL6 levels and frequency of circulating T cells, β = .04, p = .71, ΔR^2 = .002 in whole sample (data not shown).

A significant association was found between circulating IL6 levels and the frequency of senescent CD28^{-ve} CD57^{+ve} T cells, β = .37, p = .01, ΔR^2 = .13, such that patients with higher serum levels of IL6 had a higher frequency of circulating CD28^{-ve} CD57^{+ve} T cells [Figure 4.17a]. A similarly association was observed between serum TNFα levels and the frequency of circulating activated CD28^{-ve} CD57^{+ve} T cells, β = .44, p = .01, ΔR^2 = .19 [Figure 4.17b], but not for serum IL1β levels, β = .26, p = .13, ΔR^2 = .06 (data not shown).

Elevated pro-inflammatory cytokines , such as IL6 have been proposed as an underlying cause of immune activation in patients with depressive symptoms[637]. On examining associations between IL6 levels and T cell activation status a significant association was found between serum IL6 and frequency of circulating activated HLADR^{+ve} T cells, β = .38, p = .01, ΔR^2 = .15, such that patients with higher serum levels of IL6 had a higher frequency of circulating HLADR^{+ve} T cells [Figure 4.17c].

However, there was no association between serum IL6 levels and frequency of circulating activated CD69^{+ve} T cells, β = .16, p = .30, ΔR^2 = .02. There was also no association between serum TNFα and IL1β levels and activated T cells (HLADR^{+ve}, CD69 $^{+ve}$, CD25^{+ve}; data not shown).

p = .04
$R^2 (35) = .12$
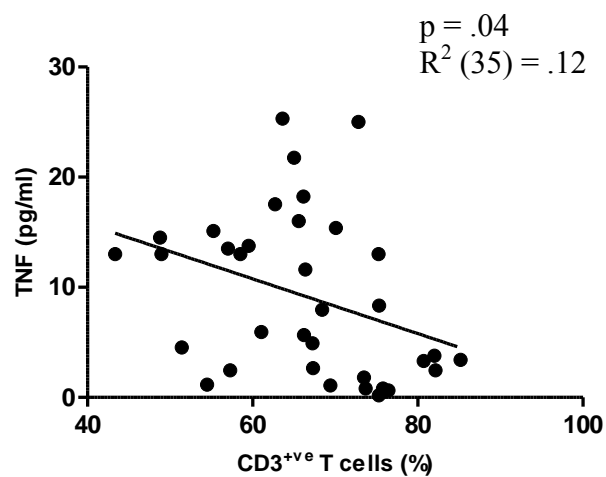
Figure 4.16 Association between circulating TNFα levels and peripheral T cells.

