Defining the mechanisms underlying reduced immunity to *Streptococcus pneumoniae* with age

by

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A thesis submitted to the
University of Birmingham for the degree of
DOCTOR OF PHILOSOPHY

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September 2018

Abstract

This thesis tested the hypothesis that immunosenescence contributes to reduced immunity to *Streptococcus pneumoniae.* The effect of age on neutrophil and monocyte responses to *S. pneumoniae* and on $CD4^+$ T cell polarisation during health, pneumococcal carriage and clinical pneumonia infection were determined.

Older adult's neutrophils produced less ROS in response to serotypes 19A and 23F, but not 4, and increased NETs towards 23F. However, neutrophils of older pneumonia patients produced high levels of ROS to all three serotypes but had impaired NET release. Older patients also had immature granulocytes and $CD16^{high}CD62L^{dim}$ neutrophils in blood. CCR2 and CD11b expression, TNF-α and IL-6 production by monocytes were unaffected by age.

Pneumococcal colonisation of the nasopharynx is an immunising event. The effect of age on carriage was tested using a human carriage model. Older adults had elevated Th1 and lower Th17 frequencies and failed to generate Th17 memory. During pneumonia, pro-inflammatory subsets increased with age, but Treg frequency and function were maintained.

In conclusion, failure of pneumococcal carriage to generate immune memory, together with altered neutrophil responses to *S. pneumoniae* and high frequencies of inflammatory Th subsets in older adults who succumb to infection, could contribute to their increased susceptibility to pneumococcal infection.

For Mom and Dad
Thank you for everything

Acknowledgements

First and foremost, I would like to thank my supervisors Professor Janet Lord and Professor Tim Mitchell for providing me with the opportunity of doing a PhD with them. I would specially like to thank Professor Lord, for the unique support, advice and encouragement throughout my PhD, for which I am very grateful. Special thanks must also go to Professor Mitchell for the advice, discussion and ideas.

I would like to thank my funders the CAPES Foundation, for the financial support. My thanks also go to all the Lord Group members and Pneumococcal lab for making my time in the lab enjoyable. A big thanks go to Mrs Hema Chahal for all sorts of advice, from lab work to life, and to Dr. Jon Hazeldine for his advice and patience in answering lots of questions. Thank you to all the volunteers and patients involved, without whom this work would not have been possible.

I would like to thank Dr Daniela Ferreira for the opportunity of collaborating with her team. A special thanks to Simon Jochems, Jesús Reiné and Carla Solórzano-Gonzalez for all the assistance during the period I worked in Liverpool. I would like to thank Professor David Thickett, Professor Elizabeth Sapey and Dr Davinder Dosanjh for the collaboration on recruitment of patients, and to all the clinical team involved.

To my friends back in Brazil, Estevam, Felipe and Ayrton for always being there. Thank you, guys, for the companionship, the laughter and online games. Thank you to all the good friends I made here in England, who were always around and made these years in the UK a wonderful experience.

I would also like to thank my parents, Cristina e José Henrique, for their endless love, guidance, and support. Thank you for everything you have given me, your support always

made the difficult choices look easier. To my brother Gustavo, for his support, sense of humour and companionship. And to my grandparents, simply for being there. I hope this work makes you proud.

And finally, to Pedro, for his endless love, patience and encouragement. Thank you for your support throughout the good moments but mostly, for staying throughout the “not so good” ones. I look forward to our new adventures together.

Table of contents

List of Figures

Chapter 1. Introduction

Chapter 3. The effect of age on the innate immune response to *S. pneumoniae*

Chapter 4. The effect of age on $CD4^+$ T lymphocyte polarisation during Experimental Human Pneumococcal Carriage (EHPC) and pneumonia infection

Chapter 5. General Discussion

List of tables

Chapter 1. Introductions

Chapter 2. Materials and Methods

Chapter 3. The effect of age on the innate immune response to *S. pneumoniae*

Chapter 4. The effect of age on $CD4^+$ T lymphocyte polarisation during Experimental Human Pneumococcal Carriage (EHPC) and pneumonia infection

Appendices

Abbreviations

Ab	Antibody
AFU	Arbitrary fluorescence units
ANOVA	Analysis of variance
APC	Allophycocyanin
BAB	Blood agar base 2
BAL	Bronchoalveolar lavage
BD	Becton Dickinson
BHI	Brain Heart Infusion media
BSA	Bovine serum albumin
BV	Brilliant Violet ™
BV605	Brilliant Violet 605
BV711	Brilliant Violet 711
C5a	Complement component 5a
CbpA	Choline-binding protein A
CBPs	Choline-binding proteins
CCL2	C-C motif chemokine ligand 2
CD	Cluster of differentiation
CD40L	CD40 ligand
CD62L	CD62 ligand
CFU	Colony forming units
CM	Central memory
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CpG	5'—C—phosphate—G—3'
CXCL1/KC/Gro-α	Chemokine ligand 1 (CXCL1)
DCs	Dendritic cells
DHR	Dihydrorhodamine 123
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EHPC	Experimental human pneumococcal carriage
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
Foxp3	forkhead box P3)
FSC	Forward Scatter
G-CSF	Granulocyte colony-stimulating factor
GARP	Glycoprotein A repetitions predominant
GATA3	GATA binding protein 3
GM-CSF	Granulocyte macrophage colony-stimulating factor
HK	Heat-killed
HLA-DR	Human Leukocyte Antigen – DR
ICAM-1	Intercellular Adhesion Molecule 1
IFN	Interferons
IFN-α	Interferon alfa
IFN-β	Interferon beta-1
IFN-γ	Interferon gamma
IG	Immature granulocytes
Ig	Immunoglobulin
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
Io	Ionomycin
IRF-7	Interferon regulatory factor 7
LPS	Lipopolysaccharide

LSTM	Liverpool School of Tropical Medicine
LTB4	Leukotriene B4
lytA	Autolysin-Encoding Gene
MACS	Magnetic assisted cell sorting buffer
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous domain
mDC	Myeloid dendritic cells
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NALT	Nasal-associated lymphoid tissue
NanA	Neuraminidase A
NanB	Neuraminidase B
NETs	Neutrophil extracellular traps
NFkB	Nuclear factor kappa B
NK cells	Natural killer cells
OD	Optical density
PAFr	Platelet-activating factor receptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered salinePCV13
PCV	Pneumococcal conjugate vaccine
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PEcy7	Phycoerythrin-Cyanine7
PerCP/Cy5.5	Peridinin Chlorophyll Protein Complex/ Cyanine 5.5
PFA	Paraformaldehyde (PFA)
PI	Propidium iodide

PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pIgR	Polymeric Immunoglobulin Receptor
PKC	Protein kinase C
PLY	Pneumolysin
PMA	Phorbol 12-myristate 13-acetate
PPV	Pneumococcal polysaccharide vaccine
PSGL-1	P-selectin glycoprotein ligand-1
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RORγt	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SIGN-R1	Specific intercellular adhesion molecule-3-grabbing nonintegrin related 1
SIRS	Systemic inflammatory response syndrome
SSC	Side scatter
T-bet	T-box transcription factor,
TCR	T-cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Tregs	Regulatory T cells
WBC	Whole blood count
WHO	World Health Organization

Chapter 1

Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae, also referred to as the pneumococcus, is a Gram-positive bacterium. It is a diplococcus species, encapsulated by a protective polysaccharide capsule. Variation in the capsule carbohydrate structure determines different serotypes, of which more than 90 have been identified [1].

While the pneumococcus can inhabit the human nasopharynx as a commensal species, it can cause invasive and non-invasive diseases [2]. Serotype distribution varies worldwide, but approximately 20 serotypes are responsible for 70% of invasive pneumococcal disease [3]. In 2017 *S. pneumoniae* was added to the list of 12 bacteria species on the new Global Priority List of Antibiotic-Resistant Bacteria of the World Health Organization (WHO) [4].

1.2 Pneumonia and ageing

Pneumonia is a major cause of death that shows a remarkable age-related incidence, with those under 5 years and over 65 being the most susceptible [5] (Figure 1.1). Independent of age, *S. pneumoniae* is the main causative pathogen of community-acquired pneumonia (CAP) worldwide [6].

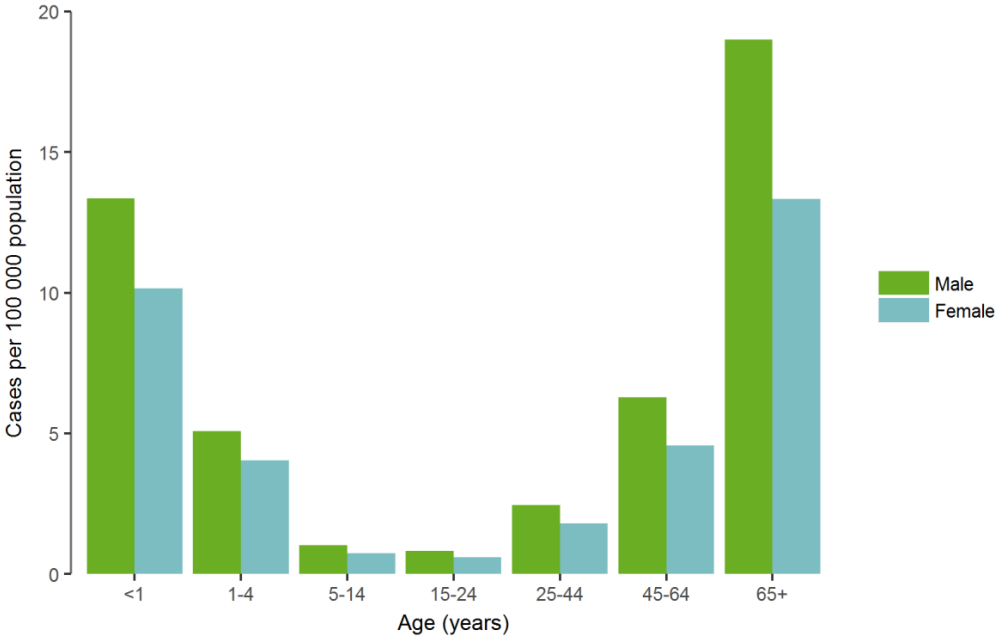

Figure 1.1. Cases of invasive pneumococcal disease by age and gender. Rate per 100 000 of confirmed cases in the European Union and European Economic Area, in 2016. The figure is taken directly from "Invasive pneumococcal disease. In: ECDC. Annual epidemiological report for 2016" by ECDC, 2018 (Ref 6).

According to the World Health Organization [8], respiratory diseases are among the five major causes of premature death in people aged 60 years and over, with chronic obstructive pulmonary disease and lower respiratory infections among the most common causes of death in this age group [8–10]. Among lower respiratory infections in older individuals, CAP caused by *S. pneumoniae* is associated with a high mortality [11,12], which increases with advancing age, rising from 7.8% of deaths of people aged 65 to 69 years, to 15.4% in people aged 90 years or older [13]. Moreover, following discharge, older patients have a 16% reduction of quality-of-life and six-fold increased mortality within a year [14].

Increased susceptibility of older adults to pneumonia is multifactorial [15], and includes lack malnutrition [16], smoking habits and coexisting medical conditions [17], such as diabetes, congestive heart failure, respiratory or renal diseases and cancer [13], but also social factors, such as being resident in care or nursing homes [13,18].

As a disease preventive strategy, two types of pneumococcal vaccines are in use today. These generate humoral protection against subsets of the 90 capsular serotypes, based on the immunogenicity of the polysaccharide capsule. The 13-valent protein-conjugated polysaccharide vaccine PCV13 (against serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) and the 23-valent polysaccharide vaccine PPV23 (against serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). PCV13 is recommended for children under 2 years and is a conjugated vaccine that uses CRM197, a nontoxic variant of diphtheria toxin, to elicit a T-cell-dependent response [5,19]. PPV23 induces a T cell-independent response, generated by antibody-producing B cells, and is recommended for adults over 65 years [5,20].

These strategies reduce the incidence of invasive pneumococcal disease (IPD) in both children and adults [19,21]. However, although pneumococcal vaccines are effective in children, they have reduced efficacy in older adults [19,22]. Moreover, while IPD cases caused by serotypes in the PCV vaccines have declined suggesting that vaccination of children can elicit herd protection of adults [20,21], the introduction of PCV vaccines has changed the serotype distribution in vaccinated populations [23,24]. This has led to an increase in disease cases caused by non-vaccine serotypes [2,25], *i.e*. 19A, that was not present in PCV7 increased its distribution globally, until it was later added to PCV13 [2,26]. Also, reports from different countries show that serotypes 1, 3, 19A, 19F and 23F are commonly isolated in older adults during IPD and CAP cases [19,22,27–30], and that some of these serotypes, 1, 3 and 19A, were found in both bacteraemic and non-bacteraemic pneumonia [31].

1.3 Population ageing

Life expectancy continues to increase, and in the coming decades the older population is expected to increase significantly across the developing and developed world. There were 962 million people aged over 60 years in 2017 and this number is expected to more than double by 2050, reaching 2.1 billion [32]. For comparison, in the United Kingdom, 11.8 million were aged 65 years, corresponding to 18% of the population in 2016 [33], while in Brazil, although 14.6% of the population was over 60 years of age in 2017, this percentage corresponds to 30.2 million people [34]. Of concern is that the increase of life expectancy may not be associated with increased healthy life expectancy and a recent paper suggested that the proportion of adults living with multimorbidity will increase significantly in the coming years [35]. The increase in lifespan thus represents a great achievement for medicine and public health, but the growth of this age group also raises concerns for health care services, as this sector of the population is at greater risk for many chronic diseases including life threatening infections such as pneumonia. Understanding the reasons for increased risk of infections could have great benefits for the individual and society.

1.4 Immunosenescence

In the last decade, it has become clear that the immune system undergoes changes during ageing that compromise its function and integrity, and this may be a major contributor to increased susceptibility to pneumonia in older adults [36]. This phenomenon, termed immunosenescence, encompasses phenotypical alterations and loss of function of a broad range of immune cells.

Age-related changes in parameters of the immune system have also been used a predictor of mortality in older adults, determined by the "Immune Risk Phenotype" (IRP). It includes a reduced CD4/CD8 ratio, infection by cytomegalovirus, and accumulation of $CD8^{+}CD28^{-}$ T cells [37,38]. Conversely, longevity has been associated with preservation of a high CD4/CD8 ratio and a low number of $CD8^{+}CD28^{-}$ T cells [38]. Haematopoietic stem cells also change with ageing, marked by lower self-renewal capacity and skewing towards production of myeloid cells [39,40].

Alongside these changes, inflammaging, a low grade chronic inflammation that consists of raised serum levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-8, IFN-γ, C-reactive protein (CRP)) and low levels of anti-inflammatory IL-10, is present in healthy older adults [37,41–44]. This rise in pro-inflammatory mediators has been associated with age-related increase risk of dementia [42,45], atherosclerosis [43], frailty [46,47], chronic diseases [44,48,49] and mortality [37] and is thought to be a major cause of the age-related phenotype.

Age-related changes in the immune system are discussed in the sections below and summarised in Table 1.1.

1.4.1 Ageing of the innate immune system

Innate immunity encompasses both cellular and humoral elements as well as physical and chemical barriers that provide immediate host defence against pathogens and that undergo structural and functional changes with ageing.

1.4.1.1 Mucosal barriers and changes in the respiratory system with age

Loss of integrity in mucosal barrier structure and function are associated with ageing. Changes in both nasal and lung epithelium in older adults can also contribute to susceptibility to *S. pneumoniae* [50,51]. These include reports of reduced nasal mucociliary clearance in older individuals, which delays clearance of airway spaces [52,53]. Aspiration of oropharyngeal secretion during sleep also contributes to transport of contaminated secretions to the lungs, and in older and debilitated individuals, such as dementia patients, can increase the risk of infection [18,54].

Levels of antimicrobial peptides human surfactant protein (SP) A and SP-D in the alveolar lining fluid of older adults and aged mice are increased, but hydrolase levels are reduced in the fluid [55], possibly compromising its protective function. Studies in aged mice demonstrated that lung tissue (blood vessel, bronchi and alveoli) have elevated A20 production, a cytosolic inhibitor of nuclear factor kappa B (NFkB) and mitogen-activated protein kinase (MAPK) cascades [56]. NFkB regulates surface expression of Polymeric Immunoglobulin Receptor (pIgR) and Platelet-Activating Factor receptor (PAFr), which are elevated in lungs of aged mice [57,58], allowing increased binding of the pneumococcus to respiratory epithelium [58]. In humans, analysis of bronchoalveolar lavage of healthy older individuals revealed an age-related increase in absolute number and frequency of neutrophils in the air spaces, as well as higher levels of IL-8 and neutrophil elastase [59], providing more evidence that the air spaces undergo changes that may affect susceptibility to pneumococcal infections.

1.4.1.2 Complement system

The complement system is a group of fluid phase proteins that via its activation promotes direct lysis of pathogens, opsonisation and production of anaphylatoxins C3a and C5a, inflammatory mediators produced by cleavage of C3 and C5 components. Studies concerning the effect of ageing on the complement system are few and contradictory. Serum levels of C3, C4 have been reported as unaltered [60] or increased with ageing [61,62]. Haemolytic activity of alternative and classic complement pathways in the elderly were comparable to young adults [60], but classical pathway activity has been reported as increased by other groups [61,62].

1.4.1.3 Dendritic cells

Dendritic cells are present at low frequencies in the blood, around 1% of peripheral blood mononuclear cells (PBMCs) [63]. Dendritic cells (DCs) are divided into two subsets, based on lineage predecessors: myeloid dendritic cells, the conventional DCs (mDCs), which express CD1c (mDC1), and a less frequent subset, identified by expression of CD141 (mDC2); and lymphoid-derived dendritic cells, plasmacytoid DCs (pDCs). mDCs can initiate activation and differentiation of naïve T lymphocytes following presentation of antigens, while pDCs have a role in anti-viral host response, through production of large quantities of type I interferon (IFN) [63].

Despite the low frequency of DCs in peripheral blood, most studies in humans report findings in this tissue due to its accessibility. The effect of ageing on the frequency of mDC1 is uncertain, as there are reports of reduced [64–66] or unaltered frequency [67–69]. Concerning pDCs, some studies report no changes in the frequency of this subset with ageing [66,68], but a larger number of studies demonstrate frequency reduction [64,65,67,70,71]. Low frequencies of both mDCs and pDCs in older individuals following immunisation with live-attenuated yellow fever (YF) vaccine were associated with low YF-specific $CD4^+$ and $CD8^+$ T cells [65].

mDC1

A decline in stimulatory ability of DCs in old subjects has been proposed in the literature, demonstrated by poor induction of T cell proliferation in mice [72] and humans [65,73]. Additionally, upon activation of pattern recognition receptors (PRR) Toll-like receptors (TLR) 1/2, TLR2/6, TLR3, TLR5 and TLR8, mDCs of old individuals produced lower levels of TNF-α, IL-6, IL-12(p40) and IFN-α [73,74]. In frail older adults, production of IL-12(p70) and IL-23 by DCs after TLR4 and TLR7/8 stimulation was also decreased, compared to young subjects [75]. Such age-related decline in mDC cytokine production was associated with reduced antibody levels following influenza vaccination [74]. However, expression of co-stimulatory molecules CD80 and CD86 were not affected by ageing [66,69,76,77].

Phagocytosis and chemotaxis also decline in mDCs with ageing, and this defective function is associated with impairment of the phosphoinositide 3-kinase (PI3K) signalling pathway [68,72,78]. As PI3K is required for migration and phagocytosis and is a negative regulator of

TLRs, the authors suggest that altered PI3K function may underlie reduced DCs activities in aged subjects [68].

mDC2

mDC2, a subset of mDCs expressing CD141, are found in blood and tissues at very low frequencies, usually corresponding to 10% of the frequency of mDC1, and are distinct for their ability to cross-present antigens to $CD8^+$ via major histocompatibility complex (MHC) class I and promote CD4+ T-helper 1 (Th1) responses via IL-12 secretion [79].

Studies of the effects of ageing on this subset are few. One report showed that distribution, migration and maturation of mDC2 in lung and intestines were conserved with ageing [80], but another study reported decrease of mDC2 frequency in the bloodstream of old participants [81].

pDCs

Similar to mDCs, cytokine production by pDCs declines with age. pDCs of older adults produced lower levels of TNF-α, IL-6, IL-12(p40) and IFN-α in following activation of TLR7 and TL9 [74], and stimulation of pDCs with influenza virus generated lower levels of IFN- α, IL-6 and TNF- α in old donors, compared to young [69]. Age-related decline in IFN-I and IFN-III production [70,82] was associated with impaired phosphorylation of the IFN regulatory factor (IRF)-7 transcription factor [82,83]. Lower levels of pDC-secreted IFN were associated with impaired induction of cytotoxic enzymes in CD8 T cell of old donors [82].

1.4.1.4 Monocytes and Macrophages

Monocytes are among the main innate mediators of inflammatory response. They migrate from the bloodstream to infection or injury sites and promote antimicrobial activities, such as phagocytosis, killing and cytokine release. In the tissues, they differentiate into macrophages, and by production of cytokines alongside antigen presentation via MHC, can mediate functions in both innate and adaptive immunity.

Overall monocyte frequency is reported as increased with ageing [64,66,84], but total numbers remain unaltered [84,85]. Based on surface expression of CD14 and CD16, monocytes can be divided into three subsets: the classical, $CD14^{++}CD16^{-}$ subset, intermediate $CD14^{+}CD16^{+}$ and the non-classical $CD14^{+}CD16^{++}$. Classical monocytes correspond to 80-90% of the monocyte pool, are competent phagocytes and producers of reactive oxygen species (ROS). The intermediate phenotype corresponds to 2-8% of the pool and also mediates pro-inflammatory responses, ROS production and angiogenesis. Lastly, the non-classical subset, corresponds to 2-10% of the monocyte population, are producers of pro-inflammatory cytokines, but also of IL-10 [86,87]. With age, there is an increase of intermediate and non-classical monocytes [85,87–89], indicating a change in the proportion of classical to non-classical monocytes.

Expression of adherence molecule CD62L and CX3CR1 in monocytes of old subjects is reduced with ageing [88,90], which could contribute to impaired chemotaxis [91]. Furthermore, monocyte phagocytosis [88,91] and ROS generation [92,93] are reduced with ageing. These functional declines could be attributed to age-related changes in TLR expression and signalling. Decreased expression of TLR1 and TLR4, but not TLR2, were

reported in monocytes from old compared to young adults, and decreased TNF-α and IL-6 production was detected after TLR1/2 ligation [94]. Monocytes from advance-aged frail elderly showed impaired TNF-α, IL-6, IL-1β and IL-8 expression following stimulation with TLR2 and TLR4 agonists [64,95]. Stimulation of TLR4 and TLR7/8 in monocytes of older adults also generated lower IFN-α, IFN-γ, IL-1β, CCL20, and CCL8 [96]. Decline in cytokine production has been associated with reduced PI3K-AKT signalling [95], reduced phosphorylation of p65 NFκB, JNK and p38 MAPK [97], as well as lower cytoplasmic levels of Jun N-terminal kinase (JNK) and p38 [98] have been demonstrated.

Moreover, monocytes of old individuals had TLR-induced expression of CD80 and CD86 diminished [99], as well as decreased surface expression of HLA-DR (MHC class II) [85], which could contribute to an impaired antigen presentation and activation of T cells.

Tissue macrophages are among the main phagocytic cells of the immune system, and the effect of ageing on these cells has been mostly reported in murine models. In aged mice, phagocytosis promoted by peritoneal macrophages was reported as reduced [100]. In macrophages from the spleen marginal zones of old mice, reduced phagocytosis was associated with lower expression of phagocytosis-promoting receptors MARCO and SIGN-R1 [101]. In humans, reduced phagocytosis was reported in $CD14^+$ monocytes of older individuals [88]. Ageing also impairs cytokine production in macrophages following TLR activation, with stimulation of TLR1/2, TLR2/6, TLR3, TLR4, TLR5, and TLR9 in macrophages producing lower levels of TNF-α and IL-6 [98,102] and lower production of pro-IL-1β following TLR4 activation [103]. Furthermore, macrophages of aged mice had lower expression of MHC class II following stimulation with IFN-γ, compared to young mice and such alteration could contribute to impaired antigen-presenting function [104]

Alveolar macrophages have a distinct phenotype than interstitial macrophages, such as expression of CD11c and CD205, and have a higher ability to cross-present antigens than other macrophage populations [105,106]. In aged mice, decreased production of pro-inflammatory cytokines by alveolar macrophages was linked to elevated presence of A20, a cytosolic suppressor of NFκB [56] and with lower phosphorylation of p65 NFκB, JNK and p38 MAPK following TLR2 activation [97]. With ageing, alveolar macrophages showed reduced self-renewal in the lungs of aged mice, reduced phagocytosis of particles and of apoptotic neutrophils [107]. Also, adoptive transfer of alveolar macrophages from healthy young mice to old mice infected with influenza virus reduced lung damage in the later [107].

In the elderly, the absolute number of macrophages were elevated in bronchoalveolar lavage fluid [108]. In contrast, immunohistochemical analysis of lung tissue of patients with pneumococcal pneumonia showed older adults had lower frequencies of macrophages, compared to young patients [109]

1.4.1.5 Neutrophils

Neutrophils originate from myeloid-derived precursors and are the most abundant leukocyte in the blood. They are the first immune cells to arrive at the site of injury and infection and capture a wide range of microorganisms and mediate different bactericidal functions. As fast responders to antigens, neutrophils are an early source of pro-inflammatory cytokines, which mediate activation of other cell types and modulate the microenvironment [110].

Production

Under physiological conditions, neutrophils are short-lived cells with an 8-hour half-life in the circulation and are produced at a range of 1-2x10^{11} cells per day by the bone marrow [110]. Production is regulated by granulocyte colony stimulating factor (G-CSF) [111], and while the numbers of neutrophil precursors in the bone marrow are not reduced with ageing, proliferative activity following G-CSF is reduced, whilst proliferation with GM-CSF and IL-3 stimulation remain similar to young [112]. The number and frequency of circulating neutrophils are unaltered with ageing under physiological conditions [38,85,113], and neutrophilia during infection is comparable to that of young subjects [114]. However, there is evidence that centenarians have higher numbers of circulating neutrophils, in comparison with 55 years old adults [38].

Neutrophil heterogeneity

During health, the neutrophil pool comprises mostly of mature, $CD16^{+}$ neutrophils [115]. Neutrophils age in the time they circulate in the bloodstream [116], and migration from and to the bone marrow is controlled by CXCR2 and CXCR4 [116]. Aged neutrophils have increased phagocytosis and ROS production [117]. During sepsis or systemic inflammatory response syndrome (SIRS), the bone marrow intensifies the production of neutrophils and the frequency of immature neutrophils, expressing $CD16^{dim}$ and with hyposegmented nuclear morphology, increases [115]. In recent years, a new population of neutrophils was identified in the blood of adults after LPS challenge and severe injury, which expressed $CD16^{bright}$/$CD62L^{dim}$ and hypersegmented nuclear morphology [118]. However, whereas this phenotype had only been found in injured patients or during endotoxin challenge [118–120] a recent study found this population in the circulation of healthy older adults [121], indicating that ageing also affects the circulating neutrophil pool.

Migration

During infection, neutrophils leave the circulation to migrate towards the inflamed site. Neutrophil adherence to activated endothelial cells is mediated by integrins CD11b and CD11a, whose expression on the surface of neutrophils in older subjects has been reported as unchanged [121–123] or increased [124]. In contrast, chemotaxis is reduced in neutrophils of older adults towards a wide range of stimuli: including the chemokines IL-8 [124–126], LTB4, C5a and CXCL1 [126] and chemoattractants lipopolysaccharide (LPS) [127,128] and N-formyl-methionyl-leucyl-phenylalanine (fMLP) [124,127–129]. Interestingly, neutrophil chemotaxis towards IL-8 was improved by physical activity in an older cohort [125]. Reduced chemotaxis itself may be detrimental not because neutrophils may take longer to reach a site of infection; indeed, during infection with *S. pneumoniae* older patients had higher neutrophil infiltration in the lung, when compared to young patients [109]. Instead, reduced chemotaxis may be detrimental because neutrophils may release more proteases, such as neutrophil elastase, as shown by increased expression of the marker CD63 [126], during a lengthy migration, potentially increasing tissue damage and inflammation in older pneumonia patients [126]. Interestingly, constitutive PI3K activity was also found to underlie this chemotaxis defect and reduced chemotaxis was corrected *in vitro* by inhibition of PI3K, specifically the delta isoform (PI3Kδ) [126].

Phagocytosis and production of reactive oxygen species

Activation of neutrophils via TLRs, Fc receptor CD16, complement receptors, such as CR3 (CD11b/CD18), initiate a process of phagocytosis, followed by killing of microorganisms [130]. Most authors report reduced phagocytosis in response to microbial

challenge in neutrophils from older adults [131]. Phagocytosis of beads [128,132], opsonised *Escherichia coli* [121,122,129,133] and *Staphylococcus aureus* [129] were all reduced with ageing, whereas phagocytosis of antibody-opsonised Group B *Streptococcus* was not [134]. These differences could be associated to age-related changes in the receptors for complement opsonins or antibodies; however current data indicate no altered expression of complement receptors CR1 and CR3 [62], whist reduced expression of antibody receptors [122] is not a uniform finding, with no difference in neutrophil FcγRII and FcγRIII (CD16) expression with age [62].