Representation of correlation between serum TNFα levels and circulating T cells in hip fracture patients (n = 35).
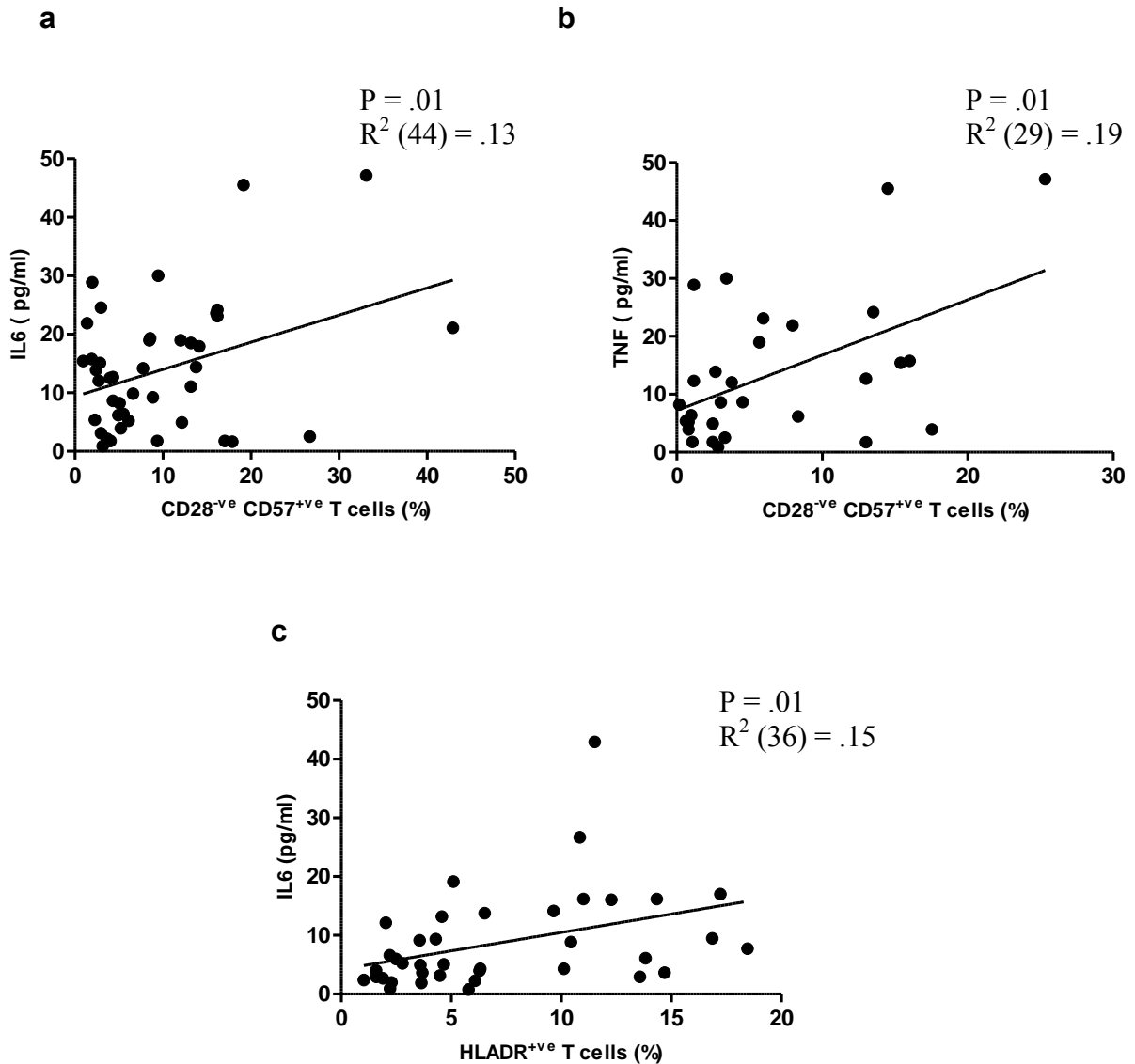
Figure 4.17 Association between circulating pro-inflammatory cytokines and senescent and activated T cells.

Representation of correlation between (a) serum IL6 levels and circulating CD28[-ve] CD57[+ve] T cells (n = 44) (b) serum TNFα levels and circulating CD28[-ve] CD57[+ve] T cells (n = 29) (c) serum IL6 levels and circulating HLADR[+ve] T cells (n = 36).

4.4 Discussion

In the previous chapter, the immunosuppressive effects of depressive symptoms in hip fracture patients on innate immune cells were described. In this chapter, it was reported for the first time that the development of depressive symptoms in hip fracture patients was accompanied by a decline in total circulating T cells and an accumulation of senescent and activated T cells. Further on examining the effect of depressive symptoms on regulatory immune cells, the data show no effect on the frequency of T_{regs}, but an increase in IL10 production in CD4 T cells. Interestingly, both numerical defects and impaired IL10 production was observed in B_{regs} in hip fracture patients with depressive symptoms. For the majority of the observations, as with innate immunity, compromised function was restricted to those patients who developed depressive symptoms and was not seen hip fracture alone.

The reduced T cell frequency and absolute numbers in hip fracture patients with depressive symptoms compared with healthy controls is in agreement with previous reports of reduced circulating T cells in depressed individuals [486, 638, 639]. Although the exact mechanism responsible has not been explored in this thesis, a few possibilities can be proposed. Firstly, T cells in depressed individuals have been shown to undergo accelerated apoptosis, especially CD4 T cells [640, 641]. Further, glucocorticoids are known inducers of T cell apoptosis [624, 642] and the hip fracture patients with depressive symptoms had higher serum cortisol levels [522]. However, there was no association between circulating T cells and serum cortisol levels, suggesting that additional mechanisms may be involved. TNFα is also known to induce T cell apoptosis [643, 644] and elevated serum TNFα was seen here in hip fracture patients with depressive symptoms (described in chapter 3). Interestingly, we have reported an association between circulating TNFα levels and peripheral T cells in this study. Unlike T cells, neither the frequency nor absolute B cell numbers were affected in hip fracture

patients with depressive symptoms, similar findings have been reported in studies of depressed individuals [645, 646].