Phagocytosis or activation of pattern recognition receptors (PRRs) trigger the production of reactive oxygen species (ROS) [130]. These are produced via NADPH oxidase system which converts molecular O_2 into O_2^- (superoxide) by electron transfer. Although superoxide itself is toxic to bacteria, it is involved in the generation of a wider range of oxygen free radicals, as O_2^- is converted into H_2O_2 by the superoxide dismutase. The reaction between O_2^- and H_2O_2 produces $OH\cdot$, and H_2O_2 can be converted into other oxygen species, such as HOCl and HOBr. O_2^- can also be produced by nitric oxide synthases, following conversion of substrates such as L-arginine into O_2^- and NO. By reacting together, both species produce peroxynitrite (OONO-) that decomposes into oxidants $OH\cdot$ and NO_2 [130]

Interestingly, ROS generation data are conflicting depending on the stimuli used. Neutrophil production of ROS is reduced with ageing following stimulation with fMLP [121,127,135–137], PMA [136] and *S. aureus* [129], but not to *E. coli* [129] and *Candida albicans* [136,137]. Thus, there is a suggestion that older adults may have divergent responses to Gram-positive and Gram-negative bacteria. Although there was no difference in the phagocytosis of *E. coli* and *S. aureus*, production of ROS in response to *S. aureus* was reduced in neutrophils of old

donors [129]. Moreover, with ageing, priming of neutrophils with agonists of TLR1/2, TLR4, TLR7/8 induced lower levels of ROS following secondary stimulation with PMA or fMLP [121]. Impaired TLR signalling has been proposed to be one of the causes for diminished phagocytosis and ROS production in neutrophils of older individuals. A study of the distribution of IRAK-1 and MyD88 in lipid rafts in neutrophils showed altered redistribution of these molecules in the lipid raft microdomains following LPS stimulation with ageing [127]. However, most studies covering age-related changes in TLR signalling were performed in monocytes and macrophages [95,98,127].

Another neutrophil mechanism for bacterial control is the release of proteases extracellularly, known as degranulation [130], which was reported as reduced in neutrophils with age [138,139]. However, a more recent study indicates an increase, supported by the increased expression of CD63 in the surface of neutrophils of older donors, a marker for degranulation [126].

Neutrophil extracellular traps

Neutrophils can also trap and kill bacteria extracellularly by a mechanism called neutrophil extracellular traps (NETs). Here DNA is extruded triggered by ROS generation and the NET produced contains neutrophil granule proteins, such as myeloperoxidase, elastase and lactoferrin [140]. NET generation has been shown to be reduced in response to both IL-8 and PMA in TNF-primed neutrophils from old donors [141].

Table 1.1. Summary of age-associated changes in innate and adaptive immunity

Cell type and function	Species	Effect	References
Levels of pro-inflammatory cytokines in the circulation	Human	↑	[37,41–44]
Haemolytic activity of alternative and classic complement pathways	Human	↑ or =	[60], [61,62]
Dendritic cells			
mDC1			
Frequency in the circulation	Human	↓ or =	[64–66], [67–69]
Cytokine production upon TLR activation	Human	↓	[73,74, 75]
Expression of CD80 and CD86	Human	=	[66,69,76,77]
Phagocytosis and chemotaxis	Human	↓	[68,72,78]
pDC			
Frequency in the circulation	Human	↓	[64,65,67,70,71]
Cytokine production after TLR activation	Human	↓	[69,70,74,82]
Monocytes and macrophages			
Monocytes			
Frequency in the circulation	Human	↑	[64,66,84]
Phagocytosis	Human	↓	[88,91]
ROS production	Human	↓	[92,93]
Cytokine production after TLR activation	Human	↓	64,94,95,96]
Expression of CD80 and CD86	Human	↓	[102]
Expression of HLA-DR	Human	↓	[85]
Macrophages			
Frequency in BAL	Human	↑	[108]
Cytokine production after TLR activation	Mice	↓	[98,102,103]
Phagocytosis	Mice	↓	[100,101]
Neutrophils			
Numbers in circulation	Human	=	[38,85,113]
Chemotaxis	Human	↓	[124,127–129]
Phagocytosis	Human	↓	[121,122,128,129,132,133]
ROS production	Human	↓ or =	[121,127,135–137], [129, 136,137]
Degranulation	Human	↓ or ↑	[138,139], [126]
NET generation	Human	↓	[141]
T lymphocytes			
Frequency of naïve T cells	Human	↓	[146,147]
T cell proliferation	Human	↓	[150,151]
Expression of surface CD28	Human	↓	[152,153]
Expression of surface CD40L	Mice	↓	[178,179]
Frequency of Th1 subset	Human	↑	[156–158]
Frequency of Th17 subset	Human	↑	[147,159–161]
IL-17 production by memory Th17 cells	Human	↓	[147]
Frequency of thymically-derived Tregs	Human	↓	[164]
Frequency of induced Tregs	Human	↑	[167–169]
Treg function	Human	↓ or =	[174,176], [175]
B lymphocytes			
Frequency of naïve B cells	Human	↓	[181,184]
Class switch recombination	Human	↓	[181,189]
Antibody levels	Human	↓ or =	[190]
Antibody functionality	Human	↓	[62,191]

1.4.2 Ageing of the adaptive immune system

B and T lymphocytes comprise the adaptive immune system. Its hallmarks are highly antigen specific responses that assist in the neutralisation of toxins or pathogens by antibody binding, killing of pathogen-infected cells, and induction of memory cells, all of which are altered by ageing, contributing to increase of autoimmunity, infections and reduced vaccine response.

1.4.2.1 T lymphocytes

The thymus is the organ in which T cells originate and mature. Thymic involution starts in puberty and leads to reduction of output of naïve T cells with age [142]. This mechanism is thought to have evolved to limit the output of naïve cells to avoid autoimmunity while keeping a broad repertoire of memory cells [142,143]. However, thymic atrophy is one of the factors associated with reduced vaccine efficacy and increased infection rates in the old [144,145]. Ageing is also accompanied by an accumulation of memory cells [146,147], which is associated with thymic atrophy, but also driven by repeated exposure to antigens throughout life and proliferation of the peripheral T cell pool [143]. There is also reduction of T-cell receptor (TCR) diversity, due to accumulation of clonal cells in naïve and memory compartments [148].

The frequencies of mature T lymphocytes, which are divided in $CD4^+$ and $CD8^+$, undergo a change in their proportion, with a lower CD4/CD8 ratio in older adults [38,149]. Decline in T cell proliferation also occurs with ageing and is associated with shortened telomeres [150,151] and loss of expression of the co-stimulatory receptor CD28 [152,153]. Reduced IL-

2 production has been reported in aged mice [154] and could also contribute to reduced proliferation. Loss of CD28 is more predominant in $CD8^+$ T cells and is a rare event in $CD4^+$ T cells [155]. In addition to impaired antigen-induced proliferation, $CD28^-$ T cells have shorter life-span and $CD8^+CD28^-$ T cells have enhanced cytotoxicity, but are often specific for cytomegalovirus [153].

In older adults, there is a trend of differentiation of $CD4^+$ T-helper subsets towards Th1 and Th17. Frequency of Th1 (IL-2 and IFN-γ secreting cells) [156–158] and of Th17 (IL-17 producers) [147,159–161] are increased with ageing. However, earlier studies report a shift of Th1 subset to Th2 (IL-4, IL-5, and IL-13 producers) in older adults [157,162,163]. An interesting finding is that memory $CD4^+$ T cells from older adults produced lower levels of IL-17 than naïve cells, compared to young [147].

Due to thymic involution, the number of thymically-derived regulatory T cells (Tregs) declines with ageing, but it is suggested that expansion of peripheral Tregs and conversion of non-regulatory T cells into this phenotype compensate for the loss of thymic output [164]. This population regulates immune activation by secretion of IL-10 and TGF-β and by cell-to-cell contact, inducing apoptosis by release of Granzyme B [165,166]. The frequency of Tregs has been reported as increased in older humans [167–169] and mice [170,171]. However, such accumulation of Tregs with ageing can promote reactivation of chronic infections [169] and poor response to vaccination [172]. The effect of age on the function of Tregs is not conclusive, as reports in mice [169,173] and humans [174–176] have described cytokine release as reduced [174,176], increased [169,173] or unaltered [175]. An interesting finding is that while Tregs from aged mice successfully suppressed IFN-γ^+ $CD4^+$ T cells, they failed to control IL-17 release from $CD4^+$ cells compared to young mice [177].

Ageing also compromises $CD4^+$ T cell-mediated B cell responses, by reduced CD40L (CD154) expression by T cells of old mice [178,179].

1.4.2.2 B lymphocytes

Another consequence of the decline of the quality of the immune response in older adults is the decrease in antibody levels, which could be a consequence of the poor expansion of B cells in the smaller germinal centres found in older adults [180]. The overall population of B cells is reduced [181,182], associated with reduced numbers of B cell precursors in the bone-marrow [183]. Similar to T cells, naïve B cell are reduced with ageing [181,184], but the effect of age on the memory B cell pool is still debated. Absolute numbers and frequencies of memory B cells and plasma cells have been reported as declined with ageing [181,185], as well as increased [186,187]. One report on regulatory B cells has reported both their frequency and IL-10 secreting function to decline with age [188].

Class switch recombination is negatively affected by ageing [181,189], but reports of age-related changes of serum and salivary antibody levels are not definitive in reporting higher or lower levels in older individuals [190], although studies from different groups show antibodies have reduced functionality with ageing [62,191].

1.5 Pneumococcal colonisation

Pneumococcal nasopharyngeal colonisation (carriage), is often asymptomatic in adults [192], but it is also regarded as a reservoir for transmission of *S. pneumoniae* and a precursor of disease [193]. Commensal pneumococcal carriage is common during infancy and decreases

with ageing, an observation associated with the development of specific immunity with increasing age [194]. However, these findings contrast with the high incidence of pneumococcal pneumonia in older adults [5]. For comparison, a study found that between the age of 0-2 years, 50% of children were carriage-positive, as were 45% of children aged 3-4 years, with the frequency decreasing to 1-10% in adults [195]. In older adults, carriage rates are even lower, present in 0.85% in individuals living in their own home, increasing to 1.45% in nursing-home residents, as reported by a Slovenic study [196], and 2.3%, reported in a Portuguese cohort [197].

Studies in infants and children show that disease was associated with acquisition of new serotypes [198,199], and could be a possible explanation for disease in older adults in the absence of carriage. In a Portuguese study [197], the most prevalent serotypes were 19A, 6C, 22F, 23A, 35F, 11A, and 23B, while a study from the Netherlands reported that carriage of serotypes 1, 3, 6A/B, 9A/V, 19A, or 23F were present during influenza-like illness in older adults [200]. Serotypes vary in their potential to cause disease, and this "invasiveness potential" was shown to be inversely correlated to carriage duration [201]. However several serotypes found in colonised older individuals, such as 1, 3, 19A and 23F, were also isolated from several IPD and CAP cases in this age group [19,22,27–30], suggesting a role for the ageing immune system in this context.

1.5.1 Changes in the nasopharyngeal microbiota with age

Carriage of *S. pneumoniae* by itself is not a determinant factor for infection, since the pneumococcus is present in the nasopharynx of healthy humans [202]. However, microbiota

diversity in the adult upper respiratory tract is significantly reduced in older adults, with the oropharyngeal area highly populated by different *Streptococcus* genera [203]. Bacterial diversity in the upper respiratory tract of young and adult mice is also diminished after *S. pneumoniae* infection and clearance of infection was shown not to reconstitute the baseline microbiota [204].

Changes in bacterial density also influence interaction between species, and synergistic interactions can turn into competition [205]. Colonisation by *S. pneumoniae* was found to occur more frequently in patients carrying *Haemophilus influenzae* than in individuals carrying *S. aureus* [202]. This prevalence of *S. pneumoniae* in the presence of *H. influenzae* appears to be due to the evasion of opsonophagocytic killing by *S. pneumoniae* [206]. Moreover, *S. pneumoniae* and *S. aureus* compete for the same niche in the upper respiratory tract and a dominance of *S. pneumoniae* during colonisation leads to a decrease of *S. aureus* [202]. These studies highlight how the presence of *S. pneumoniae* in the nasopharynx alters the dynamic of the microbiota in the upper respiratory tract.

1.6 Invasion of the host by the pneumococcus

S. pneumoniae is found in the mucosal microbiota of healthy individuals, but for the pneumococcus to start a respiratory infection it is necessary to colonise the upper respiratory tract. Colonisation begins in the nasopharynx and oropharynx and following inhalation or aspiration of the pneumococcus to the lungs, the bacteria can initiate infection in the bronchioles and alveoli [207]. Soon after entering the nasopharynx the pneumococcus encounters mucus which has a protective effect. One of the most important virulence

factors of *S. pneumoniae* is its polysaccharide capsule, which can help the bacterium to evade mucus [208]. The variable polysaccharide composition of the capsule determines the greater than 90 serotypes of *S. pneumoniae*, presenting significant challenges for a successful vaccination strategy and for host immunity. Furthermore, the pneumococcus controls capsule expression, making it opaque or transparent, with the opaque phenotype determined by the augmented concentration of polysaccharide in the capsule [209,210]. During contact with and invasion of host cells, the pneumococcus capsule is reduced [210], whereas absence of a capsule reduces colonisation [211].

The key elements of the infection process are summarised in Figure 1.2. Colonisation starts with adherence of the pneumococcus to epithelial cells of the respiratory tract, with the capsule facilitating access to the epithelial surface [208]. The first phase is asymptomatic and corresponds to the bacterium binding to carbohydrates (e.g. N-acetyl-glycosamine) on the surface of the epithelial cells, under non-inflammatory conditions [207], using choline-binding proteins (CBPs) that have an important role in adhesion and virulence. What influences the transition between non-invasive and invasive colonisation is unclear, but might be associated with an immunosuppressive state of the host, or to a primed epithelium, as could occur in older adults where inflammaging produces a more inflammatory microenvironment [41].

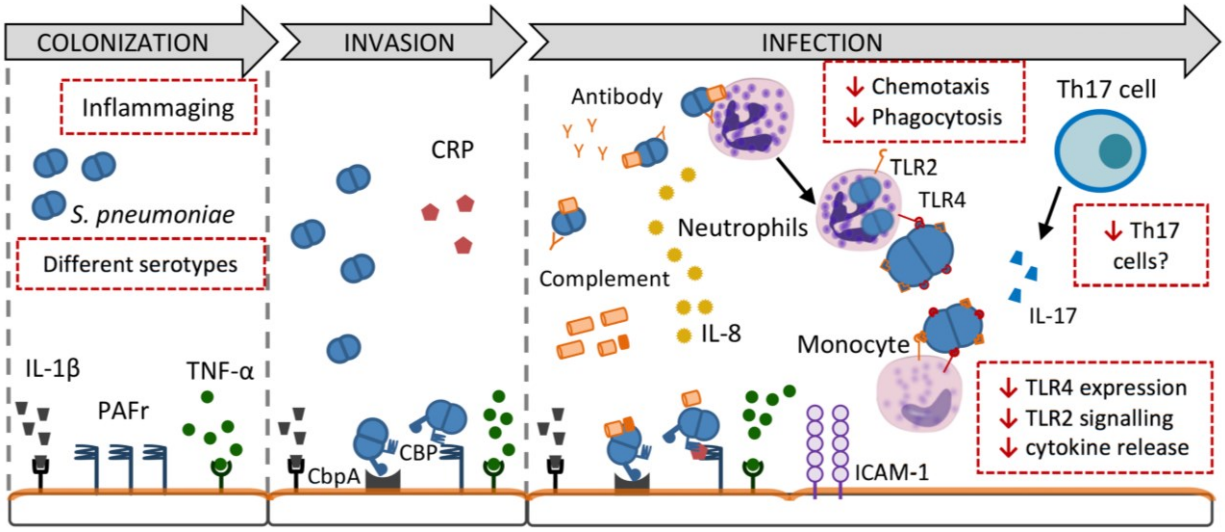

Figure 1.2. The key features of the immune response to *S. pneumoniae* infection and the changes with age. Colonisation starts with adherence of the pneumococcus to epithelial cells of the respiratory tract. In the first phase, pneumococci bind to carbohydrates on the surface of the epithelial cells. Transition to invasive disease may occur when inflammatory cytokines are produced. Inflammaging produces a more inflammatory microenvironment and may aid this process. Complement and antibody opsonise the bacteria, mediating recognition, uptake and killing by neutrophils and monocytes. Th17 based immunity provides protection via maintenance of neutrophil and monocyte recruitment by production of IL-17. Ageing results in reduced phagocyte chemotaxis, phagocytosis and killing of bacteria and reduced pro-inflammatory cytokine production by monocytes due to reduced TLR2 signalling. Differentiation of Th17 cells may also be reduced, compromising maintenance of the neutrophil response. The possible effects of age are shown in the red boxes.

When inflammatory factors, such as TNF-α, IL-1β or thrombin are produced, platelet-activating factor receptor (PAFr) expression is increased on epithelial cells and binds to the phosphorylcholine on the cell wall of the pneumococcus [212]. The same interaction occurs during the migration of the bacteria from the lung tissue to the blood [213,214]. Studies in mice have shown that PAFr has a role in induction of inflammation, as mice lacking this receptor show reduced signs of infection, with lower numbers of colony-forming units of *S. pneumoniae* and reduced levels of inflammatory cytokines and chemokines in the lung, when compared to wild type (WT) animals [213]. Studies in human cells have revealed that subsequent to PAFr expression, the adhesin CbpA (also known as PspC), a protein of the CBP

family, binds glycoconjugates from epithelial [210,212] and endothelial cells to the choline in teichoic acid or lipoteichoic acid in the pneumococcus cell wall. This step is involved in advancing the colonisation to invasion via stimulation of an inflammatory response [215] as the binding of CbpA to human endothelial cells leads to the production of the chemokine IL-8 and expression of ICAM-1 [216], a molecule that binds to integrins (LFA-1, CD11a/CD18) on neutrophils, monocytes and other cells of the immune system. CbpA can also bind to human Factor H, molecule that protects C3b deposition in the host's own cells, contributing to modulate inflammation during infection [217]. Many other virulent factors from the pneumococcus are involved in binding to the host's cells, such as PspA, lytA, NanA and NanB [207,218,219]. After receptor binding, endocytosis effects pathogen internalisation [220,221]. Although *S. pneumoniae* can be killed at this stage by lysosome mediated degradation, the bacteria that manage to evade killing in this way will translocate through the cell and continue the infection of the host's tissue [222].

1.7 The Immune Response to *S. pneumoniae*

Serial colonisations with multiple serotypes through childhood result in a complex antibody and cell based immune profile in adults [223]. Complement and antibodies to the capsular polysaccharide opsonise the bacteria, mediating recognition, uptake and killing by phagocytes (neutrophils and monocytes) [62,224]. Although vaccination with conjugate vaccines based on capsular polysaccharides generates high levels of antibody that protect against colonisation [225], colonisation in humans is associated with little induction of anti-capsular antibody and clearance does not coincide with antibody generation [223].

Moreover, antibody acquired to a particular serotype confers only 30-60% reduction in subsequent colonisation [226] and Th17 based immunity is proposed to provide the remainder of protection via maintenance of neutrophil and monocyte recruitment [227,228]. Immunity to *S. pneumoniae* is thus complex: natural immunity is largely phagocyte and Th17 mediated, whilst protection by capsular polysaccharide vaccine additionally requires generation of antibody. These key elements of the immune response to *S. pneumoniae* are now described in brief.

1.7.1 Innate immune response to *S. pneumoniae*

1.7.1.1 Pattern Recognition Receptors

S. pneumoniae has characteristic virulence factors that activate pattern recognition receptors (PRRs) on immune cells as well as epithelial cells of the respiratory tract, initiating its detection by the host. Toll-like Receptors (TLRs) are a major component of the PRR detection system and studies in mice and humans have shown that TLR2 is the main TLR involved in cellular activation by *S. pneumoniae* [229,230] and this detection is assisted by membrane CD14 [231] as well as soluble CD14 [232]. TLR2 recognises lipoteichoic acid (LTA) present on the cytoplasmic membrane of *S. pneumoniae* and this leads to NFκB-dependent production of TNF-α by monocytes/macrophages [232]. However, TLR2 does not mediate bacterial clearance in the lungs but promotes inflammation and recruitment of further phagocytes (neutrophils) with the production of a range of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, KC/Gro-α and MIP-2 by alveolar macrophages [233]. Although TLR2 is the main receptor for *S. pneumoniae* lipoproteins [229] co-operation with other

receptors enhances the inflammatory response. Knockout mice for TLR2 and TLR4 or TLR2 and TLR9 showed a significant decrease in production of cytokines and chemokines in the spleen after infection with *S. pneumoniae*, when compared with mice lacking only one of these receptors [234].

Pneumolysin, another virulence factor of the pneumococcus, is recognised by TLR4 [235]. It is a cytoplasmic protein that induces cell death by pore formation in cell membranes [215]. The production of pro-inflammatory mediators, TNF-α, IL-6 and IL-8 after TLR4 activation drives further neutrophil recruitment. Intracellular TLR9, that recognises unmethylated CpG motifs, can also offer protection against *S. pneumoniae* via production of IL-8 [236]. Studies in TLR9 knockout mice showed that activation of TLR9 was also necessary for control of bacterial burden by activating phagocytosis by lung macrophages in the early stages of infection, but it was not involved in the control of nasopharynx colonisation [237].

The scavenger receptor SIGN-R1, expressed by macrophages of the spleen marginal zones [238], has a significant role in the clearance of *S. pneumoniae* in blood as shown in mouse models [239,240]. Although SIGN-R1 is not involved in the clearance of the pneumococcus in the lungs, it may still be important in the early response against encapsulated pneumococcus, as macrophages from marginal zones lacking this receptor failed to capture the pneumococcus, activate splenic B cells and generate antibody [239,240]. Another macrophage scavenger receptor, MARCO (Macrophage Receptor with Collagenous Structure), also has a protective effect against pneumococcus. Mice lacking MARCO had impaired clearance of bacteria [241], delayed transcription of mRNA of TNF-α, IL-6, IL-1β and IFN-β in macrophages, and did not promote cellular recruitment to the nasopharynx during

colonisation [242]. MARCO also enhances TLR2 signalling in response to *S. pneumoniae* in a mouse model [242].

1.7.1.2 Complement system

Complement is also fundamental to prevent systemic replication of *S. pneumoniae*. The classical pathway is the major contributor in the defence against *S. pneumoniae* and is activated by binding of C1q to natural IgM that opsonises the pneumococcus, aiding its uptake by phagocytes [243]. Complement can also be activated by C1q [244], the acute-phase protein serum amyloid P [245] and by SIGN-R1 which can bind C1q directly and assemble a C3 convertase to generate the opsonin C3b [246].

The polysaccharide capsule protects the pneumococcus from deposition of C3b [247], and C3b deposition also differs between capsular serotypes [248]. Another strategy the pneumococcus uses to evade complement is reduction of chain length, as C3b deposition is higher on bacteria in long chains [249]. Interestingly, antibody binding and subsequent agglutination of pneumococci counters this strategy by augmenting formation of bacterial clumps, facilitating opsonophagocytosis and killing [249]. Pneumococcal surface protein PspC can also inhibit complement deposition by binding of factor H [224,250], a plasma protein that inhibits the formation of C3b convertase in the alternative pathway, and this effect varies with capsular serotypes [251].

Complement opsonic function rather than its lytic ability is important for killing of pneumococcus. As demonstrated by Van Der Maten *et al.* (2017), *in vitro* incubation of *S. pneumoniae* in plasma did not promoted killing of bacteria, which was observed in whole

blood, indicating phagocytes are necessary for killing [224]. The group also demonstrated that blocking of CD11b (CR3 receptor) decreased killing of the pneumococcus [224].

In patients with bacteraemic pneumococcal pneumonia, levels of complement components, including C3, were decreased in serum [252]. Moreover, patients lacking C1q or C2 were shown to be more susceptible to pneumonia infection, and this also demonstrate that the classical pathway is vital for promoting *S. pneumoniae* opsonisation by the complement system [253].

1.7.1.3 Neutrophils

Neutrophils constitute the main cellular defence against *S. pneumoniae*, and together with monocytes and alveolar macrophages, are responsible for phagocytosis and intracellular killing of the pneumococcus [224,254]. The involvement of neutrophils is seen at all stages of pneumococcal infection [255] and neutrophil depletion in mice models results in increased susceptibility to pneumococcal infection and a higher bacteria burden in the lungs [256–258]. Infiltrating neutrophils are fundamental for pulmonary clearance of the pneumococcus and at earlier stages of pneumonia and recruitment to the lungs is dependent on IL-6 [259], TNF-α and IL-1β [260] secreted by epithelial lung cells, by IFN-γ secreted by NK cells [256] and later, also by IL-17 [261]. In aged mice, lower levels of IL-10 in the lung tissue were associated with higher neutrophil influx to this site and higher production of chemokines by neutrophils at earlier stages of pneumococcal infection [262].

Neutrophil phagocytosis of pneumococci is mediated by TLR2 [263–265] and by P-selectin glycoprotein ligand-1 (PSGL-1) [266], and can be further enhanced by surfactant proteins A

and D [267], opsonisation by complement and antibodies [268] and by stimulation with IL-17A [269]. *S. pneumoniae* can evade phagocytosis by capsule expression, and heavily encapsulated strains are more resistant to neutrophil killing [270], but if complement opsonisation is present, phagocytosis is significant increased [224,253]. For instance, serotype 6B is more susceptible to C3b deposition and opsonophagocytosis than 19F [271].

Following phagocytosis, neutrophil mediates killing of pneumococci by generation of ROS generated within the phagosome [62,272] and serine proteases cathepsin G, elastase and proteinase 3 released from intracellular granules [273]. Neutrophil ROS production by the pneumococcus is dose dependent and differs between capsulated and non-capsulated strains [272]. Neutrophil ROS generation was elevated in response to the non-capsulated R6 strain (serotype 2 avirulent mutant), compared to D39 (serotype 2 clinical isolate strain), but not to other non-capsulated mutants [272]. Such production was abrogated in response to the capsulated D39 strain by an intracellular calcium chelator, but only partially to blockage of the p38 MAPK signalling pathway, that can activate NADPH oxidase [272]. ROS production by the non-capsulated R6 was partially suppressed by inhibition of PI3K and completely abrogated by inhibition of PKC, indicating that *S. pneumoniae* can induce neutrophil ROS production by more than one mechanism [272], and that could also vary between capsular types.

However, neutrophil activation may also be detrimental, as demonstrated by a study where mice lacking neutrophils had a prolonged survival after infection with *S. pneumoniae* serotype 8 and distinct pathological features in the lungs, when compared to control mice [274]. Another interesting finding is that *S. aureus* remained viable after being internalised by mice neutrophils, whereas *S. pneumoniae* (serotype 3) did not [275]. Recently, it was

reported that *S. pneumoniae* remained viable in mice and porcine $CD169^+$ splenic macrophages and was able to replicate [276]. This could suggest a different role for neutrophils and macrophages in the containment of *S. pneumoniae.*

Neutrophils can also contain and kill pneumococci extracellularly by releasing NETs. *S. pneumoniae* induces NET release by promoting neutrophil autophagy and PI3K activation, but independent of pneumolysin [277]. Still, PLY alone can induce NET release, independent of TLR4 and only partially dependent on NADPH oxidase-derived ROS production [278]. Nonetheless, the pneumococcus has mechanisms to evade NETs, by degrading the DNA filaments with an endonuclease [279] and by expressing capsule [280].

Although neutrophils can contain *S. pneumoniae* by several mechanisms, they are susceptible to activation and lysis by PLY. In the nasal-associated lymphoid tissue (NALT), this event is beneficial as neutrophil lysis by PLY promotes antigen delivery [281], however, in the lungs, elastase release by lysed neutrophils damaged alveolar epithelial cells and impaired macrophage phagocytosis [282]. Moreover, activation of neutrophils by PLY induces a series of pro-inflammatory events, such as ROS generation [283,284], NFκB activation and IL-8 production [285], all which could aggravate damage in lung epithelial cells and worsen patient outcome.

Immunohistochemical analysis of lung tissue of patients that died of pneumococcal pneumonia revealed that older adults had significantly higher frequencies of neutrophilic infiltrates than young patients, who had more alveolar macrophages [109]. Moreover, during non-responding pneumonia, neutrophil apoptosis in BAL and blood was higher than in responding CAP patients [286], but non-responding patients that had higher neutrophil

apoptosis reached clinical stability earlier, suggesting that in these cases, such an event can be beneficial to reduce local inflammation [286]. In old individuals, impaired phagocytosis and killing of *S. pneumoniae* were associated with low levels of specific IgG in the serum of old individuals and reduced antibody functionality [62,134].

1.7.1.4 Monocytes and Macrophages

Monocytes infiltrate the lungs after neutrophils and can differentiate into macrophages, which have enhanced phagocytic and killing functions [287]. In mice models, depletion of alveolar macrophages increased susceptibility to pneumonia and mortality [288], but bacteria clearance remained controlled by neutrophils [288]. Macrophages are essential for clearance of apoptotic cells in alveolar spaces and modulate inflammation during pneumococcal pneumonia [288].

However, production of prostaglandin E2 by macrophages during clearance of apoptotic cells greatly reduced *S. pneumoniae* killing by H_2O_2 in macrophages *in vitro*, albeit not reducing its phagocytosis [289]. Macrophage apoptosis following *S. pneumoniae* incubation is also suggested to be another mechanism for control of bacterial dissemination, as apoptosis was associated with killing of pneumococci [290].

During pneumococcal carriage, phagocytosis by monocytes and macrophages contributes for clearance, after migration to the upper airway [261]. Both migration and clearance are dependent on TLR2 signalling [261], and secretion of C-C motif chemokine ligand 2 (CCL2) by activated monocytes/ macrophages recruits additional monocytes to the site of pneumococcal infection [291,292]. In infant mice, delayed CCL2 production led to late macrophage recruitment and consequent longer colonisation period [293]. Alveolar

macrophages assessed in BAL fluid following pneumococcal colonisation in humans showed no difference between the frequency of classical and non-classical phenotypes, suggesting pneumococcal colonisation does not drive polarisation in the alveolar microenvironment [294].

Activation of monocytes by *S. pneumoniae* induces the production of several cytokines, such as NFκB-dependent production of TNF-α [232], IL-6 [89] and IL-12p40 [295], and can further drive differentiation of $CD4^+$ T-helper subsets [295]. Interestingly, it was demonstrated that *in vitro*, bacteria viability was determinant for differentiation of $CD4^+$ cells into Th1 or Th17 by human monocytes, as live pneumococci induced Th1 response and heat-killed led to Th17 differentiation [295]. Furthermore, pneumococcal serotypes can induce different levels of TNF-α production by macrophages, with commonly invasive serotypes such as 4, 6B, 14, 19F and 23F inducing lower TNF-α secretion than rarely invasive serotypes, such as 7C, 24F and 37 [296], indicating this could be a mechanism for some serotypes to evade clearance by the immune system.

1.7.2 Adaptive immune response to *S. pneumoniae*

1.7.2.1 T lymphocytes

The efficient nasopharyngeal and lung clearance of *S. pneumoniae* requires both cellular and humoral immunity [257]. In mice it is well established that the $CD4^+$ Th17 cell subset is fundamental for the clearance of the infection through their production of IL-17 [257,261,297,298] and for avoiding re-occurrence of nasopharyngeal colonisation [257,261,269,297], but in humans the role of Th17 is still not conclusive [299].

Th17 response can be induced by several pneumococcal antigens, such as polysaccharide from the cell wall [300] and PsaA, PcsB and StkP [223,301]. In mice, Th17-mediated control of colonisation involves recruitment of monocytes and macrophages in the first phase, dependent on TLR2 activation, followed by a sustained phase of neutrophil infiltration [261]. IL-17 also enhances neutrophil [269] and macrophage[302] killing of opsonised pneumococcus *in vitro*.

Pneumococcal carriage also promotes expansion of the Treg population, required for controlling bacterial growth in the lungs [303]. During carriage, Tregs can be found in the human NALT and have been shown to suppress anti-pneumococcal Th1 and Th17 responses [227]. In recent years, studies in children have found a correlation between the ratio of Th17/Tregs and presence of carriage [304,305], and that as the ratio increases with age, carriage rates decrease [304].

Pneumococcal colonisation can induce Th1-mediated immunity as well as Th17 [230,306,307]. The Th1 and Th17 responses to pneumococcal infection are dependent on IL-23, as mice lacking this cytokine have decreased levels of IL-6 and IL-12p70 in bronchiolar lavage and significantly reduced neutrophil infiltration in the lungs, associated with low levels of IL-17A [308].

1.7.2.2 Antibodies

Humoral responses against pneumococcal capsular polysaccharide are the base for current vaccine strategies and can provide protection against colonisation [309]. Nasopharyngeal colonisation by the pneumococcus promotes generation of IgG and IgA [310]. At this site,

anti-capsular antibodies promote agglutination of bacteria facilitating phagocytosis and mucociliary clearance [311], and in mice, antibody generated after nasopharyngeal colonisation protected against invasive disease [297].

However, pneumococcal-specific protection against reacquisition of carriage following exposure to *S. pneumoniae* is not exclusively mediated by antibodies, but is also dependent of $CD4^+$ T cells [228,299,312]. In human volunteers, protection against acquisition of a new pneumococcal serotype following natural colonisation was achieved in those who had polysaccharide-specific memory plasma cells [313]. However, another study showed that clearance of colonisation in healthy individuals was achieved independent of anti-pneumococcal antibodies, despite their presence [314].

In contrast with anti-capsular antibodies generated by vaccination, naturally acquired antibodies are found against various pneumococcal antigens, such as PspA and PspC [315].

During infancy, development of IgG against pneumococcal proteins following nasopharyngeal colonisation started earlier than anti-capsular IgG, as the former were found in serum from 2 months of age, while the later were detected in the serum from months 6 of age [316]. However, during the first 2 years of life, colonisation episodes promoted increase in levels of anti-capsular IgG, but not of anti-protein antigens [316]. In adults, naturally acquired antibodies against pneumococcal proteins, such as PspC, corresponded to a larger fraction of naturally-acquired antibody, instead of anti-capsular antibodies [317].

In older adults levels of naturally acquired IgM and IgG against pneumococcal capsular polysaccharide were lower than in young adults, and this decline was aggravated with increasing age [318]. Further analysis revealed anti-capsular IgG antibodies had reduced

functional activity [62]. Interestingly, while levels of anti-pneumococcal polysaccharide IgM were reduced in older adults [318,319], levels and functional activity of IgM against whole-cell non-encapsulated pneumococcus and PspA were not [320]. Thus, development of vaccines targeting conserved pneumococcal proteins across serotypes are considered a new strategy to generate protective immunity against pneumococcal disease, considering the decline in levels and efficacy of anti-capsular polysaccharide antibodies reported in elderly [62,318,319].

1.8 Thesis aims and hypotheses

The central hypothesis of this thesis is that age-related changes in the immune response to *S. pneumoniae* contribute to the increased susceptibility of older adults to pneumonia. Although neutrophils and Th17 cells have a significant role on immunity against *S. pneumoniae*, several aspects of the immune response to this bacterium in older adults remain unclear. Thus, this thesis addresses the effect of age on neutrophils responses against the pneumococcus and on $CD4^+$ T-helper subsets involved in immunity against *S. pneumoniae*.

Thus, the aims of this thesis were:

1. To determine the effect of age on neutrophil activation, reactive oxygen species production and NET generation against *S. pneumoniae* serotypes dominant in older adults.

2. To determine age-related changes in $CD4^+$ T cell polarisation following pneumococcal nasopharyngeal colonisation.

3. To investigate changes in neutrophil responses to *S. pneumoniae* and frequencies of CD4+ T-help subsets during pneumonia infection in older individuals.

Chapter 2

Materials and Methods

2.1 Bacterial strains

Streptococcus pneumoniae serotypes 4, strain TIGR4, 19A and 23F were chosen for this study. The TIGR4 strain was chosen as this is a virulent, genome-sequenced strain [321,322] widely used in studies with both mice [56,293,323,324] and human cells [224,280,295,323,325], for which mutant strains are available, such as capsule-negative mutant 403j. Serotypes 19A [27,29,30,326] and 23F [27,28] were chosen due to their high incidence in older adults during community-acquired pneumonia and invasive disease [27–30,326].

The TIGR4 wild type strain and its capsule-negative mutant 403j, which will be referred as TIGR4Δ*cps*, were provided by Prof Tim Mitchell. Serotypes 19A and 23F were clinical isolates from older patients with pneumococcal pneumonia obtained from the Scottish Microbiology Reference Laboratories and were provided by Prof Tim Mitchell. For the experimental human pneumococcal carriage study (EHPC), serotype 6B, strain BHN418, was used and the heat-inactivated sample used in this study was kindly supplied by Dr Daniela Ferreira from the Liverpool School of Tropical Medicine (Liverpool, UK).

2.1.1 Bacteria growth conditions and storage

With the exception of the 6B strain, all bacteria were grown on Blood Agar Base 2 (BAB) (Oxoid, UK) plates supplemented with 5% defibrinated horse blood (E&O laboratories, UK) overnight at 37°C in 5% CO_2, from bead stocks (Microbank^{TM}, Pro-Lab Diagnostics, UK), from Prof. Mitchell's laboratory. Purity of the colony was checked by streaking *S. pneumoniae* on a BAB plate and adding a 5 μg optochin disk (Oxoid, UK) in the centre of the plate, which was

incubated overnight at 37°C in 5% CO_2 incubator. For culture storage of pneumococcal strains, bacteria were grown statically in Brain Heart Infusion media (BHI) (Oxoid, Basingstoke, United Kingdom) in water bath at 37°C, to mid log phase (OD_{660nm}, 0.55-0.65), which was measured using a spectrophotometer (Biochrom, Cambridge, UK, model WPA Biowave CO8000). Once bacteria reached mid log phase, the liquid culture was centrifuged (4000 x g, 10 minutes, 4°C) and the supernatant discarded. The pellet was resuspended in sterile BHI containing 15% glycerol and aliquoted (1 ml/ vial). Aliquots were frozen at -80°C for use in the subsequent experiments.

2.1.2 Quantification of *S. pneumoniae* stocks

To determine the concentration of the glycerol stocks, the viable counting method was used [327]. 24 hours post freezing, 1 vial of each strain was quickly thawed at 37°C in a water bath. Under sterile conditions, the content was mixed well and 20 μl of the glycerol stock was added to 180 μl of sterile phosphate buffered saline (PBS, Sigma-Aldrich, UK) in a 96-well round bottom plate (Corning, USA), and ten-fold serially diluted until a dilution of 10^{-8} was reached. A BAB plate was divided into 6 sections and for each, 3 drops of 20 μl of each dilution was placed under its identified section of the plate. Plates were left to dry and then transferred to an incubator overnight (37°C, humidified 5% CO2 atmosphere). The following day, spots from a chosen dilution containing 20-100 colonies each were counted and recorded. From these counts the concentration of the bacterial stock was calculated and expressed as CFU/ml (colony forming units/ml) using the equation:

CFU/ml = (average of colony counts from 3 spots) x (50 which gives the CFU value/ml) x dilution factor

2.1.3 Fixation of *S. pneumoniae* with 2% paraformaldehyde

S. pneumoniae TIGR4 was grown in BHI media as described in 2.1.1. Once mid log phase was reached, 20 µl was collected for viable cell count (as in 2.1.2) and culture was centrifuged at 4000 x g for 10 minutes, room temperature (RT). The supernatant was discarded and bacteria were resuspended in 2% paraformaldehyde and incubated for 1 hour at RT. Paraformaldehyde solution was centrifuged (4000 x g, 10 minutes, RT), the supernatant discarded and pellet washed twice in sterile PBS (4000 x g, 10 minutes, RT). The pellet was resuspended in PBS, aliquoted and stored at 4°C.

2.1.4 Labelling of *S. pneumoniae* with fluorescein isothiocyanate (FITC)

Fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Dorset, UK) was prepared according to manufacturer instructions. Under sterile conditions, FITC powder was resuspended in DMSO at concentration of 10 mg/ml and used immediately.

Vials of glycerol stocks with live *S. pneumoniae* TIGR4 were thawed, washed twice with PBS (13000 x g, 5 minutes, RT). Vials of live and of fixed *S. pneumoniae* TIGR4 ($2x10^8$ CFU/ml) were resuspended in 1ml of filter sterile (0.22 µm pore size, Millipore, Darmstadt, Germany) 0.1 M sodium bicarbonate buffer, pH 9. To that suspension, FITC solution was added to a final concentration of 0.3 mg/ml. Vials were incubated for 30 minutes at RT, with rotation, in the dark. Following incubation, bacteria were washed three times with PBS (13000 x g, 5 minutes, RT), live *S. pneumoniae* were resuspended in BHI with 20% of fetal calf serum (FCS)

(Invitrogen, Paisley, UK) and stored at -20°C, and fixed bacteria were resuspended in PBS (1 ml/vial), and stored at 4°C, protected from the light. The next day, a vial of live FITC-labelled *S. pneumoniae* was thawed for viable count (as described in 2.1.2).

2.1.5 Heat inactivation of *S. pneumoniae* TIGR4

Vials of glycerol stocks containing live *S. pneumoniae* TIGR4 were thawed at 37˚C in a water bath and from these $1x10^8$ CFU/ml stocks were prepared in PBS. Vials were centrifuged (13000 x g, 5 minutes, RT), and pellets were resuspended in PBS. Bacterial stocks were heat inactivated by incubation at 65˚C in a dry bath incubator (Labnet Dry Baths, Edison, NJ, USA) for 10 minutes. Vials were then stored at -20˚C until further use.

2.2 Ethical approval and participants

2.2.1 Healthy volunteers

For the study of the effect of age on the immune response to *S. pneumoniae*, 42 young (mean age 26.1 ± 3.4 years; range 21-32 years) and 39 old (mean age 72.4 ± 5 years; range 67-83 years) healthy volunteers were enrolled. Older participants were recruited from the Birmingham 1000 Elders group, which is maintained by the Institute of Inflammation and Ageing and are all clinically healthy and largely medication free with anti-hypertensives being the most common medication taken. All subjects gave written informed consent and the study was approved by the University of Birmingham Ethics committee (reference ERN_14-1166). All volunteers had no significant co-morbidity or immunological illness and were not on medications known to interfere with immune function.

2.2.2 Pneumonia patients

In total, 3 young (mean age 33±2.6 years; range 30-35 years) and 21 old (mean age 79.8±9.1 years; range 66-96 years) patients diagnosed with pneumonia were enrolled in this study. Ethical approval was given by the Coventry & Warwickshire Research Ethics Committee (reference 16/WM/0026) and written informed consent was given by the patients or their relatives at the time of recruitment. Patients were recruited at the Queen Elizabeth Hospital, Birmingham, UK, during September 2016 to February 2018. Inclusion criteria required all participants to be over the age of 18 and with a confirmed diagnosis of pneumonia infection. Clinical diagnostics of pneumonia were assessed following the guidelines of the British Thoracic Society. Exclusion criteria were pregnancy, use of immunosuppressive medications, such as inhaled corticosteroids, and immunocompromised patients. Patients with concurrent respiratory diseases, such as bronchitis, COPD and asthma, malignancies and palliative care patients were also excluded. Demographics of young and old patients are shown on table 2.1, however information such as ethnicity, comorbidities and mortality were not available on all patients.

Table 2.1.Pneumonia patient demographics and comorbidities

	Young (YP)	Old (OP)
Number of participants	3	21
Mean age (years)	33±2.6 (30-35)	77.7±9.8 (66-96)
Male (%)	1/3 (33%)	9/21 (42%)
Influenza (qPCR)	0	5/18 (27%)
Sepsis (SIRS)	0	3/19 (15%)
Pneumococcal vaccine	0	7/21 (33%)
Influenza vaccine	0	12/21 (57%)
Current smoker	1/3 (33%)	2/10 (20%)
Ethnicity		
Caucasian	2/3 (66%)	8/10 (80%)
Black or Black British	1/3 (33%)	1/10 (10%)
Asian or Asian British	0	1/10 (10%)
Comorbidities		
Angina	0	1/10 (10%)
Aortic stenosis (mild)	0	1/10 (10%)
Arthritis	0	3/10 (30%)
Atrial fibrillation	0	2/10 (20%)
Chronic kidney disease	0	1/10 (10%)
Depression	1/3 (33%)	0
Diabetes mellitus type 1	1/3 (33%)	1/10 (10%)
Diabetes mellitus type 2	0	2/10 (20%)
Guttae Psoriasis	1/3 (33%)	0
Hypercholesterolaemia	0	1/10 (10%)
Hypertension	0	8/10 (80%)
Iron deficiency anaemia	0	2/10 (20%)
Ischemic heart disease	0	3/10 (30%)
Mitral valve prolapse	0	1/10 (10%)
Osteomyelitis	0	1/10 (10%)
Secondary generalised epilepsy	0	1/10 (10%)
Vascular dementia (mild)	0	1/10 (10%)
Mortality		
30 days	0	0
90 days	0	1 (83 years old female. Cause:

		Bronchial pneumonia)

2.2.3 Experimental Human Pneumococcal Carriage cohort

For this study, stored PBMCs samples from 15 healthy young (mean age 20.73 ± 3.7 years) and 15 older adults (59.8 ± 7.1 years) from the Experimental Human Pneumococcal Carriage (EHPC) study were used. The recruitment of participants was conducted by and at the Liverpool School of Tropical Medicine (LSTM). All participants gave written informed consent at start of the study. Ethical approval was given by the NHS Research Ethics (older participants recruitment: reference 16/NW/0031, young participants recruitment: reference 14/NW/1460). This experimental model of carriage has been described in detail previously [328]. Briefly, healthy individuals aged 18-80 years old that were not natural nasal carriers of *S. pneumoniae*, were inoculated intranasally with live 6B pneumococcus (80.000 CFU/100μl/ per nostril). Nasal and blood samples were collected prior to pneumococcal inoculation and at days 2, 7, 9, 14, 22 and 29 days after inoculation. For this thesis, I had access to peripheral blood mononuclear cell (PBMC) samples from pre-inoculation and at post inoculation at day 14, for the young cohort, and day 29, for the older cohort.

2.3 Blood sampling and assays

Peripheral blood from volunteers was collected by venepuncture into BD Vacutainers (Becton Dickinson, UK) containing lithium heparin, ethylenediaminetetraacetic acid (EDTA) or clot activator.

2.3.1 Preparation of serum

Blood samples were collected into vacutainers containing clot activator and allowed to clot for 20 minutes at RT. Tubes were then centrifuged at 1500 x g for 10 minutes at 4°C. Serum was collected, aliquoted and stored at -80°C.

2.3.2 Preparation of pooled sera

Two large pooled sera batches were prepared as a source of complement to opsonise the pneumococcus for neutrophil phagocytosis and oxidative burst assays in order to minimise assay variability across the thesis studies. One contained sera from 10 healthy young and the other sera from 14 older volunteers. Serum samples were thawed in a 37°C water bath, placed in ice (4°C) and pooled together under sterile conditions. The pooled sera were aliquoted (100 μl) and stored at -80°C until further use.

2.3.3 Measurement of anti-pneumococcal antibodies levels in pooled sera

Levels of anti-pneumococcal IgGs were measured in sera samples using a multiplex assay, which was carried out by Dr Siân Faustini, at the Clinical Immunology Services of the University of Birmingham.

Briefly, pneumococcal polysaccharide capsules (serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) were conjugated with beads. Beads were sonicated and pre-diluted on coupling buffer (activation buffer (0.53g NaH_2PO_4 and 0.16g Na_2HPO_4 to 50 ml reagent grade water),

normal human serum, (15 mg in 300 µl activation buffer) and EDC, (15 mg in 300 µl activation buffer)). The mixture was added to a 96-well MultiScreen$_{HTS}$ BV filter plate (Merk-Millipore, New Jersey, USA) and incubated for 30 minutes on a plate shaker at RT, protected from light. Next, buffer was aspirated using a vacuum manifold and 150 µl of pneumococcal capsule added to each well, and incubated for 3 hours on a plate shaker at RT. The plate was then washed using a vacuum manifold, beads resuspended in blocking buffer and collected from wells. The beads were then counted ahead of use in the assay. Beads were mixed together into a mastermix solution (25µL per well) and added to a new 96-well MultiScreen$_{HTS}$ BV filter plate. After the plate was washed twice with wash buffer using a vacuum manifold, 25 µl of serum samples were added to the wells, and plate was incubated for 1 hour, protected from light, on a plate shaker at RT. Next, the plate was washed three times with wash buffer using a vacuum manifold ahead of incubation with anti-human IgG PE-conjugate antibody diluent and incubated for 30 minutes, protected from light, on a plate shaker at RT. After incubation, the plate was washed twice with wash buffer using a vacuum manifold and well contents resuspended in 125 µl wash buffer. The plate was analysed in a Luminex analyser (Bio-Rad Laboratories, Germany).

2.4 Preparation of *S. pneumoniae* for use in assays

Glycerol stocks of live *S. pneumoniae* serotypes 4, 19A, 23F and TIGR4Δ*cps* were thawed at 37°C in a water bath. From these an intermediate 1x10^8 CFU/ml stock was prepared in PBS. Preparation of FITC-labelled TIGR4 or heat-killed (HK) TIGR4 also followed the following steps. These new stocks were centrifuged (13000 x g, 5 minutes, RT), the supernatant

discarded and the pellets were resuspended in PBS. From the $1x10^8$ CFU/ml stocks, bacteria were aliquoted into a new Eppendorf tubes at the concentration required for each assay and centrifuged (13000 x g, 5 minutes, RT). The supernatant was collected, and the pellet was resuspended in assay media. Bacterial preparations were used immediately.

2.4.1 Pre-opsonisation of pneumococcal serotypes

From the $1x10^8$ CFU/ml stocks prepared as described in 2.4, bacteria ($8x10^6$ CFU total; $4x10^6$ CFU/ test) were aliquoted into a new Eppendorf, centrifuged (13000 x g, 5 minutes, RT) and resuspended according to the opsonins of interest:

- Non-opsonised control: resuspended in PBS;
- Antibody-opsonised: TIGR4 and TIGR4Δ*cps* strains were resuspended in anti-type type 4 capsule serum, 19A was resuspended in Pneumococcus Group 19 antiserum, and 23F was resuspended in Pneumococcus Group 23 antiserum (all raised in rabbit, Statens Serum Institut, Copenhagem, Denmark). All anti-capsular serum was pre-diluted 1:150 in PBS;
- Pooled sera from young volunteers (to be referred as young serum): resuspended in 50% young pooled sera prepare in 2.3.2, diluted in PBS;
- Pooled sera from older volunteers (to be referred as old serum): resuspended in 50% old pooled sera prepare in 2.3.2, diluted in PBS.

Bacteria were incubated for 20 minutes at 37°C in 5% CO_2. After incubation, samples were centrifuged (13000 x g, 5 minutes, RT) and resuspended in RPMI-1640 media (Sigma-Aldrich,

UK) supplemented with 2 mM L-glutamine (Thermo Fisher, USA) and 10% fetal calf serum (assay media) and used immediately.

2.5 Analysis in whole blood

2.5.1 Blood cell count

Whole blood cell analysis was performed using a Sysmex XN-1000 analyser (Sysmex UK, UK). This instrument generates a full blood cell count that also includes complementary parameters such as immature granulocytes (IG) and other parameters of immature granulocytes and monocytes. For Sysmex analysis, 200 µl of heparin anticoagulated blood from 31 healthy young, 27 healthy older adults, 24 older pneumonia patients and 3 young patients were analysed.

2.5.2 Measurement of neutrophil phagocytosis of *S. pneumoniae* TIGR4 in whole blood

This phagocytosis protocol is a modified version of the PhagoTest^{TM} kit (Glycotope Biotechnology, Heidelberg, Germany). For assessing phagocytosis, 100 µl of heparinised blood was aliquoted into FACS tubes and rested on ice for 10 minutes. Next, pre-opsonised FITC-labelled *S. pneumoniae (*4x10^{7}CFU/tube), prepared as described in 2.4.1 were added to blood aliquots, samples were vortexed and incubated at 37°C in a water bath for 10 minutes. Stimulations were performed in duplicate. One tube containing antibody-opsonised TIGR4 was kept on ice, protected from light, as a negative control. After incubation, samples were immediately placed on ice and ice-cold Trypan blue (1:4 diluted in PBS, Sigma-Aldrich, UK)) was added to quench the fluorescence of non-internalized bacteria. 2ml of PBS was added to tubes ahead of centrifugation (250 x g, 5min, 4°C). The supernatant was discarded, and the

wash step repeated. The supernatant was discarded, and the pellet resuspended in 2ml of 1x BD FACS lysing solution (1:10 dilution in deionized water, BD Biosciences, UK). Tubes were incubated at RT for 20 minutes, in the dark. Following incubation, samples were centrifuged (250 x g, 5min, 4°C), the supernatant discarded, and cells washed with 2 ml PBS. After centrifugation, the supernatant was discarded, and the pellet resuspended in 200 µl of propidium iodide (PI) (Sigma-Aldrich, UK) 10 µg/ml solution (prepared in 3.4 M sodium citrate buffer) and incubated for 10 minutes on ice. Samples were analysed within 1 hour using a BD Accuri™ C6 flow cytometer. Neutrophils were gated based on their forward scatter/sideward scatter properties, 15,000 cells were acquired. The amount of pneumococcus phagocytosed was measured by the median fluorescence intensity (MFI) of gated neutrophils using CFlow software (BD Biosciences, UK). The phagocytic capability of neutrophils was determined by the phagocytic index, which considers both the percentage of phagocytosing neutrophils and MFI from ingested bacteria, as shown by the equation below:

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

2.6 Isolation of immune cells from whole blood

2.6.1 Neutrophil isolation

Under sterile conditions, EDTA-anticoagulated blood was transferred to a 50 ml Falcon™ tube (Thermo Fisher, USA). 2% Dextran (diluted in 0.9% saline, Sigma-Aldrich, UK) was added to the blood at the proportion of 1 ml for every 6 ml of blood and mixed gently. Blood was left to rest for 40 minutes at RT to allow erythrocytes to sediment. Next, the layer on top of

the erythrocytes, containing plasma and leukocytes, was collected and layered on top of a Percoll (Scientific Lab Supplies, UK) density gradient. The Percoll gradient was prepared by carefully adding 2.5 ml of 80% Percoll under 5 ml of 56% Percoll. After transferring the serum on top of the gradient, the tube was centrifuged for 20 minutes, 220 x g at RT, with no brake. Following centrifugation, the neutrophils at the 56%-80% Percoll interface were collected and transferred to a new Falcon tube and resuspended in RPMI-1640 media (Sigma-Aldrich) supplemented with 2 mM L-glutamine (Thermo Fisher) and 10% fetal calf serum (Sigma-Aldrich) (assay media). Neutrophils were washed (220 x g, 10 minutes, RT) resuspended in assay media at a concentration of $1x10^6$ /ml for the following experiments.

2.6.2 Peripheral blood mononuclear cell (PBMC) isolation

Heparinised blood was transferred to a sterile Falcon™ tube (Thermo Fisher) and diluted 1:1 in assay media. Diluted blood was then layered on top of 6 ml of Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and centrifuged for 30 minutes, at 400 x g, RT, with no brake. Following centrifugation, the layer of mononuclear cells was collected and transferred to a universal tube (Sarstedt, Leicester, UK), in which cells were resuspended in magnetic assisted cell sorting buffer (MACS; PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA and 0.09% azide; Miltenyi Biotec, Gladbach, Germany). Cells were pelleted (400 x g, 10 minutes, RT) and washed once again in MACS buffer. After the supernatant was discarded, the PBMCs were resuspended in assay media at a concentration of $1x10^6$ cells/ml and used for subsequent experiments. PBMCs destined for T lymphocyte stimulation were frozen as described in 2.6.3.

2.6.3 Freezing and thawing of PBMCs

For immunophenotyping of T lymphocytes, PBMCs isolated as described above were resuspended in 1ml of freezing media (FCS containing 10% dimethyl sulfoxide (DMSO), Sigma-Aldrich), following the last wash with MACS buffer and stored in cryovials. The cryovials were placed into a freezing container (Mr Frosty, Sigma Aldrich) and stored at -80°C for future experiments. Thawing of samples was performed by placing the cryovials at 37°C in a water bath. The contents were then transferred to a 15 ml Falcon^{TM} tube (Thermo Fisher, USA) and warm assay media supplemented with 50 µg/ml DNase (reconstituted in 0.15M NaCl solution; Sigma-Aldrich) was slowly layered on top of the freezing media [329]. PBMCs were centrifuged (300 x g, 10 minutes, 23°C) and the supernatant was discarded. Pellet was resuspended in warm assay media supplemented with DNase as before and centrifuged again. The supernatant was discarded, and cells were resuspended in 2 ml of complete RPMI to which Benzonase® Nuclease (Merk-Millipore, New Jersey, USA) was added at the proportion of 1 µl per 10^6 of cells frozen. PBMCs were incubated for 1 hour at 37°C in 5% CO_2. This step was performed to enhance cell viability following the thaw process, as nuclease digests cell clumping, allowing an increased recovery of viable cells [329]. Following nuclease incubation, cells were washed with assay media (300 x g, 10 minutes, 23°C), the supernatant was discarded, and PBMCs were resuspended in assay media at a concentration of $5x10^6$ cells/ 5 ml. Cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2.

Thawing of PBMCs from the EHPC cohort did not undergo the nuclease incubation step, as these samples were frozen using the CTL-Cryo™ ABC Media kit (ImmunoSpot, Cleveland, US), that allow recovery rates higher than 70% and high cell viability post-thawing. The next day,

cells were centrifuged (300 x g, 10 minutes, 23°C), re-suspended in assay media at a concentration of $1x10^6$ cells/ml and used in the subsequent experiments.

2.7 Analysis of neutrophil and monocyte function following stimulation with *S. pneumoniae*

2.7.1 Measurement of neutrophil reactive oxygen species (ROS)

Generation of ROS by isolated neutrophils was assessed by incubating $1x10^5$ neutrophils in a 96-flat bottom well plate (Corning, USA) together with pre-opsonised live *S. pneumoniae* serotypes strains ($4x10^6$ CFU/well) (prepared in 2.4.1) in duplicate. As a negative control, a well containing only neutrophils was used, and as positive control, neutrophils were stimulated with PMA (1.62 μM). The plate was incubated for 15 min at 37°C in a 5% CO_2 incubator. Next, dihydrorhodamine 123 (DHR 123) was added at a concentration of 100 μM/well and neutrophils were incubated for 15 min at 37°C in a 5% CO_2 incubator. After incubation, neutrophils were transferred to FACS tubes and 2ml of PBS was added, tubes were centrifuged at 250 x g for 5 minutes at 4°C. Supernatants were discarded and pellets resuspended in PI solution (10 μg/ml) (prepared as described in 2.5.2) for 10 minutes, on ice, protected from light. 1% paraformaldehyde was then added to tubes, for fixation of remaining live bacteria, and samples were incubated for 10 minutes on ice. ROS generation was measured within 30 minutes, using a BD Accuri™ C6 flow cytometer. Acquisition of 15,000 neutrophils was done by gating on their forward scatter/sideward scatter properties. Analysis of ROS production was assessed using CFlow software as explained in section 2.5.3.

2.7.2 Generation of neutrophil cell-free DNA

To assess neutrophil extracellular trap (NET) generation in response to *S. pneumoniae* strains, a modified version of a published protocol was used [141]. Neutrophils ($2x10^5$/well) were added to a 96-flat bottom well and cells that were to be incubated with bacteria were pre-treated with 10 µg/ml Cytochalasin D (Sigma-Aldrich, UK), for 15 minutes at 37°C in 5% CO_2, as described previously [280], to inhibit phagocytosis, by inhibiting actin polymerization. Following incubation, *S. pneumoniae* strains ($2x10^6$ CFU/well), prepared as described in section 2.4, were added to neutrophils. Unstimulated neutrophils were used as a negative control, and 25nM PMA (Sigma-Aldrich) was used as a positive control for NET generation. Wells containing bacteria alone in assay media were also used, for measurement of any DNA release by the pneumococcus. All stimulations were performed in duplicate. The plate was then incubated for 3 hours at 37°C in 5% CO_2. Within 30 minutes of incubation, 100 µg/ml gentamicin (Thermo Fisher) was added to all wells. This high dose of gentamicin was used to inhibit *S. pneumoniae* growth in culture, a strategy previously used by other groups [295,330]. Gentamicin is also a non-lytic antibiotic [331], which could reduce DNA release from *S. pneumoniae* following death. The susceptibility of all the pneumococcal strains used, to the dose of 100 µg/ml gentamicin, was confirmed before these experiments were carried out. Neutrophils and bacteria untreated with gentamicin were included as controls. Following 3 hours of incubation, cell-free DNA release was measured by fluorimetry. Briefly, the supernatant was carefully collected and transferred to 500 µl Eppendorfs and centrifuged at 2200 x g for 10 minutes at 4°C to pellet bacteria and cells. Following centrifugation, 150 µl of cell-free supernatant were transferred to a black 96-flat bottom well plate (Corning). To these, 1 µM of SYTOX Green stain (Thermo Fisher, Life Technologies,

UK) was added for staining of extracellular DNA, and incubated for 10 minutes, protected from light, at RT. Next, fluorescence was measured using a BioTek® Synergy 2 fluorometric plate reader, with excitation and emission set at 485 nm and 528 nm, respectively. All samples were analysed in duplicate.