The proportion of CD4 and CD8 T cells and the CD4:CD8 ratio did not differ between hip fracture patients with and without depressive symptoms and healthy controls. Our findings are in line with a few previous reports showing that the CD4:CD8 ratio was unaltered in depressed individuals [413, 647]. However, there are also reports showing an increase in the CD4:CD8 T cell ratio in depressed individuals [637, 646]. It is possible that these inconsistent findings might be a result of differences in the severity and chronicity of depression in different studies, in particular it has been suggested that the increase in the CD4:CD8 ratio is related to severity of depression [648]. Furthermore, hip fracture alone or in synergy with depressive symptoms did not have an effect on naïve: memory ratio of T cells. These findings contradict a previous study reporting an increase in memory T cells in depressed patients [648, 649]. This discrepancy might be due to the differences in cell surface markers used to identify memory T cells; Maes *et al* identified memory T cells as CD45RA^{-ve} T cells, whereas in this study both CD45RA and CCR7 were used to identify T cell subsets.

Ageing is accompanied by an accumulation of CD28^{-ve} T cells (described in chapter1). On examining the additional effect of chronic distress on CD28 expression, a significant increase in the percentage of CD28^{-ve} T cells was seen, especially in CD8 T cells in hip fracture patients with depressive symptoms. Moreover, an increase in CD57^{+ve} T cells, again most marked in CD8 T cells, was also observed in hip fracture patients with depressive symptoms, which is in line with previous studies in depressed individuals[648]. Interestingly, poor mental health associated with reduced job satisfaction has also been characterised by an increase in CD57^{+ve} CD8 T cells [612]. The lack of CD28 expression or gain of CD57 expression has been identified as markers for replicative senescence [632]. The majority of the

CD57^{+ve} T cells are also CD28^{-ve} [650], therefore it is not surprising that an accumulation of CD28^{-ve}CD57^{+ve} T cells was seen in hip fracture patients with depressive symptoms and this has also been reported in depressed individuals [651]. CD28^{-ve} CD57^{+ve} T cells are characterised by critically shortened telomeres and impaired ability to undergo cell-cycle division and an expansion of this subset has been reported during immune activation [200]. The accumulation of these expanded T cells might reduce T cell repertoire in hip fracture patients with depressive symptoms and reduce their immune response towards novel pathogens and vaccines [652, 653].

TNFα is known to induce down regulation of CD28 expression on T cells [654]. Ageing and inflammatory diseases have been associated with elevated levels of pro-inflammatory cytokines [290, 655] and accumulation of CD28^{-ve} T cells [192, 656]. In the previous chapter, elevated serum levels of IL6 and TNFα in hip fracture patients with depressive symptoms were described. Interestingly, there was a correlation between circulating CD28^{-ve} CD57^{+ve} T cells and serum IL6 and TNFα levels. Thus, it is tempting to suggest that the pro-inflammatory environment in hip fracture patients with depressive symptoms might be contributing towards accumulation of CD28^{-ve} CD57^{+ve} T cells.

Cytomegalovirus (CMV) is a β herpes virus that is highly prevalent in over 65 year olds [14, 657] and is a source of continuous antigenic stimulation [188]. CMV seropositivity has been associated with senescence in the T cell compartment and accumulation of late differentiated CD28^{-ve} CD57^{+ve} T cells [658, 659]. As individuals with higher antibody titres against CMV exhibit higher symptoms of depression, it is possible that rather than CMV infection itself the magnitude of CMV-specific immune responses is associated with depressive symptoms [651, 660]. Although, CMV seropositivity has not been tested in our hip fracture patients, it is

possible that there might be an association between CMV titres in our hip fracture patients and accumulation of CD28^{-ve} CD57^{+ve} T cells in hip fracture patients.

Furthermore, chronic viral infections (such as CMV) often result in terminal differentiation of pathogen specific CD8 T cells into exhausted T cells, which have upregulated expression of inhibitory surface receptor PD-1 [661]. T cell exhaustion is characterised by a loss of effector T cell function , including cytokine production , proliferation capacity and ex vivo killing [662, 663]. Limited studies examining PD-1 with age have reported an increase in PD-1 expression on T cells of aged mice [453, 663]. However, no study has so far has examined the effect of exposure to prolonged stress on exhausted T cells and future studies could test the possibility that in addition to an accumulation of senescent T cells there would be an increase in exhausted PD-1^{+ve} T cells in hip fracture patients with depressive symptoms.

In addition to being an inflammatory disorder, depression is also characterised by activation of cell-mediated immunity [664]. Although this study failed to report signs of monocyte activation in hip fracture patients with depressive symptoms (discussed in chapter 3), an increase in T cells expressing the activation markers CD69 and HLADR was seen in hip fracture patients with depressive symptoms. These findings are consistent with previous reports of increased activated HLADR^{+ve} T cells in depressed individuals [486, 649, 665]. In contrast , the frequency of cells expressing CD25 did not differ between hip fracture patients and healthy controls, but these findings do not agree with a previous report showing an increase in CD25^{+ve} activated T cells in depressed patients [665].