2.7.3 Visualisation of neutrophil extracellular traps using fluorescent microscopy

This methodology was adapted from the one described in Dr Jon Hazeldine's doctorate thesis and as published by Halverson *et al.* (2015) [332]. For NET visualisation, $2x10^5$ isolated neutrophils (200 µl) resuspended in assay media, were seeded onto 24-well flat bottom plates (Corning) and incubated for 30 minutes at 37°C and 5% CO_2 atmosphere, to allow neutrophils to adhere. Next, 10 µg/ml Cytochalasin D (Sigma-Aldrich) was added to wells and incubated for 15 minutes at 37°C in 5% CO_2. *S. pneumoniae* TIGR4 (section 2.4), was resuspended in assay media and added to neutrophils at a proportion of 10:1 ($2x10^6$ CFU/100 µl) and 100:1 ($2x10^7$ CFU/100 µl). PMA (25nM)-treated neutrophils served as positive control. Assay media was added to unstimulated neutrophils to reach a final volume of 300 µl/well. The plate was then centrifuged at 800 x g for 10 minutes, at 23°C, and then incubated for 3 hours at 37°C in a humidified 5% CO_2 atmosphere. Within 30 minutes of incubation, gentamicin (100 µg/ml) was added to wells containing *S. pneumoniae*. Following stimulation, neutrophils were fixed with 4% paraformaldehyde (20% PFA stock, diluted into wells to final concentration of 4%) and incubated for 30 minutes at 37°C in 5% CO_2. Next, the culture media was collected, and the contents of the wells washed with 500 µl of PBS for 5 minutes, at RT. The PBS was collected, and this step repeated. After PBS removal, cells were

permeabilised with 0.1% Triton X-100 (200 µl) (Sigma-Aldrich) for 1 minute ahead of a 5 minute PBS wash. Next, cells were stained with 1 µM SYTOX Green stain (200 µl) (Thermo Fisher, Life Technologies) for 5 minutes at RT, protected from light. After the stain solution was collected, wells were washed with PBS for 5 minutes and a drop of mountant was added to each well (ProLong Diamond Antifade, Thermo Fisher) and a coverslip applied. Images were acquired with a LEICA DMI 6000 B microscope at X20 objective (Leica microsystems, Milton Keynes, UK).

2.7.4 Stimulation of neutrophils and monocytes for assessment of receptor expression and cytokine production

Neutrophils ($2x10^5$) isolated from EDTA-anticoagulated blood and PBMCs ($2x10^5$) isolated from heparin-anticoagulated blood were transferred into FACS tubes or 96-well round bottom plates. PBMCs were used to assess monocyte activation. Both neutrophils and PBMCs were incubated with *S. pneumoniae* strains TIGR4, 19A, 23F, TIGR4Δ*cps* or heat-killed TIGR4, prepared as described in 2.4, at a multiplicity of infection of 40:1 ($8x10^6$ CFU/test). Neutrophils or monocytes were left unstimulated as a negative control and lipopolysaccharide (LPS) (50 ng/ml) (Sigma-Aldrich) stimulation was used as positive control. After 30 minutes within the incubation time, Gentamicin (100ug/ml) was added to tubes containing *S. pneumoniae*, to stop bacterial growth. Cells were stimulated for different purposes:

- For analysis of surface receptor expression and intracellular cytokine production: Brefeldin A (10µg/ml; Sigma-Aldrich) was added to neutrophils and PBMCs in FACS

tubes, and cells were stimulated for 4 hours at 37°C, with 5% CO_2. At the end of the incubation, samples were processed for immunostaining and flow cytometry analysis.

- For analysis of pro-inflammatory and other cytokine production by neutrophils: neutrophils were stimulated with LPS (50 ng/ml) or pneumococcal strains in a 96-well round bottom plate for 18 hours at 37°C in a humidified atmosphere of 5% CO_2. Once the incubation had ended the plate was centrifuged for 5 minutes 250 x g, at 4°C. Supernatants were collected, aliquoted into Eppendorfs and stored at -80°C for later assay of cytokine content.
- For analysis of IL-17A production by neutrophils: neutrophils were stimulated with LPS (50 ng/ml) or pneumococcal strains in FACS tubes for 18 hours at 37°C in a humidified atmosphere of 5% CO_2. BD GolgiPlug™ (1:100 then 1:100 into wells, BD Biosciences, UK) was added to cells 16 h before the end of incubation. After the incubation, neutrophils were stained for IL-17 and flow cytometry analysis.

2.7.4.1 Measuring surface receptors expression and intracellular cytokine production by neutrophils and monocytes

Following a 4-hour incubation with *S. pneumoniae* strains, neutrophils and PBMCs stimulated as described in section 2.7.4, were washed with 1 ml of PBS and pelleted by centrifugation (5 minutes, 250 x g, 4°C). PBMCs were used to assess monocyte activation following stimulation with the pneumococcus. The supernatant was dispensed, and the cells were resuspended in the residual volume left in the tubes. A cocktail of primary antibodies, detailed in Table 2.1, were added to FACS tubes containing neutrophils or PBMCs. The tubes

were vortexed and incubated for 20 minutes, at 4°C, protected from light. After incubation, 1 ml of PBS was added to tubes for washing unbound antibody (5 minutes, 250 x g, 4°C). The supernatant was dispensed, and pellets resuspended in 100 µl of Fixation Medium (Medium A, Fix and Perm kit, Invitrogen, UK), and were incubated for 30 minutes in the dark, at RT. Post-fixation, cells were washed with 1 ml of PBS and centrifuged (5 minutes, 250 x g, 4°C). The pellet was resuspended in 100 µl of Permeabilisation Medium (Medium B, Fix and Perm kit, Invitrogen) and intracellular antibodies, as shown in Table 2.1. Samples were incubated at RT, protected from light, for 30 minutes. Next, cells were washed with 1 ml of PBS and pelleted by centrifugation (5 minutes, 250 x g, 4°C). Finally, cells were resuspended in 250 µl of PBS and acquired using a BD Fortessa flow cytometer (BD Biosciences, UK). Neutrophil acquisition was performed by counting 20,000 events on the $CD15^+$ gate. Monocyte acquisition was achieved by counting up to 5000 events on the $CD14^+$ gate. Analysis of neutrophil expression of CD11b, CD62L, TLR2 and TLR4 and IL-8 production, and of monocyte expression of CD11b and CCR2 and production of IL-6 and TNF-α were done using FlowJo software (version 10.4.1, FlowJo, LLC, Ashland, Oregon, USA).

Table 2.2 Antibodies used for neutrophils and monocytes staining for flow cytometry

Panel 1: Neutrophils	**Clone**	**Company**	**Volume per tube**
CD15 eFluor® 450	MMA	eBiosciences	3 µl
CD11b APC	ICRF44	eBiosciences	1 µl
CD62L PE-Cyanine7	DREG56	eBiosciences	1 µl
TLR2 FITC	TL2.1	eBiosciences	5 µl
TLR4 PE	HTA125	eBiosciences	5 µl
IL-8 PE-CF594	G265-8	BD Biosciences	2 µl
Panel 2: Monocytes	**Clone**	**Company**	**Volume per tube**
CD14 eFluor® 450	61D3	eBiosciences	3 µl
CD11b APC	ICRF44	eBiosciences	1 µl
CCR2 PE-Cyanine7	357212	Biolegend	1 µl
IL-6 PE	MQ2-13A5	Biolegend	2 µl

TNF-α FITC	MAb11	Biolegend	2 µl

2.7.4.2 Staining for neutrophil IL-17A production by flow cytometry

To investigate if neutrophils could produce IL-17A in response to *S. pneumoniae* strains, following stimulation of isolated neutrophils as in 2.7.4, neutrophils were washed with 1 ml PBS and pelleted by centrifugation (5 minutes, 250 x g, 4°C). Next, pellets were resuspended in 25 µl of viability dye (diluted 1:500 in PBS, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain, Invitrogen) and incubated at 4°C, for 15 minutes. Next, anti-human CD15-eFluor® 450 antibody (3 µl, clone: MMA, eBiosciences, UK) was added, and neutrophils incubated for 15 minutes at 4°C. Next, tubes received 1 ml of PBS and were centrifuged (5 minutes, 250 x g, 4°C). Once supernatant was discarded, neutrophils were fixed with 100 µl of Fixation Medium (Medium A, Fix and Perm kit, Invitrogen), for 30 minutes in the dark, at RT. Cells were washed with 1 ml of PBS and pelleted (5 minutes, 250 x g, 4°C) before resuspension in 100 µl of Permeabilisation Medium (Medium B, Fix and Perm kit, Invitrogen). Anti-human IL-17-APC (2 µl, clone: eBio64DEC17, eBiosciences) was added and cells were incubated for 30 minutes, at RT, in the dark. Neutrophils were washed with 1 ml of PBS, pelleted by centrifugation (5 minutes, 250 x g, 4°C) and resuspended in 250 µl of PBS for flow cytometry analysis. Acquired was performed using the BD Fortessa flow cytometer (BD Biosciences), by gating on live $CD15^+$ neutrophils and counting 20,000 events. Analysis of neutrophil IL-17A production was done using FlowJo software (version 10.4.1, FlowJo, LLC, Ashland, Oregon, USA).

2.9 Measurement of cytokines levels in serum and in neutrophil supernatants

Levels of cytokines in serum and supernatants of neutrophil cultures (described in 2.7.4) of healthy young, healthy older adults and pneumonia patients were determined using multiplex technology and commercial kits according to the manufacturer's instructions (Bio-Rad Laboratories, Germany). The cytokines measured were IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, TNF-α, IL-17A, IL-21, IL-23 and IL-33. Sera were diluted 1:4 in Sample Diluent provided by the kit and supernatants were diluted 1:2 in assay media ahead of assay.

2.10 Immunophenotyping by flow cytometry

2.10.1 Staining of neutrophils and monocytes subsets

Aliquots (100 µl) of fresh heparinised blood were transferred to a FACS tube. To the aliquot destined for neutrophil immunophenotyping, a cocktail containing anti-human CD15-FITC, CD16-eFluor® 450 and CD62L-Phycoerythrin (PE)-Cyanine7 (PEcy7) was added to the blood. For monocyte immunophenotyping, cells were stained with a cocktail of antibodies against human CD16-eFluor® 450, CD14-allophycocyanin (APC) and CCR2-PEcy7. Information about antibodies used is detailed in Table 2.2. Tubes were incubated for 20 minutes on ice, protected from light. After incubation, 1 ml of PBS was added to FACS tubes ahead of centrifugation (5 minutes, 250 x g, at 4°C). The supernatant was dispensed, and pellet was resuspended in 2 ml of 1x BD FACS lysing solution (1:10 dilution in deionized water, BD Biosciences). Samples were incubated for 20 minutes, at RT, in the dark. Following lysis of erythrocytes, cells were pelleted by centrifugation (250 x g, 4°C, 5 minutes) and resuspended in 1 ml PBS, for another centrifugation (250 x g, 4°C, 5 min). Next, supernatant was

discarded, cells resuspended in 250 µl of PBS, and stored at 4°C until acquisition by using a BD Fortessa flow cytometer (BD Biosciences). Acquisition of neutrophils was performed by setting the acquisition gate on CD15 positive cells and counting 10,000 to 15,000 neutrophils within this gate. Acquisition of monocytes was performed by counting up to 15,000 events within the $CD14^{+}$ gate. Post analysis of $CD16^{bright}CD62L^{dim}$ neutrophil population and $CD14^{+}CD16^{+}$ monocytes subsets were performed using the FlowJo software (version 10.4.1, FlowJo, LLC, Ashland, Oregon, USA).

Table 2.3. Antibodies used for immunophenotyping of neutrophils and monocytes by flow cytometry

Panel 1: Neutrophils	**Clone**	**Company**	**Volume per tube**
CD15 FITC	HI98	Biolegend	5 µl
CD16 eFluor® 450	eBioCB16	eBiosciences	3 µl
CD62L PE-Cyanine7	DREG56	eBiosciences	2 µl
Panel 2: Monocytes	**Clone**	**Company**	**Volume per tube**
CD14 APC	61D3	eBiosciences	3 µl
CD16 eFluor® 450	eBioCB16	eBiosciences	3 µl
CCR2 PE-Cyanine7	357212	Biolegend	2 µl

2.10.2 Staining of dendritic cells

This protocol was established by Dr Simon Jochems, from the LSTM and the experiment were performed by me at Dr Daniela Ferreira's laboratory, at the LSTM.

Following thawing of pre-colonisation PBMCs of the EHPC cohort (section 2.6.3), $1x10^{6}$ cells were transferred to FACS tubes. Cells were washed with 1 ml of PBS (5 minutes, 440 x g, RT). The supernatant was discarded, and tubes blotted on absorbant towel to reduce residual volume. The pellet was resuspended in 20 µl viability dye (diluted 1:200 in PBS, LIVE/DEAD™ Fixable Violet Dead Cell Stain, Invitrogen, UK) and incubated for 15 minutes at RT. Next, cells

were stained with a cocktail of anti-human antibodies for targets described in Table 2.3, and incubated for 15 minutes, at RT. Following staining, cells were washed in 1 ml of PBS (5 minutes, 440 x g, RT). The pellet was resuspended in 200 µl of PBS and acquired in a BD LSR II flow cytometer (BD Biosciences).

Table 2.4. Antibodies used for staining of dendritic cell

Antibody target	Clone	Company	Volume per tube
Violet LIVE/DEAD™ dye	Fixable dye	Invitrogen	20 µl of 1:200
Lineage cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56) FITC	SK7, MφP9, 3G8, SJ25C1, L27, NCAM16.2	BD Biosciences	10 µl
CD1c (BDCA-1) PE	F10/21A3	BD Biosciences	5 µl
CD303 (BDCA-2) APC	AC144	Miltenyi Biotec	5 µl
CD141 (BDCA-3) BV711	1A4	BD Biosciences	4 µl
HLA-DR PE-Cyanine7	L243	Biolegend	1 µl
CD40 BV605	5C3	Biolegend	5 µl

2.11 Assessment of phagocytosis by $CD16^{bright}CD62L^{dim}$ neutrophils from pneumonia patients

Analysis of phagocytosis in neutrophils from pneumonia patients was performed using *E. coli* and the PhagoTest™ kit (Glycotope Biotechnology, Heidelberg, Germany). Due to the raised numbers of circulating neutrophils in these patients the volume of blood added to the assay has to be adjusted to maintain the ratio of bacteria to neutrophils to within the range suggested by the manufacturers. The whole blood count (WBC) of the patients was firstly assessed by the Sysmex analyser. The average value of 4.921, calculated based on the WBC of healthy subjects was use as reference to determine the volume of blood from patients required for the assay. This value was divided by the WBC count from the patient. If the result was higher than 1, 100 µl of heparinised blood was used. If lower than 1, the value

was multiplied by 100, giving the volume of blood required for assay. In this case, the volume of 100 µl was achieved by completing the volume with plasma from the patient. Plasma was obtained from centrifugation of heparinised blood (10 minutes, 400 x g, 4°C). Once the required volume of blood and plasma were transferred to FACS tubes and rested on ice for 10 minutes, 20 µl of opsonised FITC-labelled *E. coli* from the PhagoTest^{TM} kit was added and samples were incubated for 10 minutes at 37°C water bath. One control tube remained on ice, as a negative control for phagocytosis. After incubation, 100 µl of ice cold trypan blue 4% (diluted 1:4 in PBS) was added to quench fluorescence of non-internalised bacteria. Tubes were vortexed, 2 ml of PBS was added to each sample and tubes were centrifuged (5 minutes, 250 x g, 4°C). Supernatant was collected, cells stained with anti-human CD16-eFluor® 450 (3 µl, clone: eBioCB16, eBiosciences) and anti-CD62L-PE-Cy7 (2 µl, clone: DREG56, eBiosciences) antibodies, for 20 minutes, on ice, protected from light. Samples were then washed in 1 ml PBS (5 minutes, 250 x g, 4°C), before pellets were resuspended in 2 ml of 1x BD FACS Lysing Solution (1:10 dilution in deionized water, BD Biosciences) and incubated for 20 minutes, at RT, in the dark. Next, samples were centrifuged (5 minutes, 250 x g, 4°C), the supernatant dispensed, and cells washed with 2 ml PBS. Finally, pellets were resuspended in 200 µl and acquired using the BD Fortessa flow cytometer (BD Biosciences). Neutrophils were gated based on their forward scatter/sideward scatter properties, 20,000 cells were acquired. The amount of pneumococcus phagocytosed by $CD16^{bright}CD62L^{dim}$ neutrophils was assessed using the FlowJo software (version 10.4.1, FlowJo, LLC).

2.12 Stimulation of $CD4^{+}$ T lymphocytes with *S. pneumoniae*

PBMCs from healthy volunteers, EHPC participants and pneumonia patients were thawed and rested overnight (section 2.6.3). Following overnight incubation, PBMCs were centrifuged (440 x g, 10 minutes, RT), resuspended in assay media and re-counted using trypan blue to determine viability. Cells were resuspended to a concentration of $1x10^6$ cells/ml and $2x10^5$ PBMCs were transferred to a 96 flat-bottom plate for stimulations. Cells were stimulated for 66 hours. Every stimulation was performed in duplicate, accordingly:

- Mock controls: unstimulated PBMCs.
- PMA/Ionomycin: treated with 10 ng/ml Phorbol 12-myristate 13-acetate (PMA) and 50 ng/ml ionomycin (both from Sigma, UK), added at 48 hours.
- *S. pneumoniae* TIGR4: cells ($2x10^5$) were stimulated with $2x10^6$ CFU/5µl of *S. pneumoniae* TIGR4 (MOI 10) (section 2.4). 30 minutes within the incubation time, 100 µg/ml gentamicin (Thermo Fisher) were added to wells to stop overgrowth.
- *S. pneumoniae* 6B: 5 µg/ml of HK 6B (3.65 mg/ml stock, diluted to 250 µg/ml, of which 4 µl were added into 200 µl cells).

After adding bacteria to PBMCs, cells were incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO_2. After 48h, cells were re-stimulated. Untreated cells were stimulated with PMA and ionomycin, TIGR4-stimulated cells were re-stimulated with HK TIGR4 ($2x10^6$/well, prepared as described in 2.4), 6B-stimulated cells were re-stimulated with 5 µg/ml of HK 6B. 2 hours post re-stimulation, BD GolgiPlug™ (1:100 then 1:100 into wells, BD Biosciences) was added to all cells to inhibit cytokine secretion. The culture was incubated overnight at 37°C in 5% CO_2 for 16 hours. After incubation, cells were added to FACS tubes

by combining the content of 2 wells together and processed for immunostaining and flow cytometry analysis.

2.12.1 Immunophenotyping of $CD4^+$ T lymphocytes for flow cytometry analysis following stimulation with *S. pneumoniae*

Following 66-hour stimulation of PBMCs, cells were transferred to FACS tubes. To these, 1ml of PBS was added cells were pelleted (5 minutes, 440 x g, at 4°C). The supernatant was dispensed, and the tubes blotted on absorbent towel to reduce residual volume. The pellet was resuspended in 25 µl of viability dye (diluted 1:500 in PBS, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain, Invitrogen) and incubated at 4°C, for 15 minutes. Cells were then stained for $CD4^+$ T cell subsets, using three distinct colour panels, as detailed in Table 2.4. The anti-human antibodies for surface molecules were mixed together into a cocktail and added to the cells, which were incubated 4°C, for 15 minutes. Next, cells were washed with 1 ml PBS (5 minutes, 440 x g, at 4°C) ahead of incubation with 800 µl of Working Solution, prepared by diluting 1 part of Fixation/Permeabilisation Concentrate with 3 parts Diluent (both from the Foxp3/Transcription Factor Staining Buffer Set, eBiosciences). Tubes were incubated protected from light, for 30 minutes at RT. Next, 2 ml of 1x Permeabilization buffer (1:10 dilution with deionized water, Foxp3/Transcription Factor Staining Buffer Set, eBiosciences) was added to tubes ahead of centrifugation (5 minutes, 440 x g, at 4°C). Supernatant was dispensed, pellets were resuspended in residual volume left from supernatants and incubated with a cocktail of intracellular anti-human antibodies targeting intracellular

cytokines and transcription factors, according to their respective colour panel, detailed in Table 2.4. Following a 30-minute incubation at RT, cells were washed with 1 ml of 1x Permeabilisation buffer and centrifuged (5 minutes, 440 x g, at 4°C). Finally, the pellets were resuspended in 250 µl of PBS and stored at 4°C until time of acquisition by flow cytometry. Acquisition of 20,000 live $CD3^+CD4^+$ T cells was performed with BD Fortessa flow cytometer (BD Biosciences), and analysis of CD4+ T lymphocytes subsets was done with FlowJo software (version 10.4.1, FlowJo, LLC). Gates were set based on isotype controls.

Table 2.5. Anti-human antibodies used for flow cytometry staining of $CD4^+$ T subsets

Panel 1: Th1/Th2	**Clone**	**Company**	**Volume per tube**
Near-IR LIVE/DEAD™ dye	Fixable dye	Invitrogen	25 µl of 1:500
CD3 PE-Cyanine7	UCHT1	eBiosciences	1 µl
CD4 eFluor® 450	OKT4	eBiosciences	3 µl
IL-4 APC	8D4-8	eBiosciences	2 µl
IFN-γ PE	B27	Biolegend	1 µl
T-bet BV711	4B10	Biolegend	3 µl
GATA3 PerCP/Cy5.5	16E10A23	Biolegend	10 µl
Panel 2: Th17, naïve & memory	**Clone**	**Company**	**Volume per tube**
Near-IR LIVE/DEAD™ dye	Fixable dye	Invitrogen	25 µl of 1:500
CD3 PE-Cyanine7	UCHT1	eBiosciences	1 µl
CD4 eFluor® 450	OKT4	eBiosciences	3 µl
CCR7 (CD197) FITC	G043H7	Biolegend	2 µl
CD45RA PerCP/Cy5.5	HI100	Biolegend	2 µl
IL-17A APC	eBio64DEC17	eBiosciences	2 µl
RORγt PE	AFKJS-9	eBiosciences	2.5 µl
Panel 3: Treg	**Clone**	**Company**	**Volume per tube**
Near-IR LIVE/DEAD™ dye	Fixable dye	Invitrogen	25 µl of 1:500
CD3 PE-Cyanine7	UCHT1	eBiosciences	1 µl
CD4 eFluor® 450	OKT4	eBiosciences	3 µl
CD25 APC	BC96	Biolegend	2 µl
CD127 PerCP/Cy5.5	A019D5	Biolegend	1 µl
GARP PE	7B11	Biolegend	5 µl
Foxp3 FITC	PCH101	eBiosciences	5 µl
Isotype controls	**Clone**	**Company**	**Volume per tube**
Mouse IgG1k APC	P3.6.2.8.1	eBiosciences	The volume of isotype control was calculated based on
Mouse IgG1k BV711	MOPC-21	Biolegend	
Mouse IgG1k PE	MOPC-21	Biolegend	

Mouse IgG1k PE-Cyanine7	P3.6.2.8.1	eBiosciences	the same concentration as the antibody in use
Mouse IgG1k PerCP/Cy5.5	MOPC-21	Biolegend	
Mouse IgG2aκ FITC	eBM2a	eBiosciences	
Mouse IgG2bk eFluor® 450	eBMG2b	eBiosciences	
Mouse IgG2bk PE	MPC-11	Biolegend	
Mouse IgG2bk PerCP/Cy5.5	MPC-11	Biolegend	
Rat IgG2aκ PE	RTK2758	Biolegend	
Rat IgG2aκ FITC	RTK2758	Biolegend	

2.13 Assessing IL-10 and TGF-β1 production by Tregs post CD3 and CD28 stimulation

To analyse the production of IL-10 and TGF-β1 by Tregs, firstly, a round-bottom 96-well plate was pre-incubated with 50 µl RPMI solution containing 5 µg/ml monoclonal anti-human CD3 (clone OKT3) and 5 µg/ml anti-human CD28 (clone 28.2) (both from eBiosciences), for 1 hour at 37°C in a humidified atmosphere of 5% CO_2. To these wells, 150 µl of PBMCs (at $1x10^6$ cells/ml), prepared as described in section 2.6.3, were added. As controls, 150 µl of PBMCs were added to wells containing only 50 µl of RPMI. The cells were stimulated for a total of 66 h. BD GolgiPlug™ (1:100 then 1:100 into wells, BD Biosciences, UK) was added to cells 16 h before the end of incubation. Next, cells were transferred to FACS tubes and washed once with PBS for 5 minutes, 440 x g, at 4°C. After discarding the supernatant, pellet was resuspended in 25 µl of viability dye (diluted 1:500 in PBS, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain, Invitrogen) and incubated at 4°C, for 15 minutes. To these, a cocktail of antibodies targeting surface molecules, containing anti-human CD3-PEcy7, CD4-eFluor® 450, CD25-APC, CD127-PerCP/Cy5.5, GARP-PE at the concentrations described in Table 2.4, was added and cells were incubated for 15 minutes, at 4°C. Next, cells were washed once in PBS for 5 minutes, 440 x g, at 4°C, prior to incubation with 800 µl of Working Solution, prepared by diluting 1 part of Fixation/Permeabilisation Concentrate with 3 parts diluent (both from the Foxp3/ Transcription Factor Staining Buffer Set, eBiosciences). Tubes were incubated for 30

minutes at RT, in the dark, before washing with 2 ml of 1x Permeabilisation buffer (1:10 dilution with deionized water, Foxp3/Transcription Factor Staining Buffer Set, eBiosciences), for 5 minutes, 440 x g, at 4°C. Pellets were resuspended in residual volume left from supernatants and incubated with a cocktail of intracellular antibodies targeting anti-human Foxp3-FITC (5 µl, clone PCH101; eBiosciences), TGF-β1- PE-CF594 (5 µl, clone TW4-9E7; BD Biosciences) and IL-10-BV711 (5 µl, clone JES3-9D7; BD Biosciences), for 30 minutes, at RT, in the dark. Following wash with 1 ml of 1x Permeabilisation buffer for 5 minutes, 440 x g, at 4°C, the cells were resuspended in 250 µl of PBS. Acquisition of 10,000 to 20,000 live $CD3^{+}CD4^{+}$ T cells was performed with BD Fortessa flow cytometer (BD Biosciences), and analysis of $CD25^{+}CD127^{-}Foxp3^{+}$ population positive for GARP, IL-10 and TGF-β1 was done with FlowJo software (version 10.4.1, FlowJo, LLC).

2.14 Statistical analysis

Statistical analysis of all data was performed using GraphPad Prism® (version 7.0, Graph Pad, La Jolla, USA). Data distribution was checked using a D'Agostino-Pearson normality test. For normally distributed data of unpaired samples, parametric T-test was used, while unpaired nonparametric data was analysed with Mann-Whitney test. For paired analysis between normally distributed data, Paired T-test was used, and nonparametric analysis was done using Wilcoxon test. For comparison of three or more groups, a One-Way ANOVA test with Tukey's post hoc test was performed if data was normally distributed, while a Kruskal-Wallis test with Dunn's post hoc test was used when data was nonparametric. For paired analysis of three or more groups of nonparametric data, Friedman test with Dunn's post hoc test was

used. The relationship between two variables was assessed with linear regression analysis. A p value of <0.05 was considered significant.

For analysis of flow cytometry data from neutrophils and monocytes following 4-hour incubation with treatments, the level of statistical significance was adjusted manually for comparisons between unstimulated controls and LPS or *S. pneumoniae* serotypes. The threshold for statistical significance was adjusted to p<0.0084 in these analyses only, to correct for the use of multiple Wilcoxon tests (nonparametric paired T tests). Raw data and adjusted p values are shown in Table A.1 and A.2 in the appendix II.

Chapter 3

The effect of age on the innate immune response to *S. pneumoniae*

3.1 Introduction

Neutrophils are a crucial component in the defence against bacterial and fungal infections, they are the main phagocytic cells for *S. pneumoniae*, followed by monocytes [224,261]. During pneumococcal pneumonia neutrophils are present in all stages of infection, from arriving quickly to inflamed tissues and mediating anti-microbial functions, to being recruited by Th17 cells to the infected site later in the infection, and phagocytosing antibody-opsonised bacteria [257,261,297]. Neutrophil-mediated phagocytosis followed by ROS production and neutrophil extracellular trap (NET) generation are key to containing and eliminating the pneumococcus [224,273,277,333].

The incidence of pneumococcal infection and community acquired pneumonia is increased in adults over 65 years of age [8,18,334]. Despite high uptake of vaccination, the case-fatality ratio is the highest in adults over 65 years [19], in part due to vaccine effectiveness greatly decreasing in individuals older than 80 years [22]. *S. pneumoniae* serotype distribution varies around the globe, but reports are consistent that serotypes 1, 3, 19A, 19F and 23F are more common in aged individuals [19,22,27,28]. Carriage of some of these strains remains prevalent post-vaccination [22] and some, such as 3, 19A, 19F and 23F, are associated with higher risk of death due to pneumococcal pneumonia [335].

Immunosenescence, the decline of immune function in older adults, affects several immune cell types involved in the response against the pneumococcus. *In vitro* studies have demonstrated that neutrophils show reduced chemotaxis, phagocytosis, ROS production and NET generation against microbe or LPS challenge with advancing age [122,126–129,141]. Furthermore, Menter *et al.* (2014) reported that of patients who died of pneumococcal pneumonia, older adults had more infiltrated neutrophils in the lung than did the young,

who had more monocytes [109], reinforcing the hypothesis that changes in immune function with the advance of age is one of the factors underlying increased susceptibility to pneumonia in older adults. However, no study to date has examined whether neutrophil activation, ROS production and NET generation, as well as monocyte activation in response to *S. pneumoniae* serotypes changes with age and whether these functions are impaired during pneumonia infection in older adults. These are the core questions addressed in this chapter.

3.2 Aims

The aims of this chapter were:

- To analyse the effect of age on neutrophil functions involved in immunity against *S. pneumoniae*;
- To analyse neutrophil functions involved in immunity against *S. pneumoniae* during a period of infection in the old, including the impact on the neutrophil pool and monocytes subsets;
- To investigate the effect of selected *S. pneumoniae* serotypes on neutrophil and monocyte cytokine production, during health or pneumonia infection in older adults.

3.3 Results

3.3.1 Healthy Participants

In total 42 young (mean age 26.1 ± 3.4 years; range 21-32 years) and 39 old (mean age 72.4 ± 5 years; range 67-83 years) healthy volunteers were enrolled in the studies on the impact

of age on immunity. All volunteers had no significant co-morbidity or immunological illness and were not on medications known to interfere with immune function. Information about age and gender of all participants is summarized in Table 3.1. Healthy young volunteers will be referred to as HY and healthy older adults, as HO.

Table 3.1. Demographics of healthy volunteers

	Young (HY)	Old (HO)
Number of participants	42	39
Mean age and range (years)	26.14 ± 3.4 (21-32)	72.43 ± 5 (67-83)
Male (%)	19/42 (45%)	11/39 (28%)

3.3.2 Pneumonia cohort

Recruitment of patients with pneumonia was part of a pilot study coordinated by Prof David Thickett. For the work on this thesis, blood samples were collected from patients at least 24h after hospital admission. All patients recruited to this study were above 18 years of age and had confirmed pneumonia by chest X ray with presence of consolidation in the lung. As this was a pilot study, only routine investigations were performed by the hospital and these did not include a detailed diagnosis of the causative pathogen of pneumonia. A total of 24 patients were included in this study, recruited in the course of two periods: September 2016 to March 2017 and January 2018 to February 2018. During recruitment of pneumonia patients, only 3 samples were obtained from young patients. The demographics of patients in the study, including whether they had previously had a vaccination for influenza or pneumonia, are shown in Table 3.2. None of the 3 younger patients had been vaccinated. Of older patients, 33% had been previously vaccinated with pneumococcal vaccine and 57% previously vaccinated against influenza.

To predict severity and mortality of diagnosed pneumonia, CURB-65 score was used. This predictor factor accounts 1 point for the presence of risk factors as confusion, blood urea nitrogen higher than 7mmol/l, respiratory rate ≥ 30/min, blood pressure < 90 mmHg systolic or ≤ 60 mmHg diastolic and age ≥ 65 years. The risk of death is assessed based on the patient score, grouped in one of the categories: low severity (0-1 point, risk of death < 3%), moderate severity (2 points, risk of death 9%) or high severity (3-5 points, risk of death 15–40%). Figure 3.1 shows mean patient age for each CURB-65 score category, indicating that the severity of pneumonia increases with age in this study cohort.

Of patients diagnosed with pneumonia, 5 (27%) had coinfection with influenza and were over 65 years. The presence of sepsis was determined using the SIRS criteria, for which 3 older patients were positive. Of these, 2 were also positive for influenza infection, determined by qPCR assay.

During the first recruitment period, neutrophil ROS and NET release assays were performed, and on the second, neutrophil and monocyte cytokine production and subsets were assessed. Young patients will be referred to as YP and healthy older patients, as OP.

Table 3.2. Pneumonia patient demographics

	Young (YP)	Old (OP)
Number of participants	3	21
Mean age (years)	33±2.6 (30-35)	77.7±9.8 (66-96)
Male (%)	1/3 (33%)	9/21 (42%)
Influenza (qPCR)	0	5/18 (27%)
Sepsis (SIRS)	0	3/19 (15%)
Pneumococcal vaccine	0	7/21 (33%)
Influenza vaccine	0	12/21 (57%)

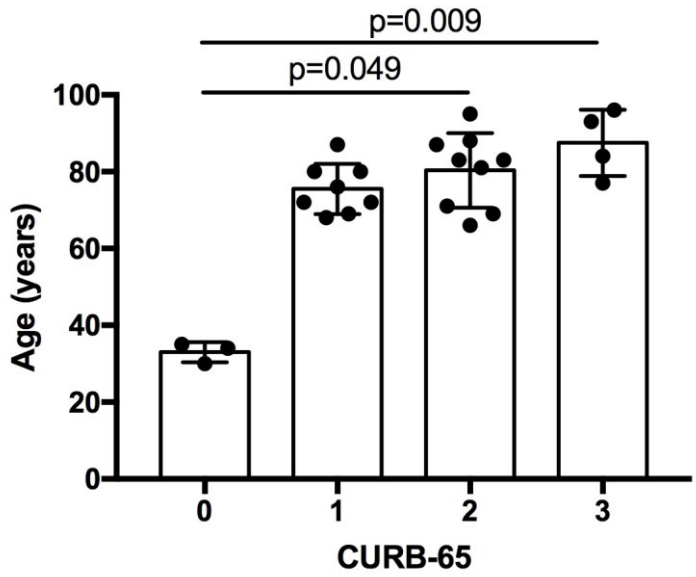

Figure 3.1. CURB-65 score according to age of pneumonia patients. Patient (n=27) age is shown as mean ± SD. Differences between groups were analysed by Kruskal-Wallis test with Dunn's post hoc test.

3.3.3 Changes in circulating leukocytes with age and during pneumonia

Analysis of leukocyte populations using peripheral blood samples from healthy donors and pneumonia patients was performed using a Sysmex XN-1000 analyser, to assess the effect of age and pneumonia infection in the leukocyte pool.

In healthy aged volunteers, the frequency of neutrophils, immature granulocytes (IGs), lymphocytes, eosinophils and basophils were unaltered by ageing, compared to young controls (Figure 3.2). The frequency of circulating monocytes was increased with age (HY, 7.52±2.64 vs HO, 9.67±2.19, p=0.002) (Figure 3.2 C), but absolute numbers were unchanged (Figure 3.3 C). The absolute numbers of the other cell types did not change with age (Figure 3.3).

During pneumonia infection, older patients had an increased frequency of circulating neutrophils (OP, 81.02±10.98 vs HO, 57.3±7.04, p<0.0001) and immature granulocytes (OP, 0.8±0.61 vs HO, 0.26±0.1, p=0.0001), whereas lymphocytes (OP, 10.66±6.85 vs HO, 29.39±6.68, p<0.0001), eosinophils (OP, 0.3±0.5 vs HO, 2.81±2.01, p<0.0001; vs YP, 2.4±1.55, p=0.002) and basophils (OP, 0.3±0.11 vs HO, 0.81±0.37, p<0.0001; vs YP, 0.56±0.25, p=0.043) were decreased, compared to healthy old and young patients (Figure 3.2). Young patients also presented lower lymphocyte frequency than healthy young (YP, 18.07±8.75 vs HY, 30.64±7.44, p=0.036) (Figure 3.2 D). Monocyte frequency was unaltered in older patients (Figure 3.2 C), but absolute numbers of monocytes were increased in the circulation of patients (OP, 0.86±0.49 vs HO, 0.54±0.15, p<0.003) (Figure 3.3 C). The absolute number of eosinophils was reduced in both young (p=0.016) and older (p=0.0001) patients during pneumonia, compared to healthy controls (Figure 3.3 E). To verify If the decline in eosinophil

numbers was associated with pneumonia severity and not ageing, patient data was plotted according to patient CURB-65 score. However, numbers of circulating eosinophils were no different between patients with moderate (scores 0-1) or severe (scores 2-3) pneumonia (Figure 3.3 E).

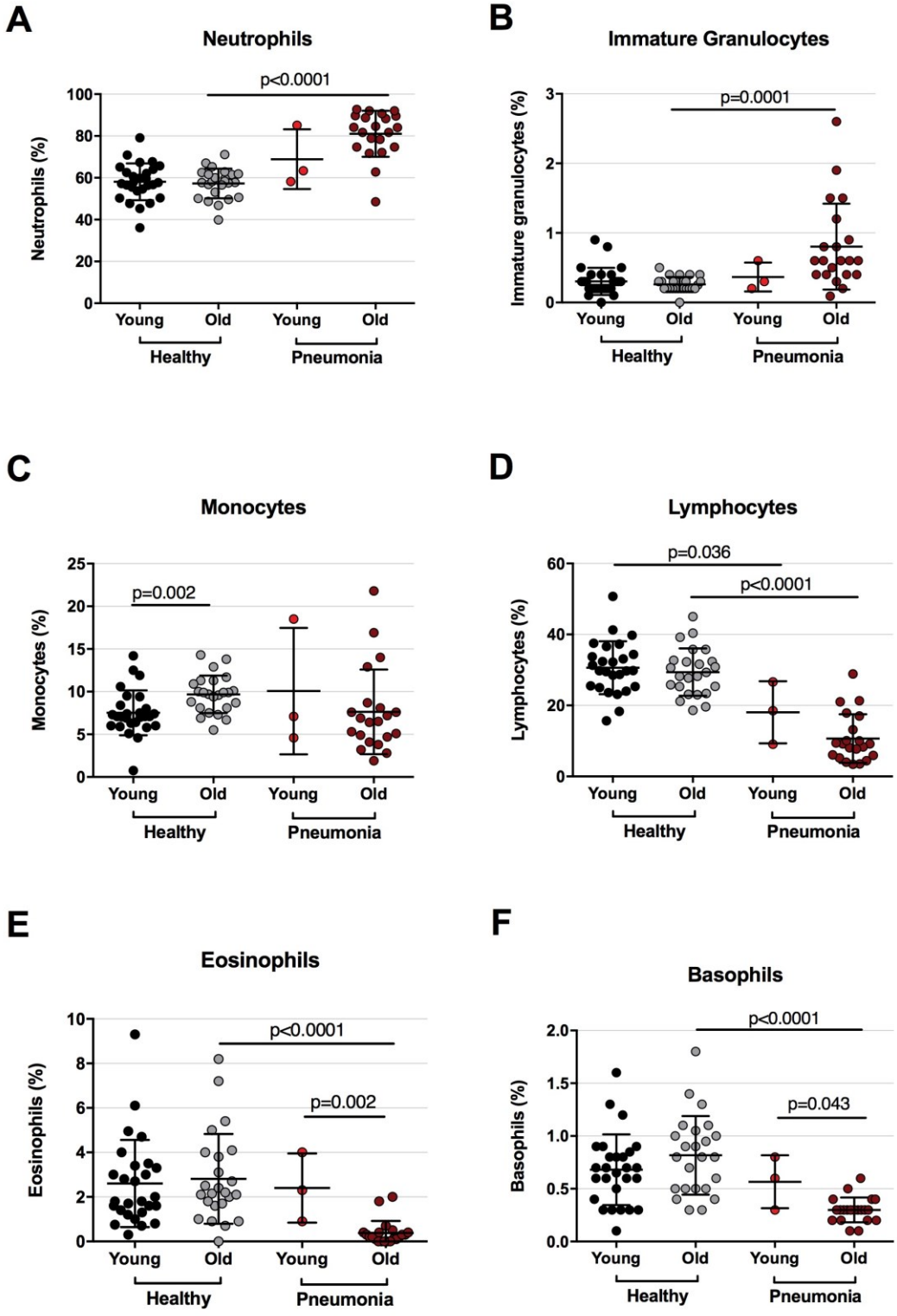

Figure 3.2. Frequency of circulating leukocytes in healthy volunteers and pneumonia patients. Whole blood analysis was performed using a Sysmex XN-1000 analyser in healthy young (n=27) and older (n=24) adults, and in young (n=3) and older (n=21) pneumonia patients. Data are shown as mean ± SD. Differences between age groups and healthy and pneumonia groups were analysed by unpaired T test. Mann Whitney test was used for comparisons with data from young patients.

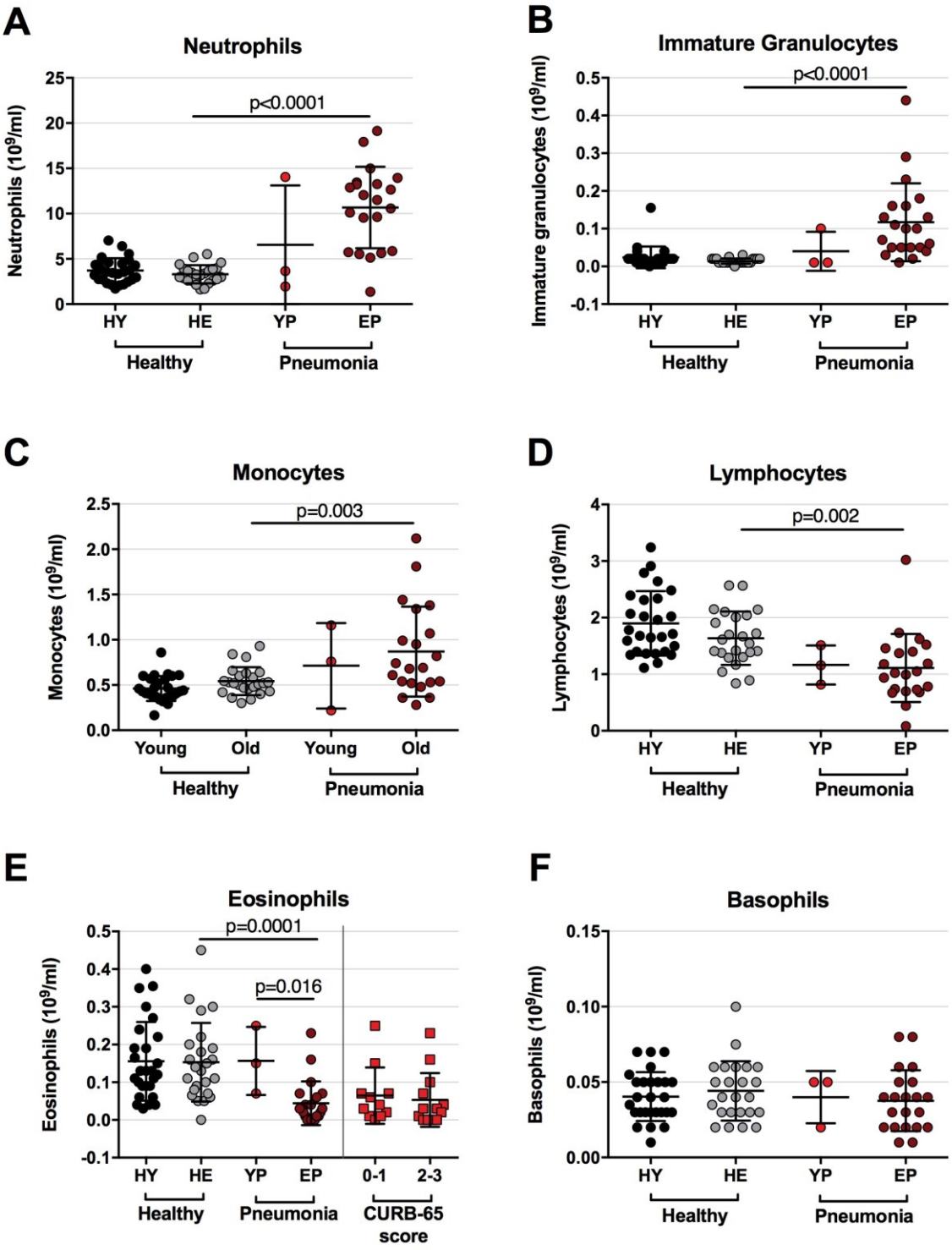

Figure 3.3. Absolute number of circulating leukocytes in healthy volunteers and pneumonia patients. Whole blood analysis was performed using a Sysmex XN-1000 analyser in healthy young (n=27) and older (n=24) adults, and in young (n=3) and older (n=21) pneumonia patients. Data is shown as mean ± SD. Differences between age groups and healthy and pneumonia groups were analysed by unpaired T test. Mann Whitney test was used for comparisons with data from young patients.

3.3.4 Analysis of neutrophil CD16^{bright}CD62L^{dim} subset in healthy ageing and pneumonia

Having found heterogeneity in the circulating neutrophil pool of older patients with pneumonia, the presence of CD16^{bright}CD62L^{dim} neutrophils was investigated in this cohort. This immunosuppressive neutrophil subset is present in patients with severe injury or acute systemic inflammation [118,119] and reported to be increased in older adults [121].

During pneumonia, the CD16^{bright}CD62L^{dim} subset was found in older patients, as shown by the representative plots in Figure 3.4 A. As reported before by Sauce *et al.* (2017) [121], the frequency of CD16^{bright}CD62L^{dim} neutrophils increased with age ($p=0.007$), and older pneumonia patients showed a trend towards an increase in this population frequency, but statistical analysis found no difference between groups of older adults (Figure 3.4 B).

Neutrophil phagocytosis was tested in samples from pneumonia patients (Figure 3.4 C), as the CD16^{bright}CD62L^{dim} subset has been shown to have a lower phagocytic capacity [121]. However, the sample size was too small to confirm if phagocytic function is low in this subset in this group.

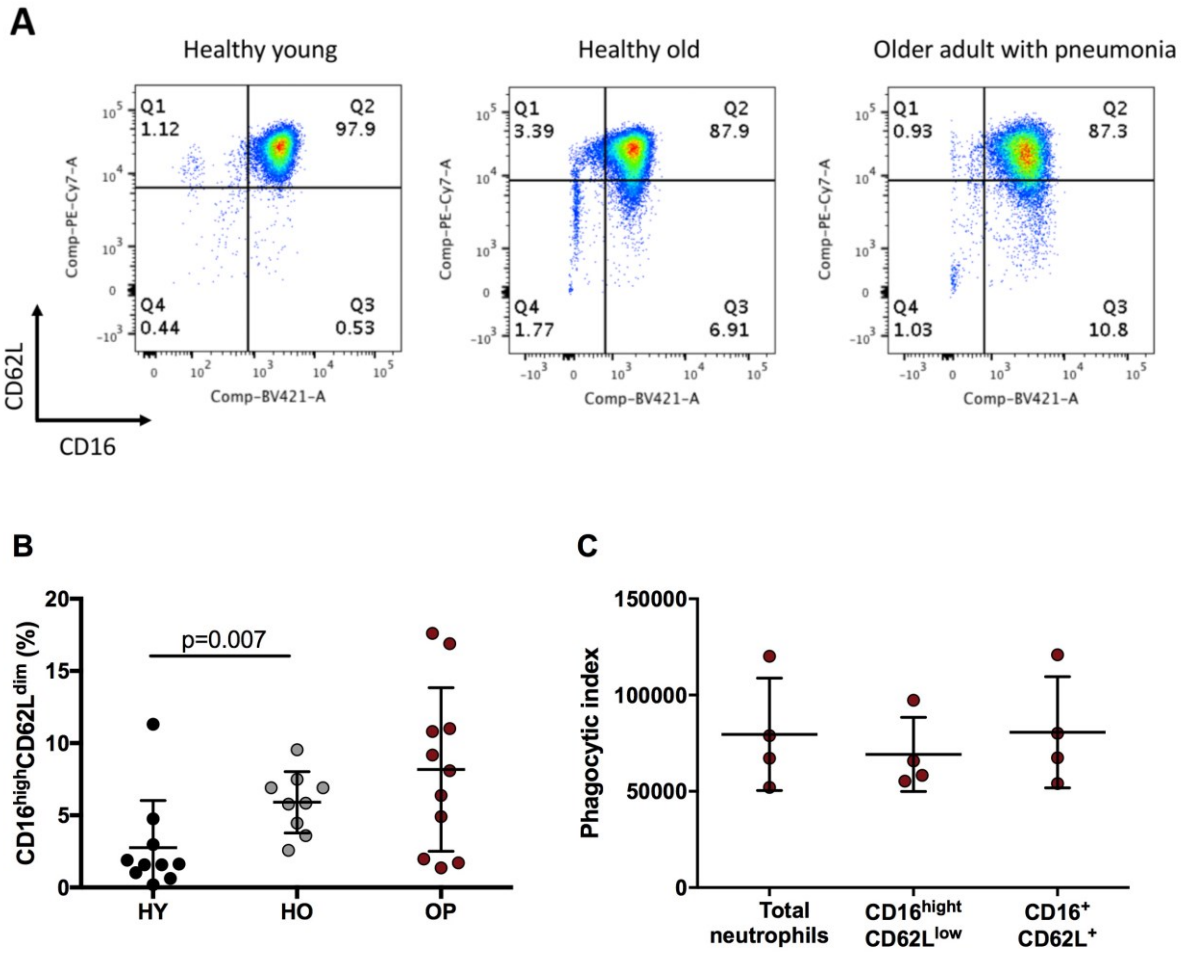

Figure 3.4. Frequency of CD16^{high}CD62L^{dim} neutrophils in healthy volunteers and older pneumonia patients. Whole blood samples were stained with anti-human CD15, CD16 and CD62L for analysis of CD16^{high}CD62L^{dim} subset. **(A)** Representative dot plots of neutrophils according to CD16 and CD62L expression in volunteers. **(B)** Frequency of CD16^{high}CD62L^{dim} neutrophil subset in healthy young (n=10), old (n=9) and in older pneumonia patients (n=11). Whole blood samples were incubated with FITC-labelled *E. coli* for 10 minutes at 37°C ahead of staining with anti-human CD16 and CD62L for assessing the **(C)** Phagocytic capacity of neutrophil populations in older pneumonia patients (n=4). Data is expressed as mean ± SD. Differences between healthy cohorts or healthy older adults and patients were analysed by Mann Whitney test. Differences between phagocytic index of neutrophils were analysed by Friedman test with Dunn's post hoc test. HY = healthy young, HE = healthy old, OP = old with pneumonia.

3.3.5 Analysis of monocytes subsets in healthy ageing and pneumonia

Parallel to neutrophil immunophenotyping, fresh whole blood was stained for monocyte subsets and CCR2 expression. CCR2 is required for monocyte transit from the blood to inflamed tissues [336]. As shown at the start of this chapter, older participants had a higher frequency of circulating monocytes than young, while older pneumonia patients had increased absolute numbers of monocytes (Figures 3.2 and 3.3). There was no age-related difference in frequency of monocyte subsets between healthy young and old participants, but older patients with pneumonia had a higher frequency of intermediate monocytes compared to healthy old ($p=0.042$) (Figure 3.5 A). Analysis of CCR2 surface expression on these subsets from all cohorts showed variation, with classical ($CD14^{high}CD16^{-}$) monocytes expressing higher levels of CCR2, followed by intermediate monocytes ($CD14^{+}CD16+$) and non-classical ($CD14^{low}CD16^{+}$) (Figure 3.5 B). CCR2 expression in non-classical monocytes was increased with age ($p=0.017$), while CCR2 expression on intermediate monocytes from older patients was higher than healthy old ($p=0.035$).

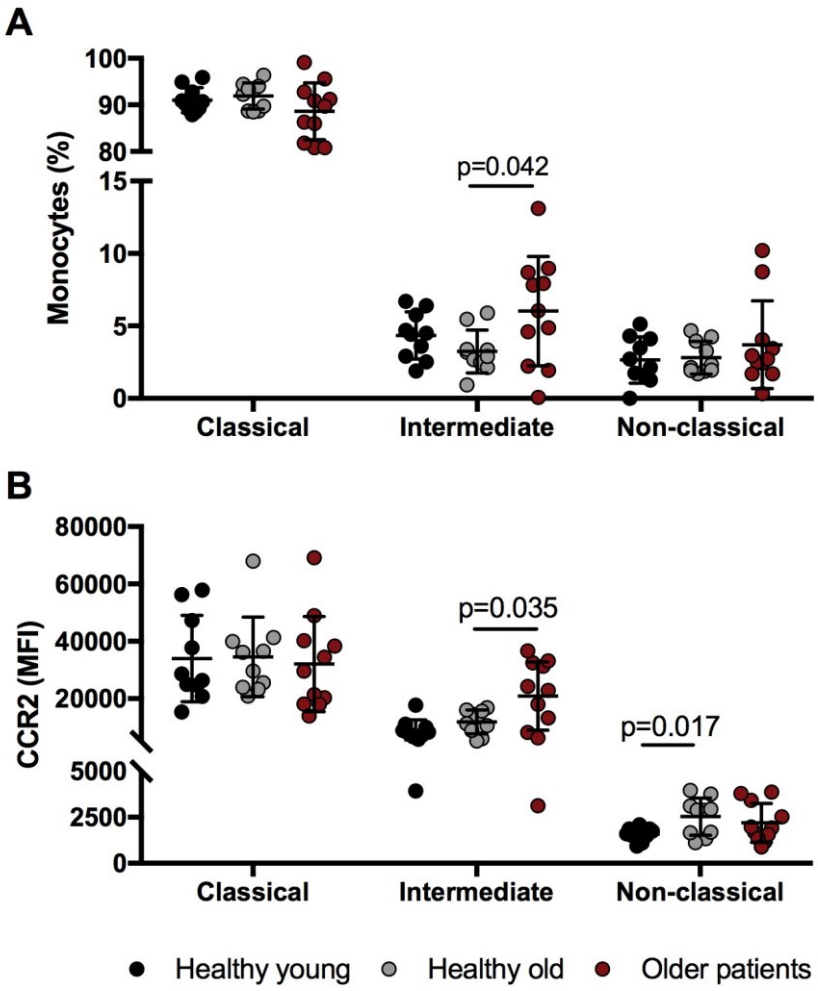

Figure 3.5. Frequency of intermediate monocytes in healthy subjects and older pneumonia patients. Whole blood aliquots were stained with anti-human antibodies CD14, CD16 and CCR2 for flow cytometry analysis of the **(A)** frequency of monocytes subsets and of **(B)** surface density (MFI) of CCR2 in monocytes subsets of healthy young (HY, n=10), healthy old (HO, n=10) and in older pneumonia patients (OP n=11). Data are mean ± SD. Differences between healthy cohorts or healthy older adults and patients were analysed by unpaired T test.

3.3.6 Cytokine levels in serum during pneumonia infection

Cytokines were measured in serum using a multiplex assay. In total, 22 serum samples were measured in each group. The cohort of young pneumonia patients was only n=3, and for these patients some of the cytokines shown in Figure 3.6 were not detected. IL-1β (Figure 3.6 A) was detected in the serum of a small number of healthy participants, but no difference was found with older subjects. There were no age-related changes in serum levels of IL-6, but IL-8 levels were increased in healthy older adults, compared to young (Figure 3.6 B and D). During pneumonia infection, both IL-6 ($p=0.005$) and IL-8 ($p<0.0001$) were elevated in the serum of older patients, compared to healthy subjects. Levels of serum IL-6 and IL-8 were not statistically different between patients with moderate (scores 0-1) and severe pneumonia (scores 2-3), as indicated by the CURB-65 score (Figure 3.6 B and D).

TNF-α was detected in the serum from all cohorts, but no significant changes were observed between subjects (Figure 3.6 C). Also, older patients showed high levels of IL-33 in serum, compared to healthy (Figure 3.6 E).

Levels of IL-2, IL-4, IL-10, IFN-γ, IL-17A, IL-21 and IL-23 were also measured but were below the detection threshold.

There was a positive trend between the levels of serum IL-6 and elevated CCR2 expression in intermediate monocytes ($p=0.017$) (Figure 3.7 A). Also, IL-8 levels in the bloodstream positively correlated with frequency of $CD16^{high}CD62L^{dim}$ neutrophils found in our cohorts ($p=0.003$) (Figure 3.7 B).