It has been previously hypothesised that cytokines such as IL6 and IL1β are the underlying cause of immune activation reported in depressive individuals [637]. Interestingly, in this chapter we have reported an association between serum IL6 levels and HLADR^{+ve} T cells,

suggesting that the pro-inflammatory environment in hip fracture patients with depressive symptoms might be contributing towards accumulation of activated T cells. Although unexplored in this thesis, soluble interleukin-2-receptor (s IL2R) which has been identified as an additional marker of T cell activation has been reported to be increased in depressed individuals [666, 667].

Regulatory cells are known to suppress unwanted immune responses. Therefore, it is tempting to hypothesise that a deficiency in regulatory immune cells might contribute towards the inflammatory state that was seen here in hip fracture patients with depressive symptoms. In this chapter, surprisingly no significant differences were observed in the frequency of circulating CD4^{+ve}CD25^{+ve}Foxp3^{+ve} T$_{regs}$ in hip fracture patients with depressive symptoms, which contradicts the findings of two previous studies showing a decline in T$_{regs}$ in mice with depression-like behaviour [668] and humans with major depression [669]. In the second chapter of this thesis, an age associated numerical deficit in B$_{regs}$ with age was described. In this chapter, a further reduction in both frequency and absolute numbers of circulating CD19^{+ve}CD24^{hi}CD38^{hi} B cells was seen in hip fracture patients with depressive symptoms.

Both T$_{regs}$ and B$_{regs}$ are known to mediate a suppressive effect on effector T cell responses via IL10 production [301, 670] . Although IL10 production by CD4^{+ve} CD25^{+ve} T cells was not considered here, it has been previously reported that CD4^{+ve} CD25^{-ve} T cells secrete very little IL10 but are known to produce IFNγ, but CD4^{+ve} CD25^{+ve} T cells on the other hand predominantly produce IL10 and produce only low levels of IFNγ and IL4 [671] . The data in this chapter showed a marked increase in IL10 production by CD4 T cells of hip fracture patients with depressive symptoms compared to healthy controls. Interestingly, a significant positive association was also found between GDS scores and IL10 production by CD4 T

cells in our hip fracture patients .Although this is the first study to examine the effect of depressive symptoms on IL10 production by CD4^{+ve} T cells , a few studies examining the effect of chronic stress such as caregiving have reported increased IL10 production [614, 619]. Interestingly, cortisol has been shown to enhance IL10 production by CD4 T cells [672, 673], though in this chapter no association was found between frequency of IL10^{+ve} CD4 T cells and serum cortisol levels.

In the case of the functional capacity of B$_{regs}$, a significant decline in IL10 producing CD19^{+ve}CD24^{hi}CD38^{hi} B cells upon CD3 stimulation was observed in hip fracture patients with depressive symptoms. Again to the best of our knowledge this is the first study to examine the effect of psychological distress on IL10 production by B cells. These data are surprising when considered with the increased IL10 production by CD4 T cells in hip fracture patients with depressive symptoms. Although the effect of glucocorticoids on B$_{regs}$ remains unexplored, it is possible that elevated cortisol levels observed in hip fracture patients with depressive symptoms had an opposite effect on IL10 production by B cells and T cells.

As mentioned in the introduction to this thesis, ageing is accompanied by extensive remodelling of the immune system, including decline in thymic output, accumulation of senescent CD28^{null} T cells and increase circulating levels of pro-inflammatory cytokines (IL6, TNFα). Similar phenotypic and functional signs of premature immunesenescence have been shown in RA patients including; elevated levels of serum proinflammatory cytokines (IL6, TNFα) [674, 675], increased proportion of autoreactive CD28^{-ve} CD4 T cells [676-678] and reduced circulating number and impairment in IL10 production by CD19^{+ve}CD24^{hi}CD38^{hi} B cells [301, 302]. Chronic psychological distress has been associated with exacerbation of autoimmune disorders [679, 680] . A higher incidence of autoantibodies has been shown in

psychiatric disorders [681], including depression [667]. The results of this chapter suggest the possibility that exposure to psychological distress challenges results in a further decrement in immunity, enhancing immunesenescence, as evidenced by even higher circulating levels of pro-inflammatory cytokines, increased senescent T cells and a further decline in circulating $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cell and their ability to produce IL10. Taken together these factors may contribute to the poorer prognosis in hip fracture patients with depressive symptoms, making them more susceptible to infection and inflammation related frailty and pathology.