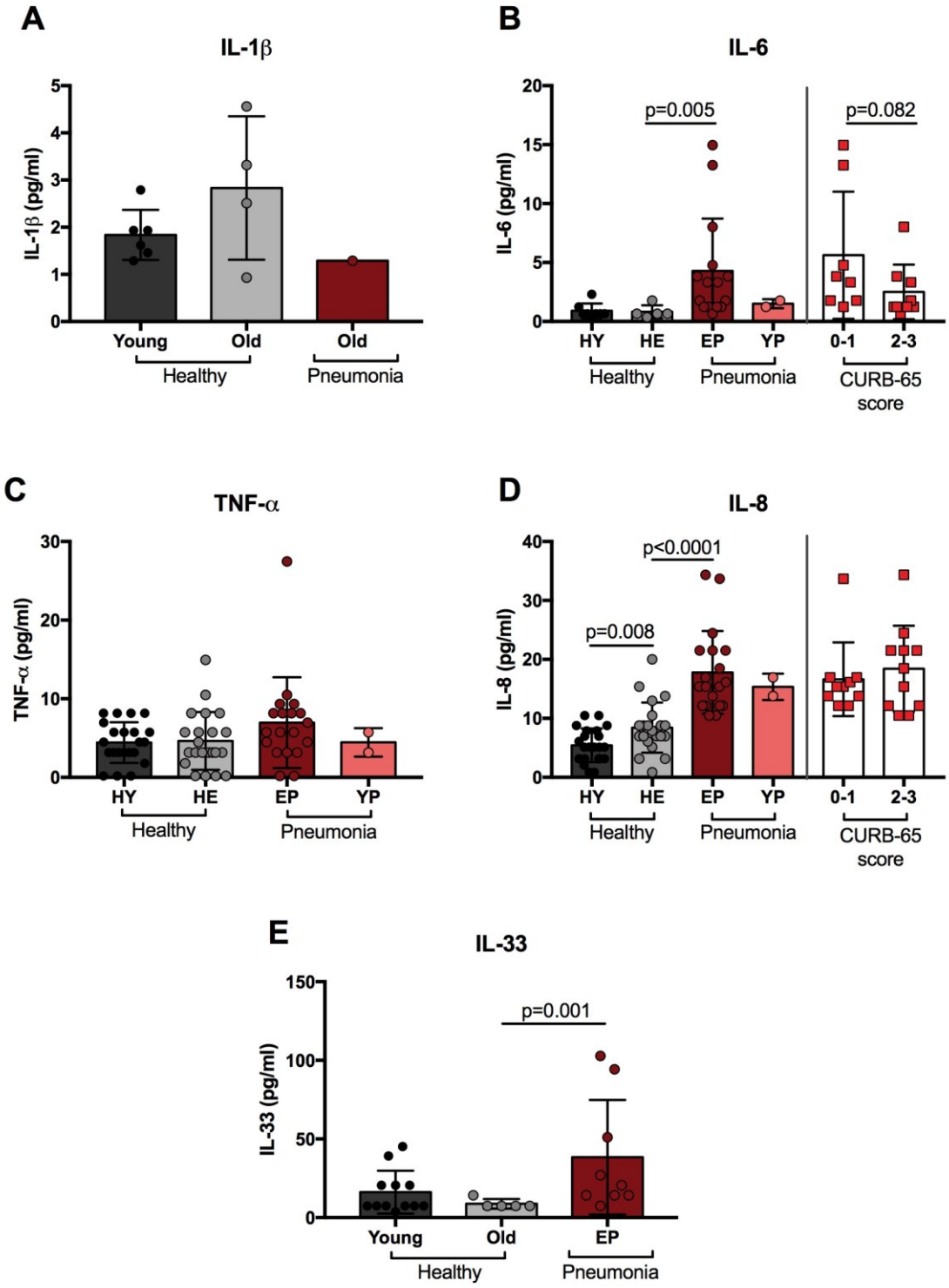

Figure 3.6. Cytokines levels in serum from healthy donors and patients with pneumonia. Serum samples were collected from healthy young (n=22) and old (n=22) donors and young (n=3) and older (n=22) patients with pneumonia and analysed for the presence of cytokines **(A)** IL-1β, **(B)** IL-6, **(C)** TNF-α, **(D)** IL-8 and **(E)** IL-33. Data are mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test

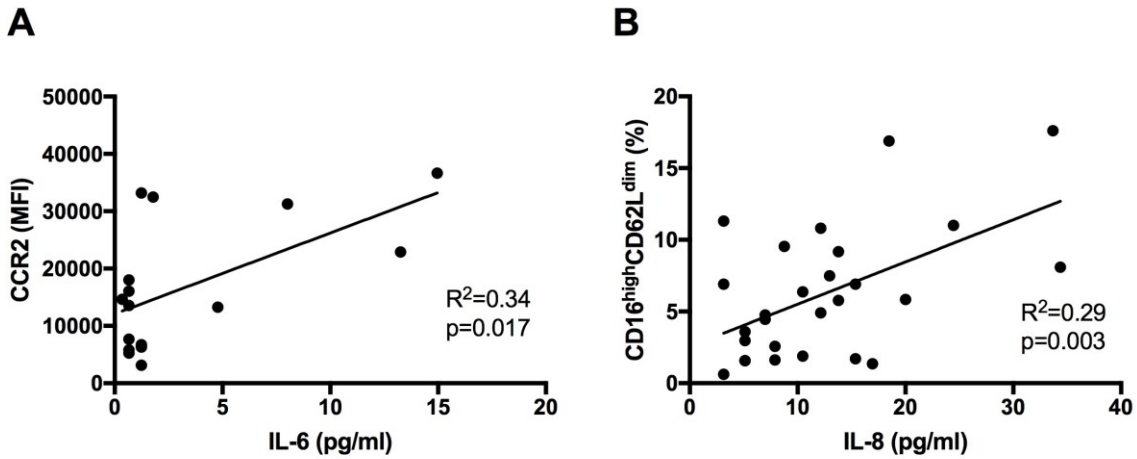

Figure 3.7. Trends between pro-inflammatory cytokine in serum and monocyte and neutrophil phenotype. (A) Correlation between CCR2 surface density (MFI) in intermediate ($CD14^{+}CD16^{+}$) monocytes and serum IL-6 (n=16); **(B)** Correlation between the frequency of $CD16^{high}CD62L^{dim}$ neutrophils and serum IL-8 (n=27).

3.3.7 Effect of age on neutrophil phagocytosis of *S. pneumoniae* in whole blood

Neutrophil function of healthy young and older donors was initially assessed in whole blood, after adapting protocols from the commercial PhagoTest™ kit. *S. pneumoniae* serotype 4, TIGR4 strain was used at a concentration of $4x10^7$ CFU/100 μl of blood. To verify if opsonisation enhanced neutrophil phagocytic activity, bacteria were pre-opsonised with anti-capsular antibody or complement, for which pooled serum from healthy volunteers (described in 2.3.2) was used as source of complement and autologous antibody.

In both groups, phagocytosis was significantly enhanced by pre-opsonisation with sera of both age groups, compared to non-opsonised bacteria (Figure 3.8 A, C and D). Also, antibody opsonisation with a specific serotype 4 antibody enhanced phagocytosis by neutrophils of young, but not by neutrophils of older volunteers (Figure 3.8 A, C and D).

However, there was no difference between neutrophil phagocytosis of TIGR4 in young and old donors. It is possible that this observation is due to the unexpectedly large variation of

fluorescence data in both groups (Figure 3.8 A). To try and determine why the data were so variable, the stability of the FITC label on the pneumococcus was examined and, as shown in Figure 3.7 B, fluorescence of FITC-labelled pneumococcus was not fully preserved during the period of data acquisition (Figure 3.8 B). Also, by using whole blood aliquots instead of a fixed number of isolated neutrophils, the MOI varied between subjects, as the number of circulating neutrophils vary across individuals. These could explain the data variability observed.

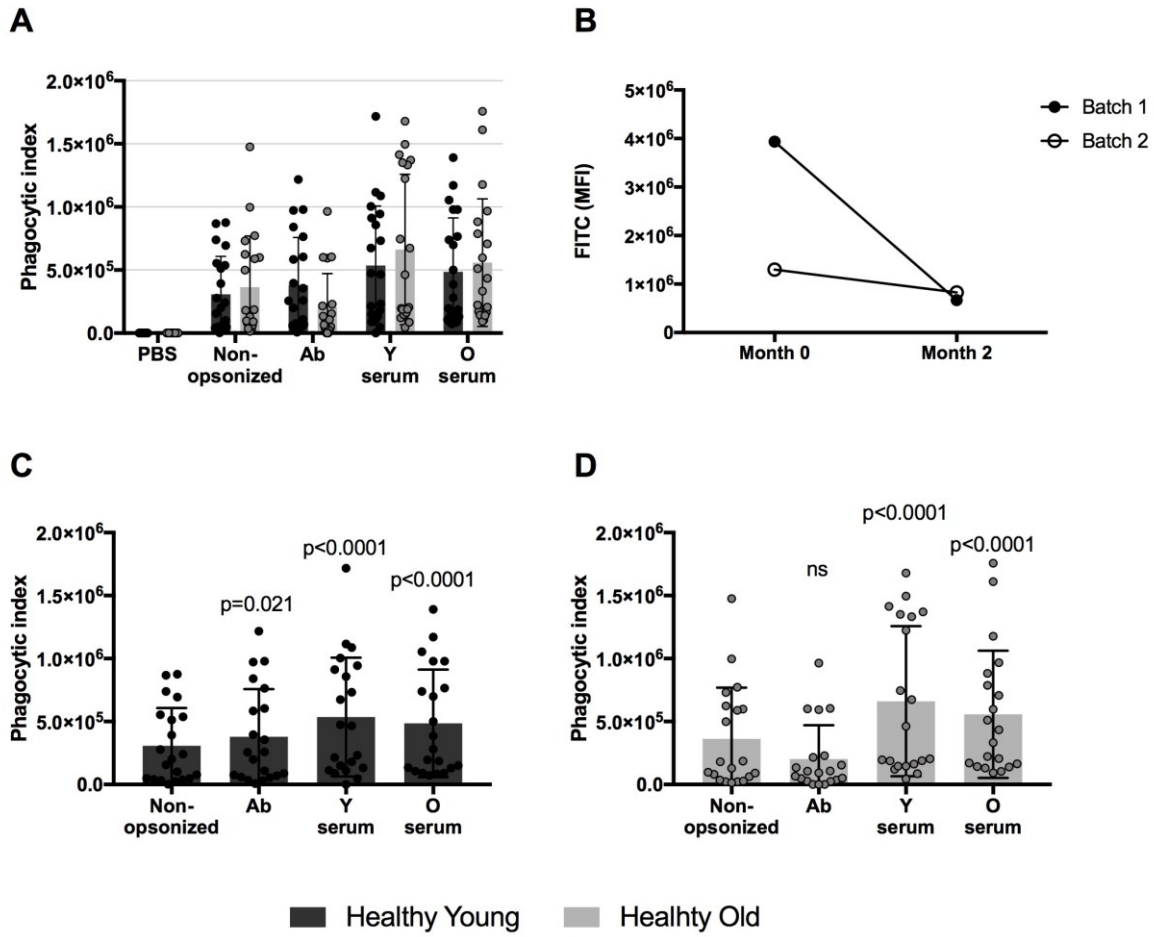

Figure 3.8. Neutrophil phagocytosis of *S. pneumoniae* TIGR4. Whole blood samples were incubated with FITC-labelled *S. pneumoniae* TIGR4 pre-opsonised or not, for 10 minutes in a 37°C water bath, ahead of flow cytometry analysis. **(A)** Neutrophil phagocytic ability of healthy volunteers. **(B)** Decay of fluorescence of FITC-labelled *S. pneumoniae* stocks, expressed as median of fluorescence intensity (MFI). Comparison of neutrophil phagocytic ability in the presence or absence of opsonins in **(C)** healthy young and **(D)** old. Data are mean ± SD. Healthy young (n=21), old (n=20). Differences between groups were analysed by unpaired T test. Comparison between non-opsonised vs opsonised

data was performed by Friedman test with Dunn's post hoc test and statistical significances are shown on top of bars.

3.3.8 The effect of age on neutrophil ROS production to *S. pneumoniae* serotypes

Epidemiological studies have shown that distribution of *S. pneumoniae* serotypes vary across age groups [19,22,337–341]. Therefore, neutrophil oxidative burst generation to different pneumococcal strains was tested, aiming to identify changes with increasing age and during pneumonia infection. Alongside laboratory strain TIGR4, isolates 19A and 23F from older patients, and TIGR4 capsule-negative mutant TIGR4Δcps were used to induce neutrophil respiratory burst, at multiplicity of infection (MOI) of 40:1 ($4x10^6$ CFU). During recruitment of pneumonia patients, only 3 samples from young patients were received, therefore not all serotypes were tested in all patient samples.

Figure 3.9 A shows no age-related changes between baseline levels of ROS production while in pneumonia patients, resting unstimulated neutrophils had high levels oxidative burst than in healthy volunteers (OP, 38964±41604 vs HO, 14795±2572, p=0.027; YP, 79312±100162 vs HY, 14113±3719 p=0.009). Upon PMA stimulation, neutrophils from all cohorts produced high levels of ROS (Figure 3.9 B). Healthy older adults showed higher PMA-induced ROS production than young (HO, 3646546±901541 vs HY, 3078740±590468, p=0.028), but neutrophils from older patients did not reach the same level of ROS production as their healthy counterparts (HO, 3646546±901541 vs OP, 2716918±1229159, p=0.04).

Overall, all serotypes, opsonised or not, were able to induce neutrophils from all cohorts to generate ROS, with the exception of TIGR4Δ*cps*, when compared to baseline ROS production.

Figure 3.10 A shows that live, non-opsonised, pneumococcal serotypes induced different levels of oxidative burst in neutrophils of all cohorts. 19A induced the highest level of oxidative burst observed, followed by TIGR4, 23F, and TIGR4Δ*cps.*

ROS generation to TIGR4 was not different between healthy cohorts, but production to 19A (HY 902711±244918 vs HO, 710882±231915, p=0.029) and 23F (HY, 212009±138497 vs HO, 48019±17860, p<0.0001) were diminished in healthy older adults, which could indicate a susceptibility to these serotypes with ageing in the non-infected state. Neutrophils from pneumonia patients had increased oxidative burst activity in response to all serotypes, including the capsule-negative mutant (TIGR4Δ*cps*), compared to healthy controls. There was no difference between ROS production by older and young patients.

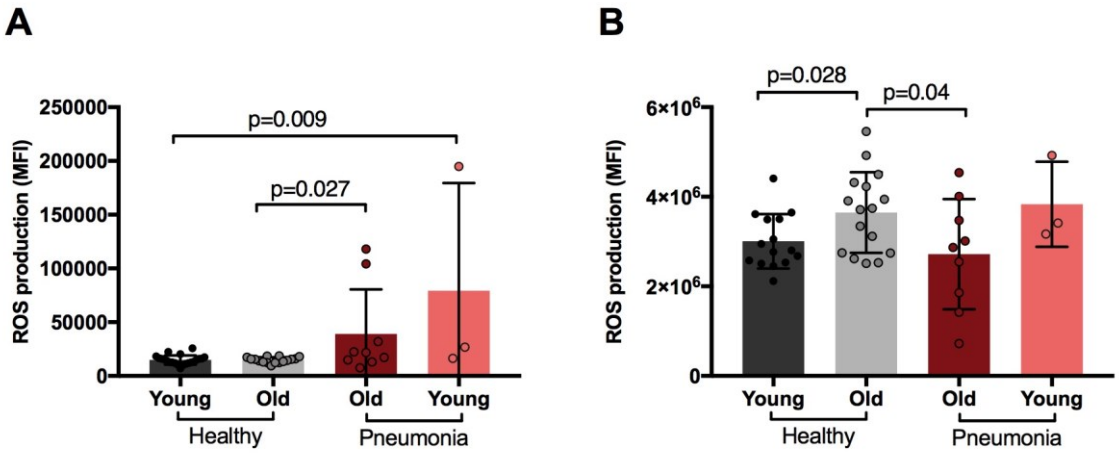

Figure 3.9. ROS production by resting and PMA-treated neutrophils. ROS production by **(A)** unstimulated and **(B)** PMA-treated neutrophils of healthy young (n=15) and old (n=15), and of young (n=3) and older (n=10) pneumonia patients. Data are mean ± SD. Differences between age groups and healthy and pneumonia groups were analysed by unpaired T test. Mann Whitney test was used for comparisons with data from young patients.

3.3.8.1 Role of opsonins

Age-related changes in neutrophil ROS production during health and pneumonia in response to opsonised pneumococcal serotypes are shown in Figures 3.10 B and Figure 3.11. The same data is presented again in Figures 3.12 and 3.13, which compare the effect of different opsonins on neutrophil ROS generation to *S. pneumoniae* serotypes.

Comparison of neutrophil ROS production induced by non-opsonised and opsonised bacteria show that this function was enhanced by all opsonins for TIGR4, but only by the presence of serum for serotypes 19A and 23F, in all cohorts tested (Figures 3.12 and 3.13). TIGR4Δ*cps* did not induced ROS production even in the presence of opsonins, compared to baseline control, in any cohort.

Levels of ROS produced following incubation with antibody-opsonised TIGR4 were the same between healthy cohorts, but pre-opsonisation with anti-capsular polyclonal antibody was unable to enhance ROS production to 19A ($p=0.032$) and 23F ($p<0.001$) in healthy older adults to the same levels as young subjects (Figure 3.10 B). In older patients, ROS generation to antibody-opsonised TIGR4 ($p=0.007$), 19A ($p=0.006$) and 23F ($p=0.02$) (Figure 3.10 B) remained higher than in healthy, even though antibody was not able to enhance ROS production against 19A and 23F, compared to non-opsonised control (Figures 3.13 C and E).

There was no statistical difference between neutrophil ROS production following opsonisation of bacteria with pooled sera of young or older donors (Figures 3.11 A and B). In neutrophils from healthy old, ROS levels were lower following stimulation with young sera-opsonised TIGR4 ($p=0.017$). Of note, ROS production by neutrophils of healthy older adults in response to 23F was lower than of young, with all opsonins tested (non-opsonised, $p<0.0001$, antibody, $p<0.001$, pooled sera from young, $p=0.001$, pooled sera from old,

p=0.002). ROS generation was again elevated in neutrophils of pneumonia patients in response to these serotypes (pooled sera from young: TIGR4, p=0.016; 19A, p=0.070 (ns); 23F, p=0.0008; pooled sera from old: TIGR4, p=0.023; 19A, p=0.021; 23F, p=0.001). However, in older patients, opsonisation was mostly enhanced by sera opsonisation (Figures 3.13 A, C, E and G).

In all cohorts, neutrophils did not produce significant levels of ROS in response to TIGR4Δ*cps*, in the presence or absence of pre-opsonisation (Figures 3.12 G and H, Figures 3.13 G and H), indicating that the pneumococcus capsule has a significant role in the induction of oxidative burst.

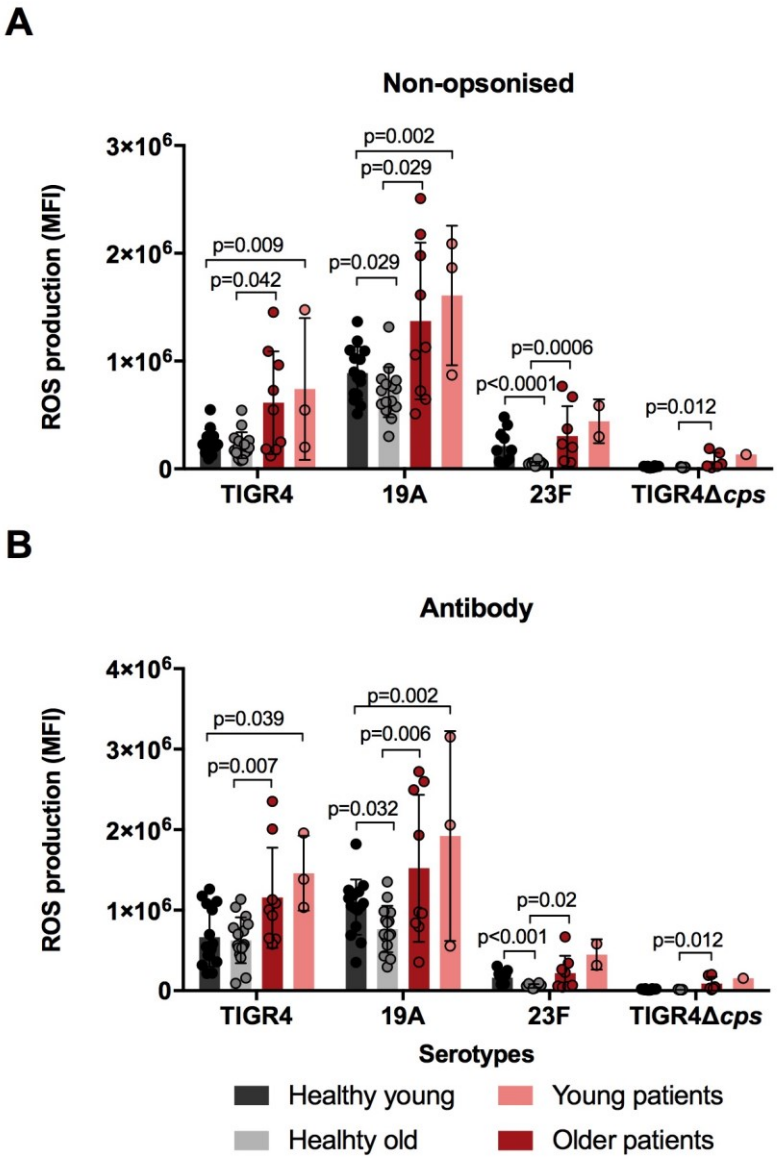

Figure 3.10. Neutrophil ROS production in healthy participants and pneumonia patients following incubation with *S. pneumoniae* serotypes. Oxidative burst generation following incubation with **(A)** non-opsonised or **(B)** antibody-opsonised pneumococcus by neutrophils ($1x10^5$) from healthy young (n=15) and old (n=15), and of young (n=3) and older (n=10) pneumonia patients. Data are mean ± SD. Differences between age groups and healthy and pneumonia groups were analysed by unpaired T test. Mann Whitney test was used for comparisons with data from young patients. No statistical analysis was performed in n<3.

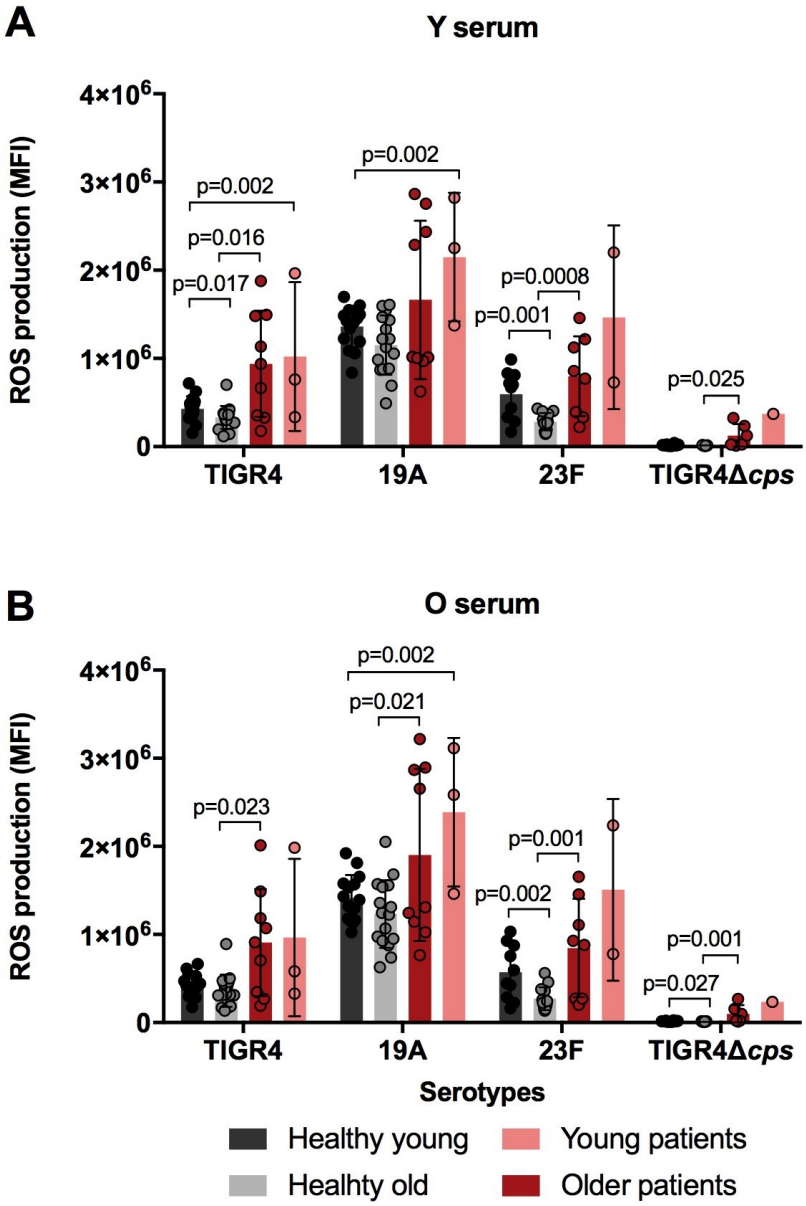

Figure 3.11. Neutrophil ROS production in healthy participants and pneumonia patients following incubation with serum-opsonised *S. pneumoniae* serotypes. Oxidative burst following stimulation of neutrophils ($1x10^5$) with pneumococcal strains opsonised with pooled sera from **(A)** young or **(B)** older donors. Healthy young (n=15) and old (n=15), young (n=3) and older (n=10) pneumonia patients. Data is expressed as mean ± SD. Differences between age groups and healthy and pneumonia groups were analysed by unpaired T test. Mann Whitney test was used for comparisons with data from young patients. No statistical analysis was performed in n<3.

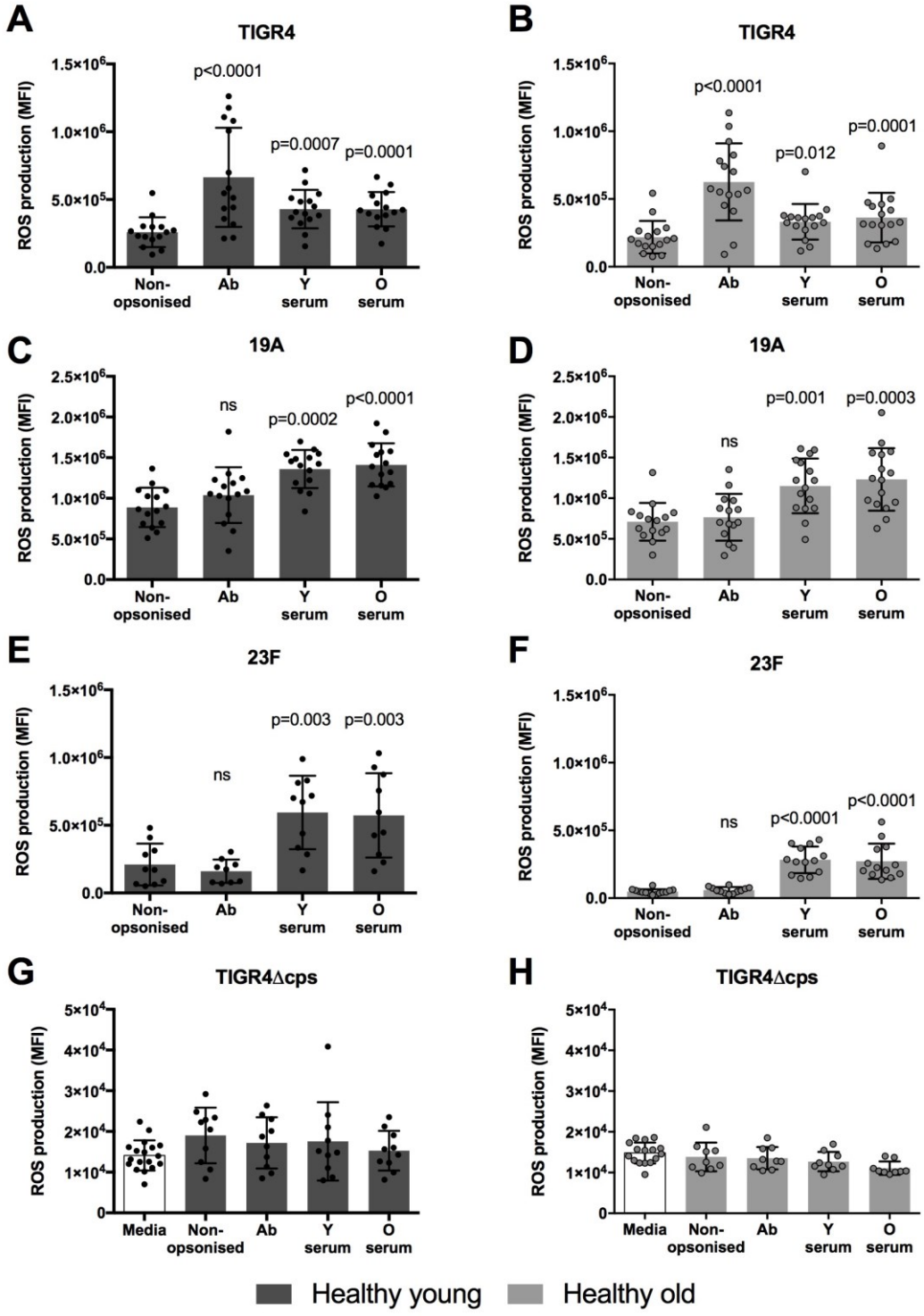

Figure 3.12. Effect of opsonisation on neutrophil ROS production to *S. pneumoniae* serotypes in healthy participants. Neutrophil ($1x10^5$) ROS production in healthy young (n=15) and older (n=15) adults following incubation with non-opsonised or antibody-(Ab), young (Y) or (O) old serum-opsonised *S. pneumoniae* serotypes. **(A and B)** TIGR4, **(C and D)** 19A, **(E and F)** 23F, **(G and H)** TIGR4Δ*cps*. Data are mean ± SD. Differences between treatments were analysed by Friedman test with Dunn's post hoc test and statistical differences with non-opsonised control are shown on top of the bars.

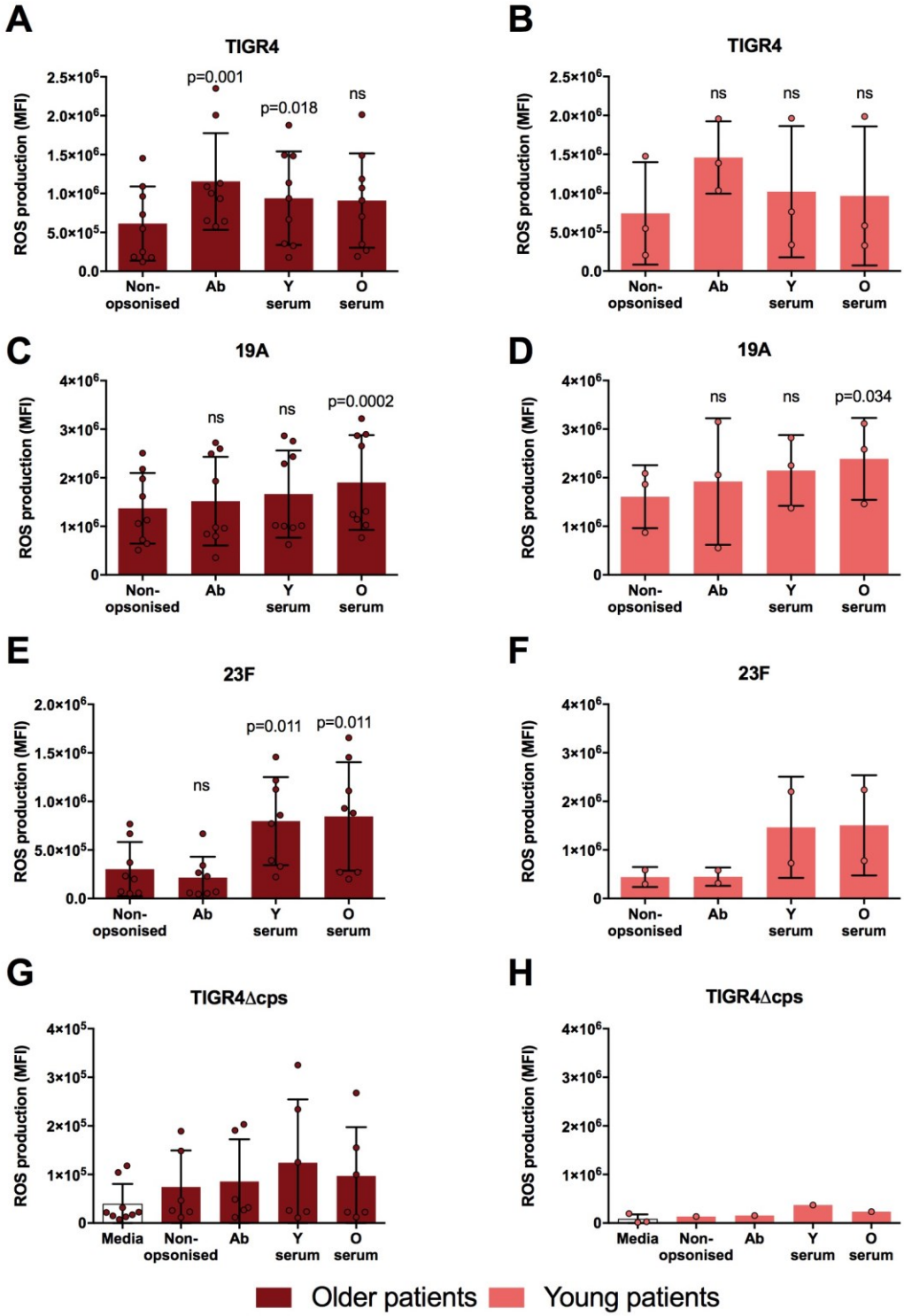

Figure 3.13. Effect of opsonisation on neutrophil ROS production to *S. pneumoniae* serotypes in pneumonia patients. Neutrophil ($1x10^5$) ROS production in young (n=3) and older (n=10) adults following incubation with non-opsonised or antibody-(Ab), young (Y) or (O) old serum-opsonised *S. pneumoniae* serotypes. **(A and B)** TIGR4, **(C and D)** 19A, **(E and F)** 23F, **(G and H)** TIGR4Δ*cps*. Data are mean ± SD. Differences between treatments were analysed by Friedman test with Dunn's post hoc test and statistical differences with non-opsonised control are shown on top of the bars. No statistical analysis was performed in n<3.

3.3.9 Characterization of pooled sera

These assays were performed by the Clinical Immunology Service at the University of Birmingham, except the assay with heat-inactive sera.

To study the effect of ageing on neutrophil phagocytosis and oxidative burst in the presence of opsonins such as the complement system, pooled human sera were used as a source of opsonins. Therefore, two pooled sera were prepared, one containing sera from healthy young donors and another with sera from older donors. Of the 14 older donors, 10 had been previously vaccinated with the PPV23 vaccine in the last 5 years. All young volunteers were between 21 to 30 years old, therefore, were not vaccinated.

Both pooled sera were tested for the presence of anti-pneumococcal IgG against serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F (Figure 3.14). All the serotypes tested are present in the PPV23 vaccine. Of those, only IgG titres against serotypes 3 and 4 of young and old pools were below the protective threshold established by WHO (0.35 µg/ml).

The activity of the classical and alternative pathways of the complement system were also tested in pooled sera, and both achieved the same levels of activity, measured by haemolytic assays (CH_{50} >45 KU and AP_{50} 117%).

Following heat inactivation of pooled sera at 56°C, to denature proteins of the complement system, *S. pneumoniae* TIGR4 and 19A were pre-opsonised with HI-sera and incubated with neutrophils of young donors as described in Chapter 2. The levels of ROS generated following incubation with bacteria opsonised with HI sera were not different from those opsonised with normal serum (Figures 3.15 A and B), suggesting that the presence of anti-

pneumococcal antibodies in the sera samples used (Figure 3.11) also participate in the neutrophil oxidative burst response to *S. pneumoniae* in the assays tested.

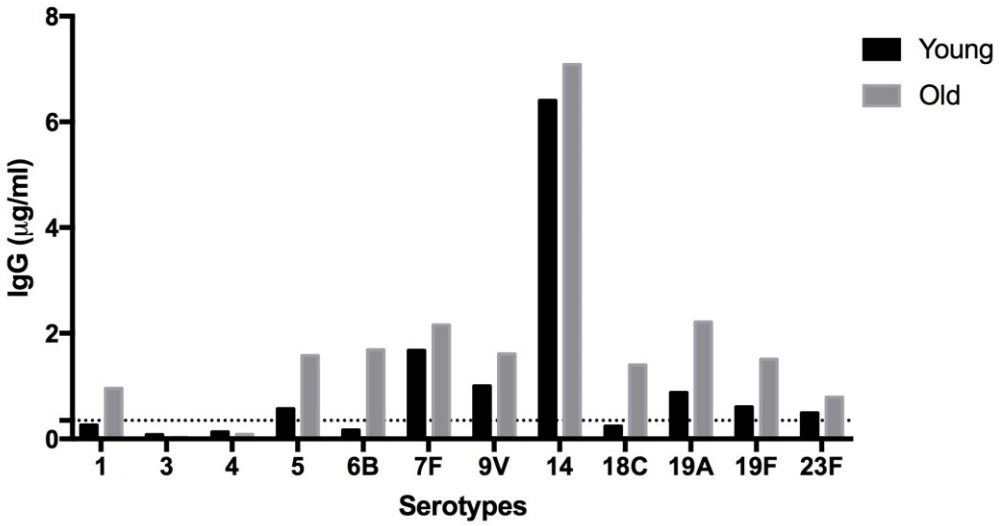

Figure 3.14. Levels of anti-pneumococcal antibodies in pooled sera of healthy participants. Anti-pneumococcal IgG levels were measured in the pooled sera used as opsonins for ROS assays. The ticked line shows the protective threshold established by WHO (0.35 µg/ml).

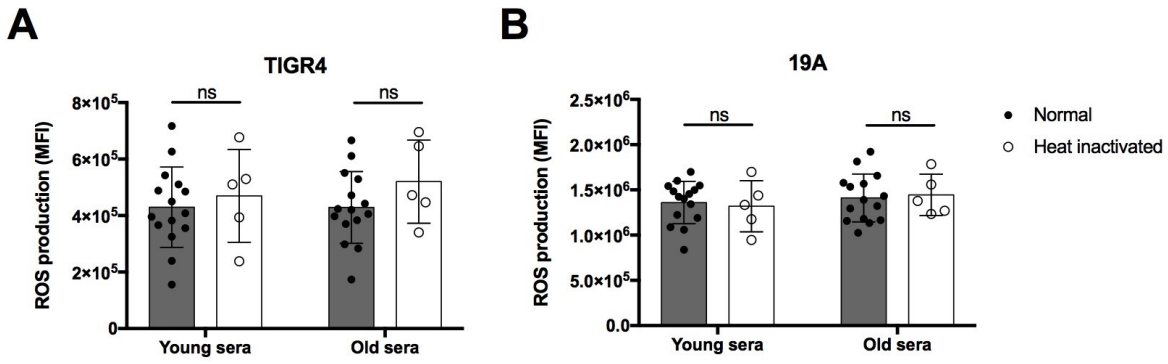

Figure 3.15. Neutrophil ROS production following incubation with *S. pneumoniae* opsonised with heat-inactive sera. Neutrophil ROS production in young donors following incubation with **(A)** TIGR4 or **(B)** 19A (MOI 40) pre-opsonised with normal pooled sera from young or older donors (n=15), or heat-inactivated pooled sera samples (n=5). Data are mean ± SD. Differences between groups were analysed by Mann Whitney test. Ns = non-significant.

3.3.10 Neutrophil extracellular traps generation in response to *S. pneumoniae*

To determine the ratio of pneumococcus to neutrophils necessary to induce cell-free DNA (cf-DNA) release and the best stimuli, NET generation was assessed in response to a range of live and fixed bacteria concentrations. Cell-free DNA measured from supernatants of neutrophils from healthy young donors (n=3) after 3-hour stimulation with live or fixed TIGR4 is shown in Figure 3.16. Based on these assays, a MOI of 10:1 ($2x10^6$ CFU) with live bacteria, but not fixed, was chosen, as only the former was able to induce significant cf-DNA release by neutrophils.

Figure 3.17 shows NET generation in response to *S. pneumoniae* serotypes compared between healthy individuals and pneumonia patients. Figure 3.18 presents the same data to compare NET generation with bacteria only controls.

PMA, TIGR4, 19A and 23F stimulation all induced NET generation though there were differences between the subject groups (Figure 3.17), while NET release was not seen following *TIGR4Δcps* incubation (Figure 3.18 B).

Stimulation of neutrophils using 25nM PMA promoted a significant increase in NET generation (Figure 3.17). However, PMA-induced NET generation seem to be lower with increasing age, as NET generation appears lower in healthy older adults, in comparison with young subjects, but that difference did not reach statistical significance. In neutrophils from healthy young donors, NET formation did not vary in response to *S. pneumoniae* capsular serotypes, but neutrophils from older adults showed a trend towards increased cf-DNA release to 23F (p=0.058) compared to HY.

During pneumonia infection, older patients had significantly lower levels of NET generation following PMA treatment than the healthy counterparts (p=0.003) (Figure 3.17). NET generation in response to TIGR4 and 19A was seen in pneumonia patients (Figures 3.18 A and D). However, 23F stimulation promoted cf-DNA release only in healthy subjects (HY, p=0.003, HO, p=0.0001, OP, p=0.324) (Figure 3.18 C). Compared to healthy, older pneumonia patients had decreased NETosis in response to serotypes 4 (p=0.034) and 23F (p=0.0006). Although only n=3, neutrophils of young patients released higher levels of cf-DNA in response to TIGR4 (p=0.028) and 19A (p=0.049) than older patients.

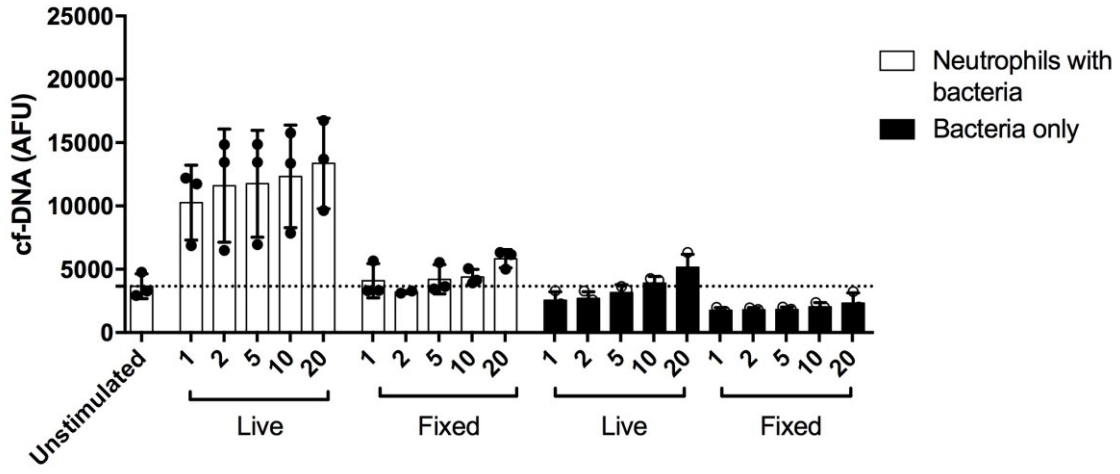

Figure 3.16. Fixed *S. pneumoniae* does not promote neutrophil NET release. Isolated neutrophils from healthy young (n=3) were stimulated for 3h with a range of MOI of live or fixed *S. pneumoniae* TIGR4. Wells containing only bacteria at the MOI tested were used as controls. Cell-free DNA released in the supernatants after incubation was measured by fluorometry. Data are presented as arbitrary fluorescence units (AFU) and are mean ± SD.

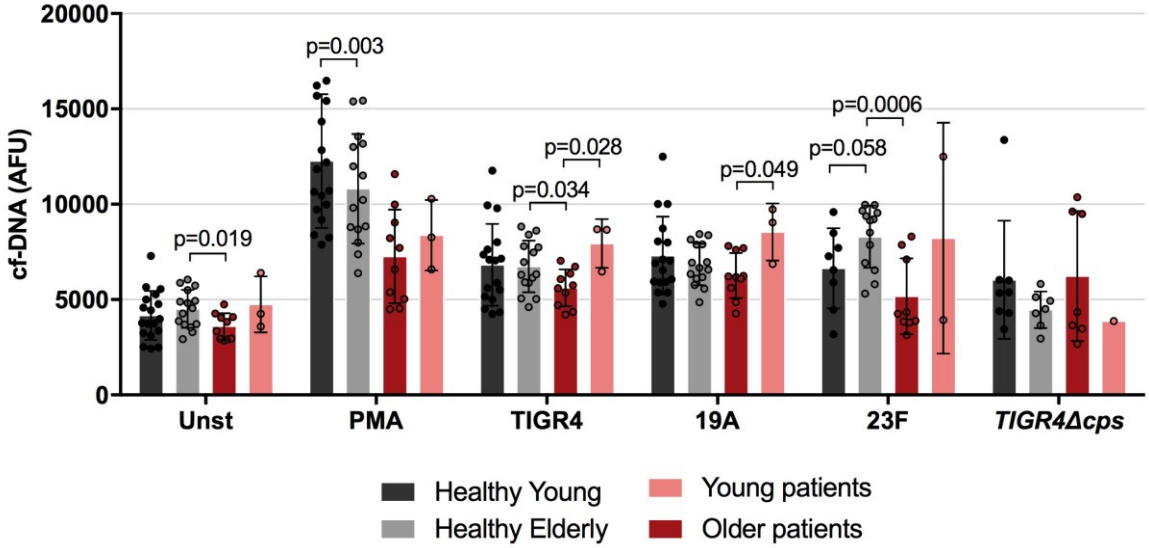

Figure 3.17. NET generation in response to *S. pneumoniae* serotypes in healthy subjects and pneumonia patients. Isolated neutrophils ($1x10^5$) from healthy donors or patients with pneumonia were incubated for 3h with *S. pneumoniae* serotypes (MOI 10). Unstimulated (Unst) or 25nM PMA were used as control treatments. Cell-free DNA released in the supernatants after incubation was measured by fluorometry. TIGR4: healthy young n=17, old n=15; young patient n=3, old n=10. 19A: healthy young n=17, old n=15; young patient n=3, old n=10. 23F: healthy young n=8, old n=13; young patient n=2, old n=9. *TIGR4Δcps*: healthy young n=8, old n=7; young patient n=1, old n=7). Data are presented as arbitrary fluorescence units (AFU) and expressed as mean ± SD. Differences between groups were analysed by unpaired T test. When n≤10, Mann-Whitney test was used for analysis. No statistical analysis was performed in n<3.

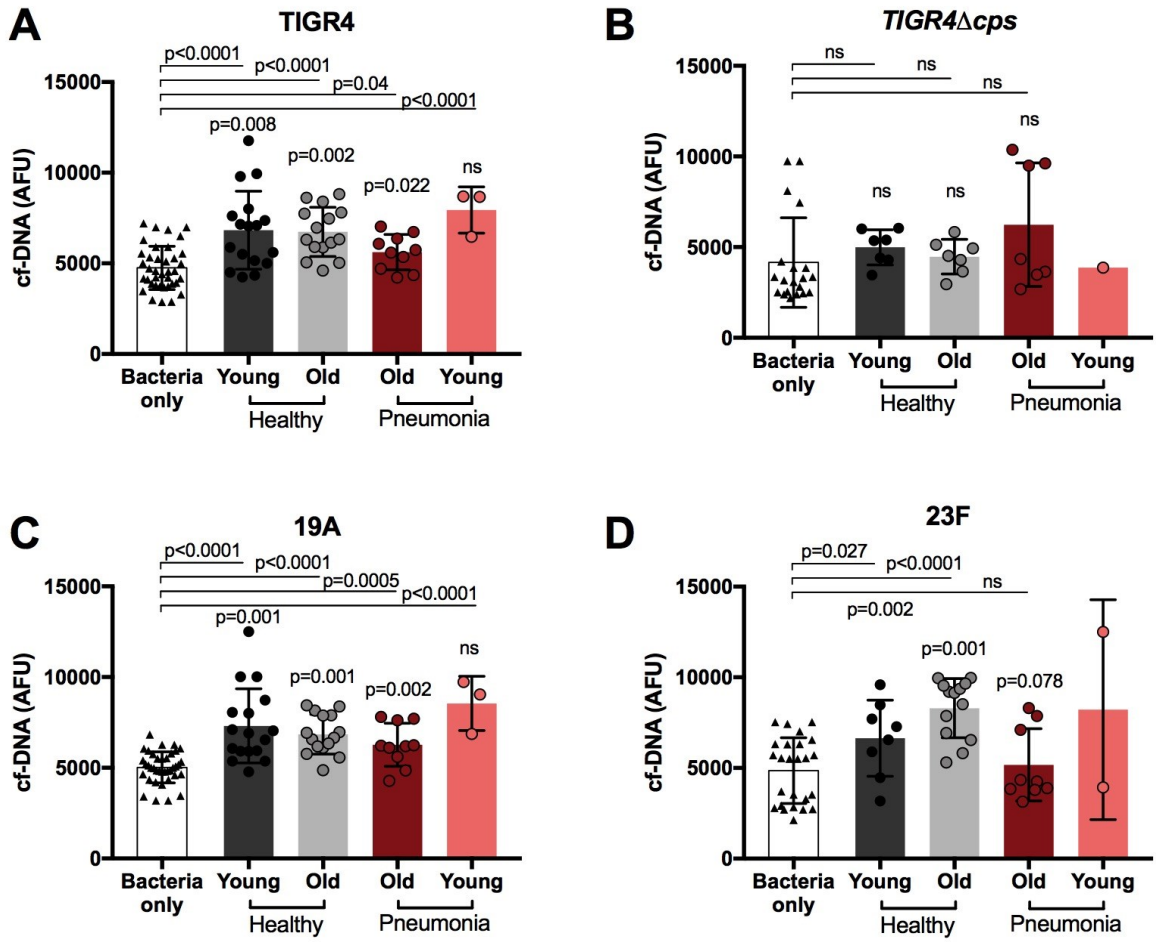

Figure 3.18. NET generation in response to *S. pneumoniae* serotypes compared to bacteria alone controls. Levels of cell-free DNA released in the supernatant of neutrophils and *S. pneumoniae* cultures or serotypes alone treated with gentamicin (100 µg/ml) were measured after 3h incubation. Cell-free DNA released in the supernatants after incubation was measured by fluorometry. *S. pneumoniae* serotypes tested were **(A)** TIGR4, **(B)** TIGR4*Δcps*, **(C)** 19A and **(D)** 23F. Data are presented as arbitrary fluorescence units (AFU) and expressed as mean ± SD. Differences between bacteria only control and neutrophil and *S. pneumoniae* cultures were analysed by unpaired T test and are shown on top of lines. When n≤10, Mann-Whitney test was used for analysis. No statistical analysis was performed in n<3. Statistical differences with unstimulated controls (not plotted) are shown on top of the bars (One-Way ANOVA test with Tukey's post hoc test). Ns = non-significant. HY, n=17. HO, n=15. OP, n=10. YP, n=3.

3.3.10.1 Visualization of NETs by fluorescence microscopy

NET formation by *S. pneumoniae* was also demonstrated by fluorescence microscopy. Neutrophils from healthy young controls showed robust NET generation with 25 nM PMA, observed by the presence of extracellular DNA (green fluorescence), while unstimulated neutrophils preserved their characteristic lobular nuclear shape. Stimulation of neutrophils pre-treated with cytochalasin D ahead of TIGR4 incubation showed that in ratios of 10 and 100, *S. pneumoniae* induced nuclei decondensation, a hallmark of NET generation. In the absence of cytochalasin D pre-treatment, TIGR4 (MOI 100) induced both nuclear decondensation and NET release, as shown in Figure 3.19.

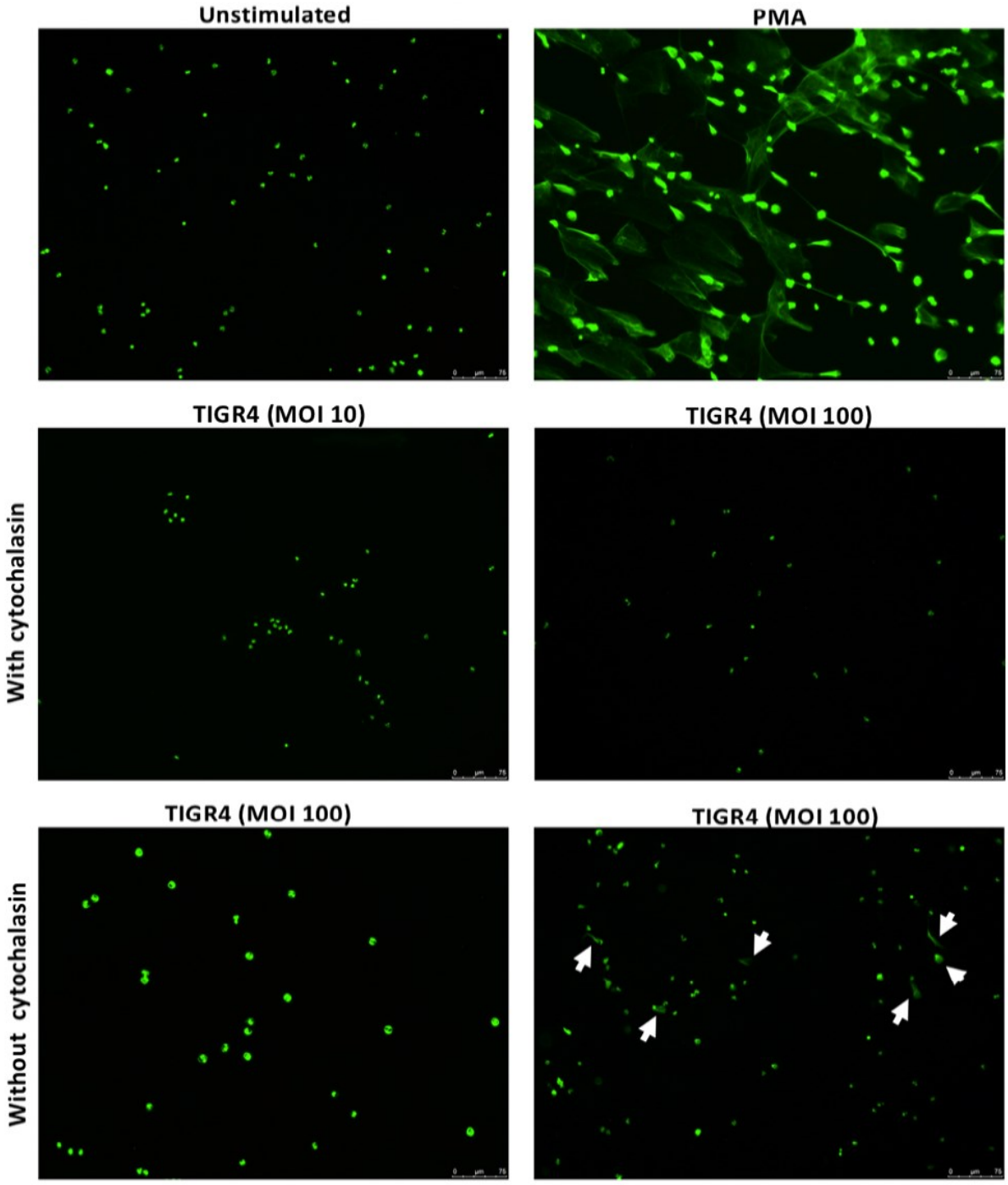

Figure 3.19. *Ex vivo* NET generation following incubation PMA or with *S. pneumoniae* TIGR4. Fluorescence microscopy of neutrophils ($2x10^5$) pre-treated or not with cytochalasin (15 min at 37°C, 5% CO_2 atmosphere) and stimulated with PMA (25nM) or *S. pneumoniae* TIGR4 (MOI 10 or 100) for 3 hours. DNA is stained green with SYTOX Green Stain. Nuclear structure is preserved in unstimulated neutrophils, while *ex vivo* NET generation is observed in response to PMA and TIGR4 at MOI 100 (white arrows) by neutrophils from a healthy young donor. All images are taken at 20x magnification.

3.3.11 The effect of age and pneumonia infection on neutrophil activation following stimulation by *S. pneumoniae*

A time course experiment was performed to determine the optimal time for production of IL-8 by neutrophils. Isolated neutrophils ($2x10^5$) healthy young donors (n=3) were incubated with LPS (50 ng/ml) or live *S. pneumoniae* TIGR4 (MOI of 40:1, $8x10^6$ CFU) for 4h and 6h. Intracellular cytokine production was detected in neutrophils following 4h incubation with increased production at 6h (Figure 3.20 A). Next, neutrophil expression of TLR2 and TLR4 was determined at 2h and 4h to test if stimulations with LPS (50 ng/ml) or live *S. pneumoniae* TIGR4 (MOI of 40:1, $8x10^6$ CFU) could modulate these receptors. TLR2 expression was increased by both stimuli at 2h and 4h, and TLR4 expression remained the same following 4h (Figures 3.20 B and C). Thus, hereafter neutrophils were stimulated for 4h, as IL-8 production was detectable at this time point and modulation of TLR2 and TLR4 expression in neutrophils remained detectable.

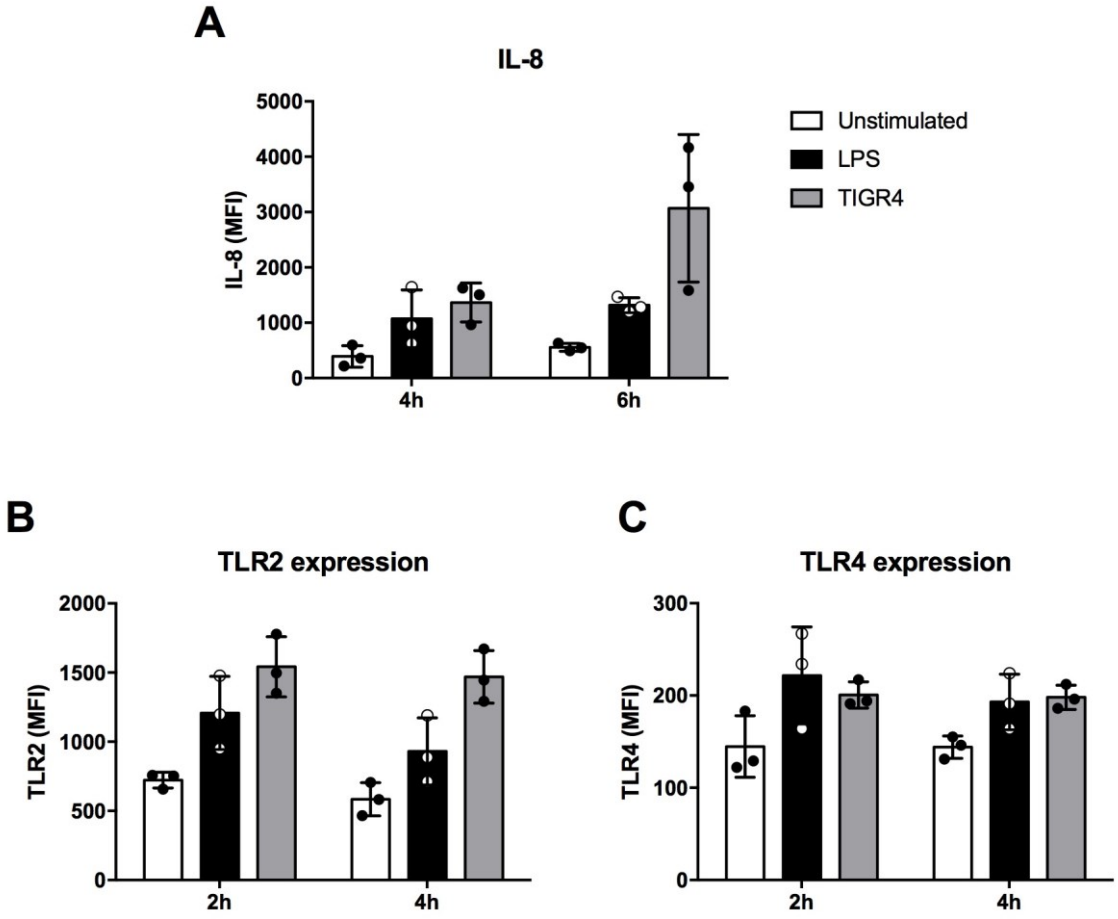

Figure 3.20. Intracellular IL-8 production and TLR expression by neutrophils. Isolated ($2x10^5$) neutrophils were incubated with or without LPS (50 ng/ml) or *S. pneumoniae* (MOI 40) for 2h, 4h or 6h and stained for flow cytometry analysis of **(A)** intracellular IL-8 production and surface expression of **(B)** TLR2 and **(C)** TLR4 by neutrophils. Healthy young, n=3. MFI values are expressed as mean ± SD.