In summary, in this chapter the data show that development of depressive symptoms in older hip fracture patients is characterised by a reduction in circulating T cells, accumulation of $CD28^{-ve}CD57^{+ve}$ senescent T cells and $HLADR^{+ve}$ and $CD69^{+ve}$ T cells and also numerical and functional defects in B_{regs}. Moreover, the suppressive effect of depressive symptoms on the frequency of B_{regs} in hip fracture patients persisted up to 6 months post-surgery.

Chapter 5

General Discussion

Organisms are challenged with a number of physiological and psychological stressors during their lifetime and the ability to handle stressors has a profound effect on health [353]. Over the years a number of models of chronic stress including life stressors such as marital stress, bereavement, caregiving, feelings of loneliness and social disruption have been reported to have a detrimental effect on immune function [357, 682], which might have clinical consequences such as increased risk of infections [683, 684] and impaired vaccine responses [358].

The worldwide prevalence and disability caused as a result of depression has made it a major public health issue [685]. Depression was rated as the fourth leading cause of disease burden in 2000 [686]. The term depression is sometimes used to refer to depressive illness, and sometime to the presence of depressive symptoms. In much of the research into these psychiatric symptoms in physical health conditions, depressive symptom indices are used, and so the term depression often refers to the presence of depressive symptoms rather than a psychiatrically-diagnosed condition. A high incidence of depression (that is depressive symptoms) occurs in individuals suffering from chronic diseases [687, 688] and the development of depressive symptoms has been identified as a potential risk factor for decrements in health and increased morbidity and mortality in these patients [689, 690]. Previous studies have reported a high prevalence of depressive symptoms in hip fracture patients post-surgery [435, 439]. In addition to being a psychiatric disorder, depressive symptoms has also been recognised as an immune disorder [691]. In this thesis, for the first time the interactive effects of physical trauma (hip fracture), psychological distress (depressive symptoms) and ageing on the immune system have been examined. The key finding was that new onset depressive symptoms post hip fracture can further suppress age associated impairments in both innate and adaptive immune responses, including a recently

identified subset of B cells with immune regulatory properties. Interestingly by 6 weeks after the hip fracture, immunity was not reduced in the patients who only had a fracture, suggesting that either the physical stressor was less potent or was transient. Hopefully, these data will result in a fresh analysis of the need to ameliorate depressive symptoms in this vulnerable patient group.

5.1 Summary of main findings

In the first study of this thesis (chapter 2) the effect of age on B cells with immune suppressive properties, namely transitional B cells and B10 cells, was examined. There was a decline in both peripheral frequency and absolute numbers of $CD19^{+ve}CD24^{hi}CD38^{hi}$ and $CD19^{+ve}CD5^{+ve}CD1d^{hi}$ B cells with age. Impairments in IL10 production were also observed in both these subsets of B_{regs} post-stimulation via CD40 or TLR pathway in healthy older donors. On investigating the mechanisms involved in suppressed IL10 production by B_{reg} cells, age-related effects on both T cells and B cells were observed, specifically CD40 ligand expression was lower in CD4 T cells from older donors following CD3 stimulation and signalling through CD40 was also impaired in regulatory B cells from older adults as evidenced by reduced phosphorylation and activation of STAT3. Also, impairments in IL10 secretion by isolated B cells on stimulation via CD40 and TLR pathway were observed. Finally, there was a negative correlation between IL10 production by $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cell and serum autoantibody (rheumatoid factor) levels in healthy individuals. It was therefore proposed that an age-related decline in $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cell number and function may contribute towards the increased autoimmunity and reduced immune tolerance seen with advancing age.

In the second major study in this thesis (chapter 3 and chapter 4) the potential synergistic effects of physical distress (hip fracture) and psychological distress (depressive symptoms) on immunesenescence was investigated. The data revealed a suppressive effect of depressive symptoms on neutrophil, monocytes and NK cell functioning in older hip fracture patients, but not of the hip fracture alone. Additionally, the suppression in monocyte and NK cell functioning in hip fracture patients with depressive symptoms remained up to six months post-surgery.

Further, the data also showed that the development of depressive symptoms was associated with HPA axis activation, resulting in elevated serum cortisol levels in hip fracture patients with depressive symptoms. Additionally, a reduction in serum DHEAS levels was also observed in these patients, resulting in an overall elevated serum cortisol: DHEAS ratio in hip fracture patients with depressive symptoms that remained elevated six months post-surgery. Cortisol is known to have immune suppressive properties and DHEAS is an immune enhancer. Thus, this elevated serum cortisol: DHEAS ratio in hip fracture patients with depressive symptoms might be one of the factors contributing towards the immune suppression seen in these patients.

Ageing is accompanied by an increase in circulating levels of pro-inflammatory cytokines (section 1.2) .In this thesis it was reported that the additional effect of psychological distress on the aged immune system resulted in a further increase in levels of pro-inflammatory cytokines, including IL6 and TNFα in hip fracture patients with depressive symptoms. Also, an increase in serum IL10 levels was observed in hip fracture patients with depressive symptoms.

Further, on examining the effect of physical and psychological distress on adaptive immune cells, there was a decline in circulating T cells in hip fracture patients with depressive symptoms, but no change in the CD4:CD8 ratio and naïve:memeory T cells ratio. Additionally, CD28^{-ve} CD57^{+ve} senescent T cells were reported to be raised in hip fracture patients with depressive symptoms and there was an accumulation of activated CD69^{+ve} and HLADR^{+ve} T cells in hip fracture patients with depressive symptoms.

Finally, on examining the effect of chronic distress on regulatory immune cells, although there were no differences in circulating levels of T$_{regs}$, a dramatic reduction in B$_{regs}$ was observed in hip fracture patients with depressive symptoms that remained for six months post-surgery. IL10 production by T cells post-stimulation was increased but IL10 production was reduced in B$_{reg}$ cells in hip fracture patients with depressive symptoms.

5.2 Expected outcome and future direction

In this thesis, it was suggested that numerical and functional defects in B$_{reg}$ cells with advancing age might be a novel contributing factor towards increased risk of autoimmunity in older adults. Although unexplored in this thesis , there have been previous reports of a decline in the frequency of circulating immunosuppressive CD19^{+ve}CD24^{hi}CD38^{hi} B cells in RA patients [302]. Thus, in the future development of therapeutic agents to improve B$_{reg}$ cell numbers and/or function in older adults could reduce incidence of autoimmune disorders. In one such approach, isolation of CD19^{+ve}CD24^{hi}CD38^{hi} B cells from older RA patients followed by *in-vitro* expansion and re-infusion of these B cells into older adults might be useful in suppressing autoimmunity. Additionally, it might also be possible to identify older adults at higher risk of developing autoimmune disorders by examining the functioning of their B$_{reg}$ cells.

The data in this thesis raise a variety of research questions that can be addressed in future studies. Although we have reported numerical and functional defects in CD19^{+ve}CD24^{hi}CD38^{hi} B cells with age, the immunosuppressive effect of IL10 on young and aged immune cells still remains unexplored. In future studies, it might be interesting to examine the effects of IL10 on production of pro-inflammatory cytokines (IFNγ) in young and old CD4^{+ve} T cells. Additionally, the effect of IL10 on suppression of pro-inflammatory cytokine production (TNFα, IL6) and antigen presentation (surface expression of MHC class II and co stimulatory molecule) by young and aged monocytes could also be examined. This will provide novel insight into the effect of age on anti-inflammatory responses to IL10. It is possible that in addition to impaired IL10 production and secretion with age the response to IL10 is also defective in older adults.

This thesis also reported that depressive symptoms post hip fracture in older adults results in poorer functional outcome, increased immune dysregulation and altered adrenal hormone balance. These findings can have several clinical implications and help improve patient care. Firstly, the findings highlight the importance of screening for the presence of depressive symptoms in hip fracture post-surgery. It is also possible that other major surgery may produce the same outcome regarding depressive symptoms and the data may therefore have more far reaching importance. Secondly, this study provides an evidence base for the use of interventions to reduce depressive symptoms as a way of reducing immune dysregulation and improving outcome in hip fracture patients. Thus, we suggest that stress management and psychological support can help improve the immune outcome post hip fracture in older adults.

The treatment of depressive symptoms in hip fracture patients with antidepressants might improve their psychological well-being and might also has a beneficial effect on their

immune cell functioning. Firstly, antidepressants are capable of restoring HPA axis activity and glucocorticoid levels, by normalisation of GR mediated feedback inhibition of the HPA axis [565, 692]. Additionally, two independent clinical studies have reported an additional improvement in NK cell activity on treatment with anti-depressants [566, 693, 694] . Interestingly, antidepressants have a suppressive effect on production of pro-inflammatory cytokines by activated monocytes and Tcells [695, 696]. In-vivo studies have also reported that treatment with selective anti-depressants, including selective serotonin reuptake inhibitors (SSRI) can normalise initially increased serum IL6 levels in depressed individuals[697, 698]. Therefore, it is not surprising that anti-inflammatory medication has been suggested as an alternative treatment option for depressive symptoms [699, 700] and might be an additional therapeutic strategy to treat depressive symptoms post hip fracture. However, caution is required as an association has been shown between the rate of falls and use of antidepressant drugs in older adults [701]. The use of antidepressants can also have negative effects on the microarchitecture of bone and increase the future risk of another hip fracture [702-704].

In contrast a range of beneficial effects of DHEA supplementation has been reported in older adults including ; improvement in arterial stiffness [705], improved physical performance [706] and exercise capacity [707] as well as reduced rates of infections [708]. Additionally, a few clinical studies have also reported that DHEA supplementation has a beneficial effect on psychological well-being of individuals, including reduction of depressive symptoms and anxiety [709, 710] and DHEA also has a mood enhancing effect [711]. Further, anti-glucocorticoid effects of DHEA have also been reported [364]. Interestingly, studies in animal models showed that treatment with DHEA ameliorated the trauma induced increase in circulating levels of corticosteroids [712]. Further, DHEA supplementation is also known to modify immune function in vivo [395], including improvement in the age-associated

dysregulation in IL6 production [407]. Immune enhancing effects of DHEAS have been reported in *in vitro* studies of neutrophil superoxide generation [575], NK cell cytotoxicity [579, 713] and T cell proliferation and cytokine production [712]. Moreover, a previous study by our group has shown that cortisol mediated suppression of neutrophil superoxide generation *in vitro* that could be prevented by coincubation with DHEAS [411].

The findings in this thesis provide a rationale for the use of DHEA supplementation as an intervention to reset cortisol: DHEAS ratio and improve psychological and physical outcome and thereby indirectly reduce immune dysregulation in hip fracture patients with depressive symptoms. DHEA supplementation might be therapeutically useful in preventing development of depressive symptoms and would avoid the side effects on bone density and falls incidence associated with the use of antidepressants. In future, intervention trials for determining an effective dosage and regime for a beneficial effect of DHEAS supplementation on emotional distress, physical frailty and immune dysregulation in hip fracture patients are required.

In addition to pharmacological interventions, counselling, psychological support, broadening social involvements [714] and maintaining healthful practices (proper diet, exercise) may also help to combat the deleterious effects of psychological distress or prevent its onset and thereby preserve immune functioning. For instance, a study has suggested a protective effect of good social support (marriage, frequent contact with friends and family) on cancer-related stress and immunity, facilitating recovery in cancer patients [715]. Beneficial effects of physical activity have been reported on depression [716, 717], with moderate exercise associated with increased DHEAS levels [718], reduced serum cortisol levels [719], reduced serum circulating IL6 and TNFα levels [719, 720] and reduced frequency of antigen experienced and senescent T cells [721] during chronic stress. Physical activity interventions

might therefore help restore cortisol and DHEAS balance and prevent immune dysregulation in hip fracture patients with depressive symptoms. Interestingly, music therapy has also been shown to a reduce stress related cortisol/DHEAS imbalance and has been shown to have a beneficial effect on immune parameters, such as NK cell activity [722], and might be an alternative stress coping strategy.

Although emotional well-being is not usually the focus of orthopaedic care, the data from this thesis suggest that early detection and treatment of psychological distress reducing would help improve the quality of life in older hip fracture patients, improve their degree of recovery of physical functioning, reduce loss of immunity and thus reduce costs of hospitalisation and social care.

5.3 Study limitations

A numerical and functional deficit in B_{reg} cells was seen with advancing age. However, it was not possible to isolate $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells from peripheral blood of our participants to determine if they showed reduced ability to suppress T cell responses, the ultimate test of regulatory function. This was mainly due to the extremely low circulating numbers of these cells in peripheral blood and the large volumes of blood that would be required. As a result, to look for age associated impairments in regulatory function IL10 secretion was assessed by ELISA on cultures of B cells rather than cultures on $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells. In future studies it would be worth doing co-culture assays of $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells and CD4 T cells to examine the ability of cells from young and old donors to suppress CD4 T cell proliferation and cytokine production (TNFα, IFNγ).

The next two chapters reported the findings from the hip fracture study. One of the biggest limitations of this study was that it was not possible to recruit a healthy older population

with depressive symptoms alone to compare our findings reported in hip fracture patients with depressive symptoms. This was because any individuals that we might be able to identify from general practitioner records would already be undergoing anti-depressant treatment, which was one of our exclusion criteria. However we do know from other work in the group that psychological distress alone, specifically bereavement, in older adults also results in reduced neutrophil superoxide generation [360]. This aspect of innate immunity may therefore be particularly susceptible to the negative effects of stress. What would also have been interesting would have been to assess immune function closer to the time of injury, such as a few days after, to determine if the physical stress did have an effect but that this was transient. At that time it may have been possible to detect additive effects of physical and psychological stress.

Secondly, our exclusion of previously depressed patients or those with cognitive impairment, or immune-related comorbidities means that the findings may not be generalised to the wider hip fracture population. However, this had to be done to exclude the possible effects of various comorbidities on immune function of hip fracture patients, to exclusively examine the effect of depressive symptoms on immunity. It is also difficult sometimes to distinguish cognitive impairment from depression, and so including people with cognitive impairment could confound the investigation into the specific effect of depressive symptoms. Also a person with a previous history of depression may have other factors driving the depressive symptoms that would have made attempts to identify causative agents much more difficult. However, if anything you would expect the immune dysregulation to be even worse in depressed hip fracture patients in the presence of additional co morbidities.

Also, we were only able to measure the serum cortisol and DHEAS levels in hip fracture patients at one time during the day, though this was kept constant. Ideally, a diurnal assessment would be undertaken to give a more a fuller picture of changes in hormone secretion. However, in this frail patient group, it was not practical or ethical to increase the number of blood samples taken.

Finally, there were a relatively high number of withdrawals in the study as many patients felt too unwell to participate, but this is common in studies of hip fracture in older patients. Thus, the sample of hip fracture patients included that have been tested at the six month time point are likely to be in relatively better health compared to those who withdrew.

5.4 Conclusions

It is well established that ageing and exposure to chronic stressors, both physical and psychological, result in immune dysregulation. Moreover, the effects of chronic stress and age can be interactive and intensify health threats in older adults. In this thesis, it has been demonstrated for the first time that development of depressive symptoms in hip fracture patients results in immune dysregulation and elevated pro-inflammatory cytokine levels, whereas the hip fracture alone had minimal impact at the time points assessed. Adrenocortical hormone imbalance was also demonstrated in hip fracture patients depressive symptoms and might be contributing indirectly to the immunosuppression seen in these patients. Additionally, an age associated numerical and functional defect in a novel subset of $CD24^{hi}CD38^{hi}B$ cells with immunosuppressive properties was demonstrated and this defect worsened in the presence of chronic psychological distress in older adults. Overall, the data supports the original hypothesis of a relationship between psychological distress and immune related health, but did not confirm synergy between psychological and

physical stress in the older adults studied. The findings are relevant to healthcare professionals and highlight the importance of routine screening for depressive symptoms and providing appropriate interventions to improve recovery post hip fracture. Furthermore, it is proposed that correcting the elevated cortisol: DHEAS levels in hip fracture patients by DHEA supplementation could help improve psychological well-being reduce physical frailty and restore immune function in this patient group.

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7.0 Appendix

7.1 List of publications

<u>Book Chapter</u>

2013 – Duggal**, N.A** and Lord, J.M. Introduction to ageing of the innate immune system. *Immunesenescence – Psychosocial and behavioural determinants.*

<u>Published Manuscripts</u>

1. **Duggal, N.A**, Upton.J, Sapey, E., Phillips, A.C and Lord, J.M. An age-related numerical and functional deficit in $CD19^{+ve}CD24^{hi}CD38^{hi}$ B cells is associated with an increase in systemic autoimmunity. *Aging Cell 2013* Oct;12(5):873-81

2.**Duggal, N.A**, Upton.J, Phillips, A.C, P.Hampson, and Lord, J.M. Depressive symptoms are associated with reduced neutrophil function in hip fracture patients. *Brain behaviour and Immunity* 2013 Jul 20.

3. Phillips, A.C, Upton.J**, Duggal, N.A**, Carroll, D and Lord, J.M. New onset depression following hip fracture is associated with increased physical frailty in older adults: the role of the cortisol: DHEAS ratio. *BMC Geriatrics* 2013 Jun 17; 13:60.

4. **Duggal, N.A**, Beswetherick.A, Upton.J, Phillips, A.C, P.Hampson, and Lord, J.M. Depressive symptoms are associated with reduced monocyte superoxide production. *Exp Gerontol*. 2014 Feb 8

<u>Manuscripts in press and preparation</u>

1. **Duggal, N.A**, Upton.J, Phillips, A.C, P.Hampson, and Lord, J.M. Depression in hip fracture patients is associated with impaired NK cell cytotoxicity which might be a result of reduced perforin expression (In press).

2. **Duggal, N.A**, Upton.J, Phillips, A.C, P.Hampson, and Lord, J.M. New onset of depressive symptoms post hip fracture surgery in older adults is associated with phenotypic and functional alterations in T cells (In preparation).