On examining neutrophil oxidative burst of healthy young and old donors as well as of pneumonia patients, ROS production was reduced in response to serotypes 19A and 23F in healthy older adults but increased in pneumonia patients (Figure 3.10 A). As pre-opsonisation enhanced oxidative burst to these serotypes by neutrophils from healthy older adults, TLR2 and TLR4 expression were measured following pneumococcal stimulation, as these receptors are involved in immunity against non-opsonised *S. pneumoniae* [229,231–235,342] and their expression and signalling impaired with age [75,94,99,343]

Briefly, isolated neutrophils from volunteers were stimulated with *S. pneumoniae* strains for 4h at 37°C. Following incubation, the cells were stained for flow cytometry analysis of CD11b, CD62L, TLR2 and TLR4 expression as well as IL-8 production. Neutrophil activation is associated with increased Mac-1 complex (CD11b/CD18) and loss of L-selectin (CD62L) [344]. Expression of both receptors was analysed following a 4-hour incubation of neutrophils, in the presence or absence of stimulation. LPS was chosen as a positive control [345,346]. The data show no age-related changes in CD11b and CD62L, as neutrophils from healthy aged volunteers had similar expression of both molecules as of young, in both unstimulated and LPS-stimulated neutrophils (Figures 3.21 A and B). Older pneumonia patients, however, showed higher CD11b ($p=0.004$) and lower CD62L ($p=0.011$) expression in unstimulated neutrophils and lower shedding of CD62L expression ($p=0.007$) following LPS stimulation than healthy old, indicating that neutrophils showed an activated phenotype even in the absence of stimulation and, when LPS stimulation is present, expression of both molecules did not reach the same levels shown by neutrophils of healthy older adults. This is likely due to the presence of inflammatory cytokines in the bloodstream which could have primed the neutrophils.

Following stimulation with the pneumococcus, neutrophils from all cohorts were activated by the serotypes. There were no differences in CD11b and CD62L expression between healthy volunteers and older pneumonia patients. Overall, neutrophil activation by TIGR4 was higher than for 19A and 23F, in all cohorts. This observation is supported by presence of increased CD11b expression following TIGR4 stimulation, compared to 23F, in young ($p=0.001$) and patients ($p=0.004$), and to 19A in healthy older adults ($p=0.034$) (Figure 3.21 A). Complementary to these data, CD62L expression following TIGR4 incubation was diminished in neutrophils of healthy participants, compared to 19A (HY, $p=0.048$; HO, $p=0.009$) and 23F (HY, $p=0.004$; HO, $p=0.022$) (Figure 3.21 B).

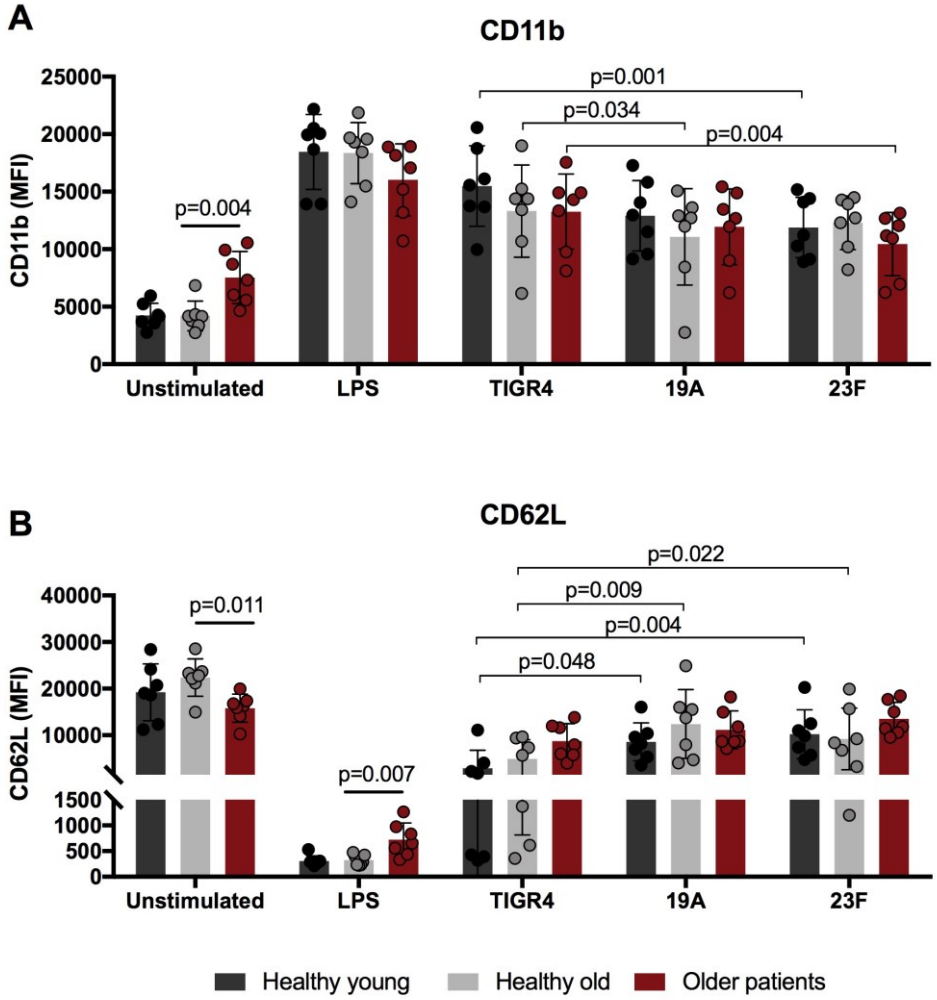

Figure 3.21. Neutrophil phenotype in healthy subjects and older patients with pneumonia. Neutrophils isolated ($2x10^5$) from healthy young (n=7), old (n=7) donors or older patients with pneumonia (n=7) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40) for 4h ahead of flow cytometry analysis. Surface density (MFI) of **(A)** CD11b and **(B)** CD62L are expressed as mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test. Comparison between *S. pneumoniae* serotypes was analysed by Friedman test with Dunn's post hoc test.

Expression of TLR2 (Figure 3.22 A) and TLR4 (Figure 3.22 B) in unstimulated neutrophils was the same between healthy participants. In older pneumonia patients, there was a trend toward increased expression of TLR2 (p=0.053) in unstimulated neutrophils, while TLR4 (p=0.024) expression was higher than in healthy old. LPS stimulation *in vitro* significantly reduced expression of TLR2 by neutrophils of healthy donors (HY, p=0.005; HO, p=0.002, Table A.1), but in pneumonia patients, levels of TLR2 (p=0.011) remained higher than in healthy older adults and TLR4 (p=0.054) showed a trend towards this effect.

Following stimulation with *S. pneumoniae*, no modulation of neutrophil TLR2 expression was detected in healthy donors, but its expression increased in patients, following incubation with serotypes 4 (p=0.002) and 19A (p=0.002) (Figure 3.22 A, Table A.1). Neutrophil TLR4 expression (Figure 3.22 B) was significantly upregulated by 23F (p=0.005), in the young. Expression of TLR4 was decreased in the old in response to 19A (p=0.011) and 23F (HY, p=0.007, OP, p=0.004), compared to healthy young and patients, however, as shown on Table A.1, TLR4 expression following stimulations with these serotypes were not different from unstimulated control in healthy old donors, meaning that these differences actually demonstrate an overall lower expression of TLR4 by neutrophils of this group.

Age-related changes in resting neutrophil IL-8 levels were observed, which were higher in neutrophils from healthy old individuals, even in the absence of stimulation (p=0.011) (Figure 3.22 C), indicating a possible feature of inflammaging. This is similar to the finding that IL-8 levels were increased in the serum of healthy older adults (Figure 3.6 D). Both LPS and *S. pneumoniae* serotypes promoted IL-8 generation by neutrophils in all cohorts, though TIGR4 strain induced higher IL-8 production than 23F (HY, p=0.001; HO p=0.048; OP, p=0.009). In neutrophils of older pneumonia patients, there was a trend towards reduced IL-

8 production following stimulations, and its intracellular levels were significantly reduced in response to 23F ($p=0.026$), compared to healthy old.

Taken altogether, these data suggest that serotype 4, TIGR4 strain is a more potent activator of neutrophils than 19A and 23F. No evidence was found that, in health conditions, ageing significantly impacted neutrophil surface integrin or TLR modulation and IL-8 production in response to the selected pneumococcal serotypes. However, during pneumonia infection in older adults, neutrophils presented an activated phenotype, as seen by CD11b, CD62L, TLR2 and TLR4 surface expression, in the absence of *in vitro* stimulation.

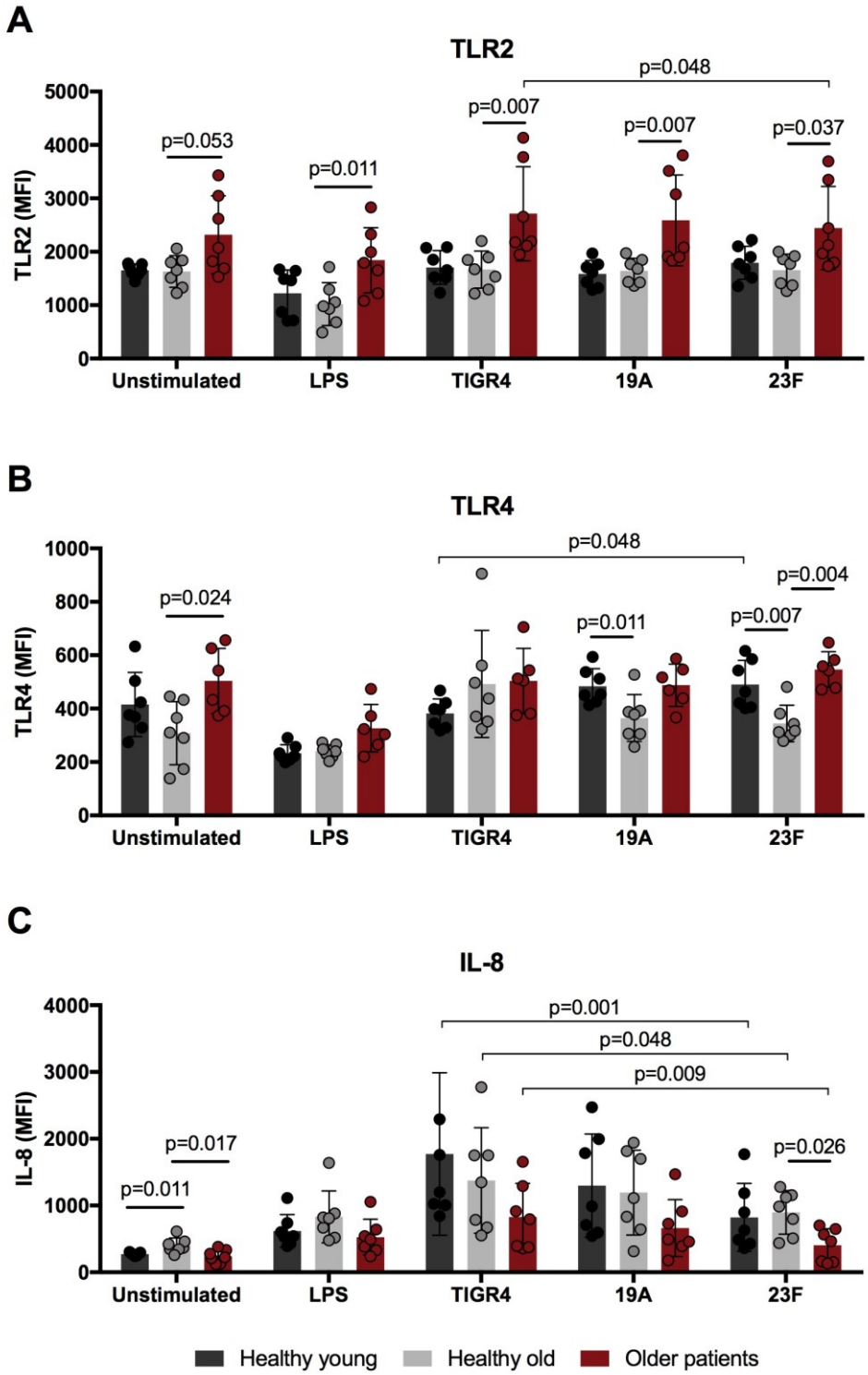

Figure 3.22. TLR expression and IL-8 production in healthy subjects and older pneumonia patients. Neutrophils isolated ($2x10^5$) from healthy young (n=7), old (n=7) donors or older patients with pneumonia (n=7) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40) for 4h ahead of flow cytometry analysis. Surface density (MFI) of **(A)** TLR2, **(B)** TLR4 and **(C)** intracellular levels IL-8 are expressed as mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test. Comparison between *S. pneumoniae* serotypes was analysed by Friedman test with Dunn's post hoc test.

3.3.11.1 Further investigation of *S. pneumoniae* TIGR4-induced neutrophil activation

As demonstrated by the data above, *S. pneumoniae* serotype 4, TIGR4 strain, successfully activated neutrophils. This strain is known to be virulent and to cause invasive disease [321,322].

Although no evidence of ageing susceptibility to this serotype was found, based on the data above and neutrophil ROS production and NET release, older patients released less cf-DNA in response to this serotype. Also, neutrophils from healthy older adults showed a trend towards lower TLR4 expression, which is the receptor for pneumolysin [235] . By using different forms of TIGR4, a further investigation of the activation of neutrophils by this serotype was performed. Previous experiments were performed with live bacteria within 30 minutes of incubation, following inhibition of growth with gentamicin. For the following analysis, neutrophil activation by live and heat-killed TIGR4, or capsule-negative mutant, TIGR4Δ*cps* were compared. Using the same strain allowed to analyse conditions in which the whole bacterium was present but its proteins inactive, by heat inactivation or in which all proteins were present, but the capsule absent (Δ*cps*). The hypothesis was that lack of capsule on TIGR4Δ*cps* could expose more pneumolysin, allowing it to interact with TLR4.

Both HK TIGR4 and Δ*cps* failed to modulate neutrophil CD11b (Figure 3.23 A). Interestingly, Δ*cps* increased CD62L expression in neutrophils from healthy old, compared to unstimulated control ($p=0.002$) (Figure 3.23 B). Neither TLR2 and TLR4 (Figures 3.23 C and D) were significantly modulated by HK TIGR4 or Δ*cps* on neutrophils from young donors or patients, but expression of TLR4 in neutrophils from healthy old volunteers was reduced following incubation with Δ*cps* ($p=0.015$). Moreover, although Δ*cps*-induced IL-8 production by

neutrophils of healthy older adults was higher than in neutrophils from young ($p=0.011$) (Figure 3.23 E), IL-8 levels were not different than unstimulated controls (Table A.1).

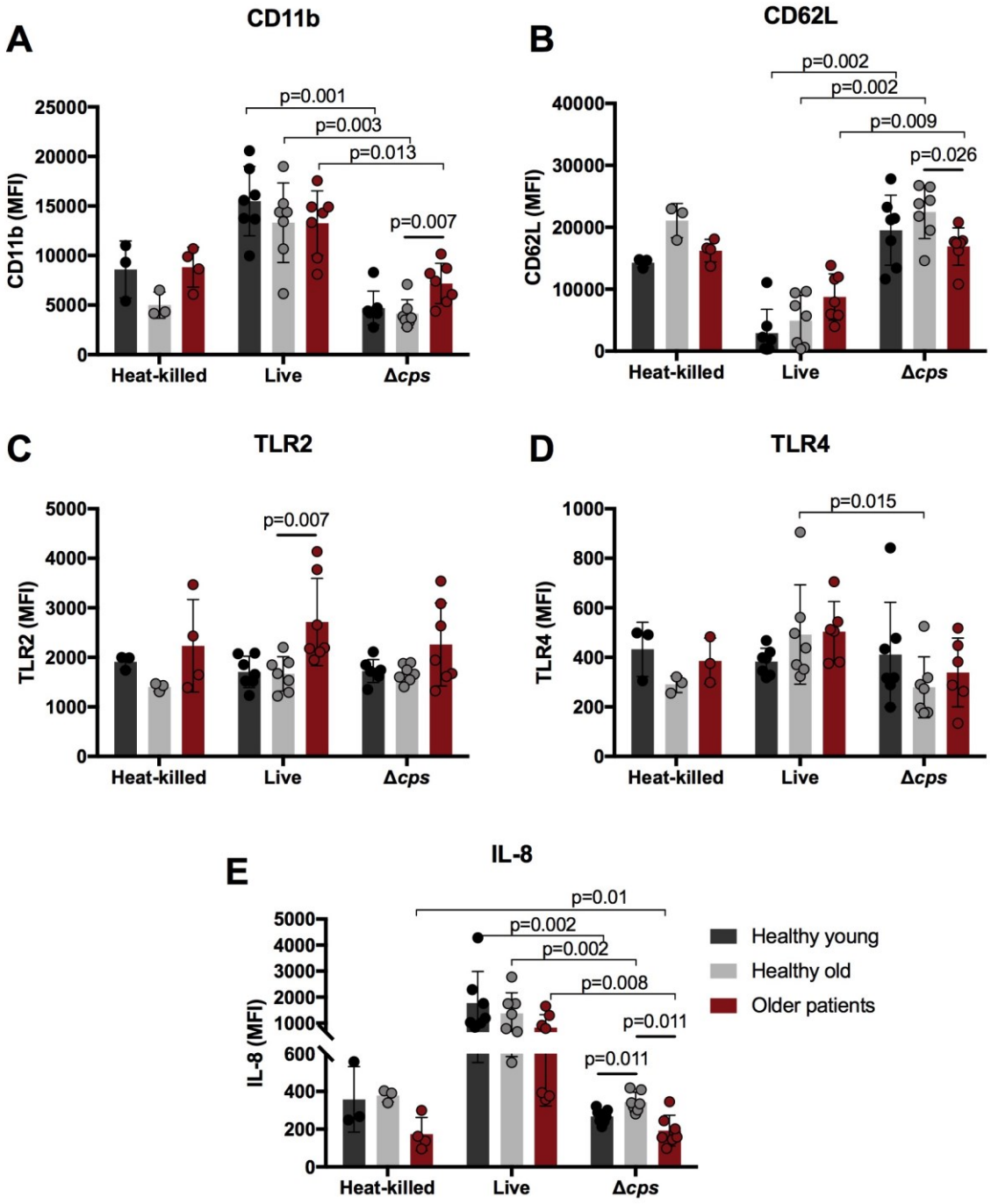

Figure 3.23. Neutrophil activation induced by *S. pneumoniae* TIGR4, live, HK or Δ*cps*. Neutrophils isolated ($2x10^5$) from healthy young (n=7), old (n=7) donors or older patients with pneumonia (n=7) were stimulated with live, heat-killed (HK) or capsule-negative (Δ*cps) S. pneumoniae* TIGR4 (MOI 40) for 4h ahead of flow cytometry analysis. Surface density (MFI) of **(A)** CD11b, **(B)** CD62L, **(C)** TLR2, **(D)** TLR4 and **(E)** intracellular levels IL-8 are shown as mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test. Comparison between *S. pneumoniae* serotypes was analysed by Kruskall-Wallis test with Dunn's post hoc test.

3.3.12 Neutrophil cytokine production following stimulation by *S. pneumoniae*

IL-17A has been reported to mediate neutrophil recruitment to infected sites during pneumococcal pneumonia. Although Th17 cells are accounted as the main producer of this cytokine, neutrophils have been reported to produced IL-17A in both human [347–349], mice [348,350] and in a mice model of *S. pneumoniae*-induced acute otitis media [351]. The hypothesis was that *S. pneumoniae* could induce IL-17A production in human neutrophils and that this production was altered in older patients during pneumonia infection. To determine if IL-17A could be detected in human neutrophils following *S. pneumoniae* stimulation, isolated neutrophils were incubated for 4h in the presence of Brefeldin A or for 18h, in the presence of GolgiPlug, ahead of immunostaining and flow cytometry analysis. An increase in intracellular IL-17A following 18h of incubation of neutrophils with TIGR4 was detected (Figure 3.24). Therefore, this length of incubation was chosen to measure neutrophil IL-17A intracellular production and release of cytokines in supernatants.

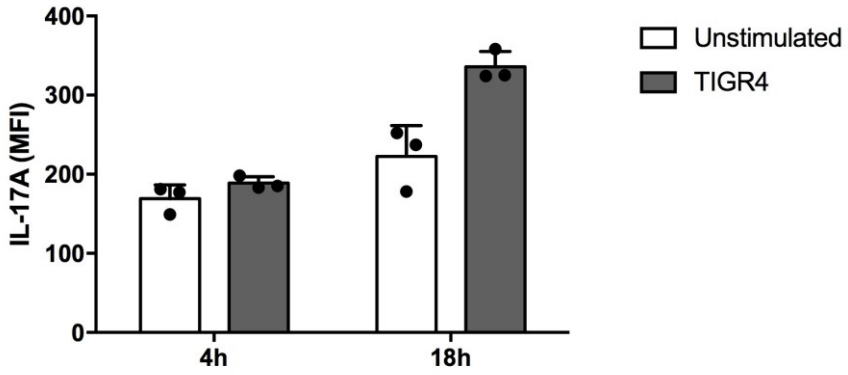

Figure 3.24. Time course of intracellular IL-17A production by neutrophils. Isolated ($2x10^5$) neutrophils from healthy young donors (n=3) were incubated with or without *S. pneumoniae* TIGR4 (MOI 40) for 4h with Brefeldin A or 18h with GolgiPlug and stained for intracellular IL-17A. MFI values are mean ± SD.

Neutrophils from healthy donors and pneumonia patients were then stimulated with *S. pneumoniae* for 18h and intracellular IL-17A production was measured by immunostaining and flow cytometry. Only a small number of assays were performed (HY, n=4; HO and OP, n=3). The frequency of IL-17A^{+} neutrophils (Figure 3.25 A) and of intracellular IL-17A (Figure 3.25 B) was low in all subjects but IL-17A-expressing cells were readily detected following stimulation with LPS and TIGR4, 19A and 23F. Due to the small sample sizes, no statistical differences were found between groups.

With a larger sample size (n=7) IL-17A release from neutrophils was assessed. Although intracellular IL-17A in neutrophils cultured with *S. pneumoniae* was detected (Figure 3.25), the levels of this cytokine (Figure 3.26 A) and of IL-21, IL-23 and IL-33 in supernatants of neutrophils were barely detectable (Figures 3.26 B, C and D). Therefore, it is not possible to confirm the secretion of this cytokine by neutrophils in response to *S. pneumoniae.*

Broader cytokine release after 18h of incubation was also measured using a multiplex assay. Inflammatory cytokines were the most abundant in the supernatant of neutrophils cultured with *S. pneumoniae* serotypes. Neutrophils from all cohorts produced IL-1β, IL-6, IL-8 and TNF-α, IL-10 and IFN-γ but there was great variation among participants (Figure 3.27). Among healthy older adults, neutrophil cytokine production was similar to young, but neutrophils from aged adults produced more IFN-γ than young, in response to serotype 23F ($p=0.03$). Overall, during pneumonia infection neutrophils from older patients seem to produce less cytokines than during health. Patients showed lower levels of neutrophil derived IL-1β following LPS treatment ($p=0.026$) and IL-6, following incubation with pneumococcal strains (TIGR4, p=0=036; 19A, $p=0.046$; 23F, $p=0.073$).

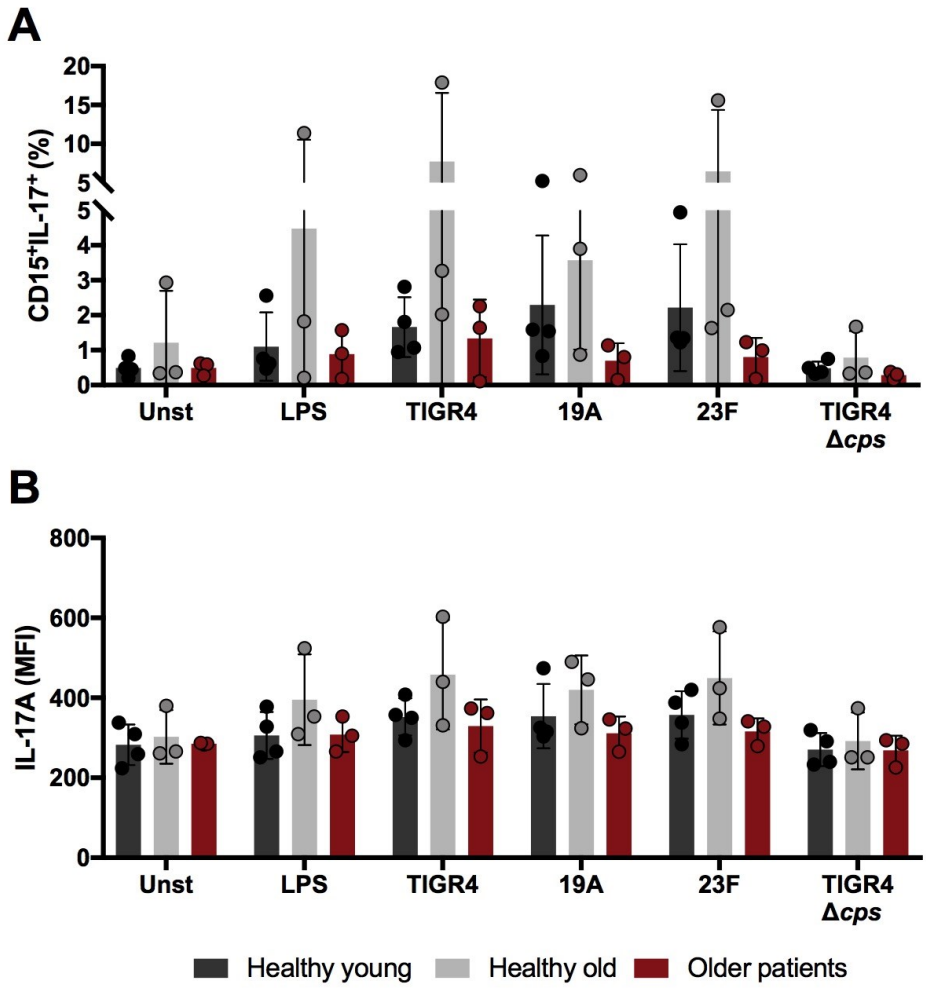

Figure 3.25. IL-17A production by neutrophils in response to *S. pneumoniae*. Neutrophils isolated ($2x10^5$) from healthy young (n=4), old (n=3) donors or older patients with pneumonia (n=3) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40) for 18h ahead of immunostaining and flow cytometry analysis. **(A)** Frequency of $CD15^+IL\text{-}17A^+$ neutrophils and **(B)** levels of intracellular IL-17A in neutrophils. Data are mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test.

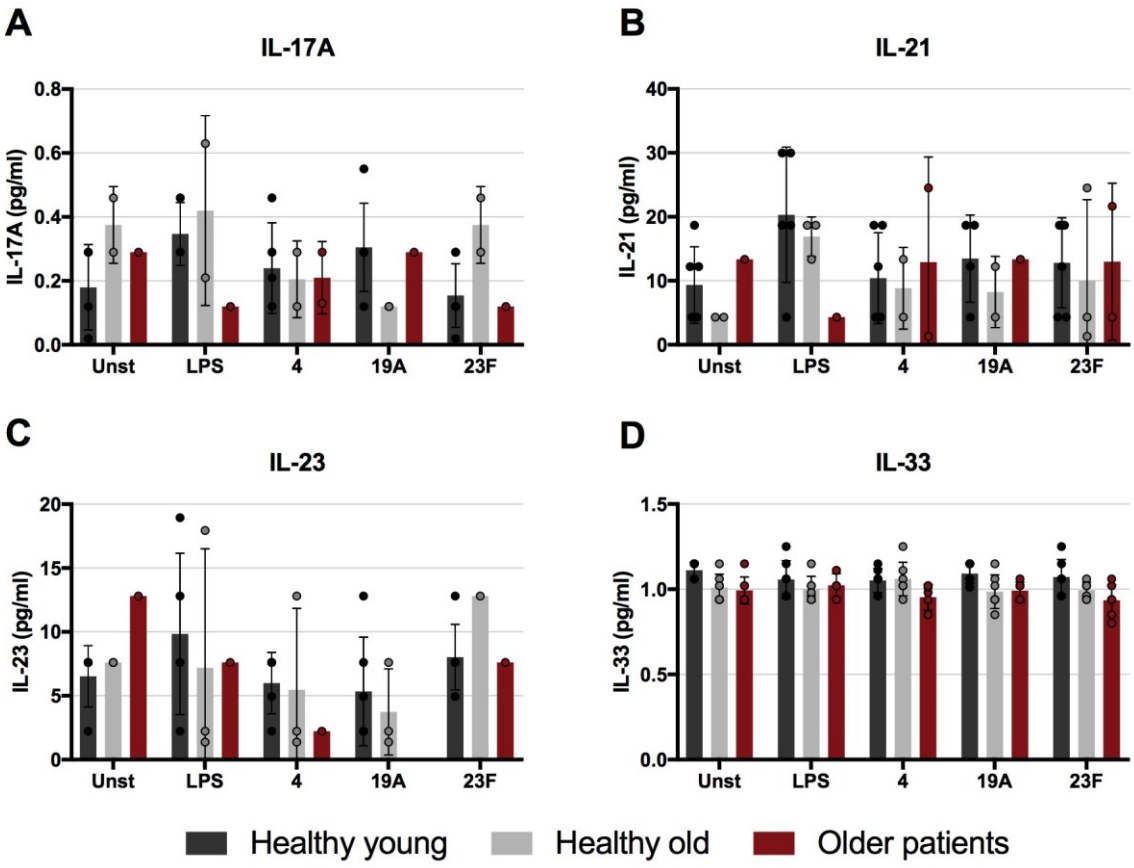

Figure 3.26. Presence of Th17 family cytokines and IL-33 in supernatants of neutrophils challenged with *S. pneumoniae*. Neutrophils isolated ($2x10^5$) from healthy young (n=7), old (n=7) donors or older patients with pneumonia (n=7) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40). Cytokines were measured in culture supernatants after 18h. Levels of **(A)** IL-17A, **(B)** IL-21, **(C)** IL-23 and **(D)** IL-33 are shown in pg/ml. Data are mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test.

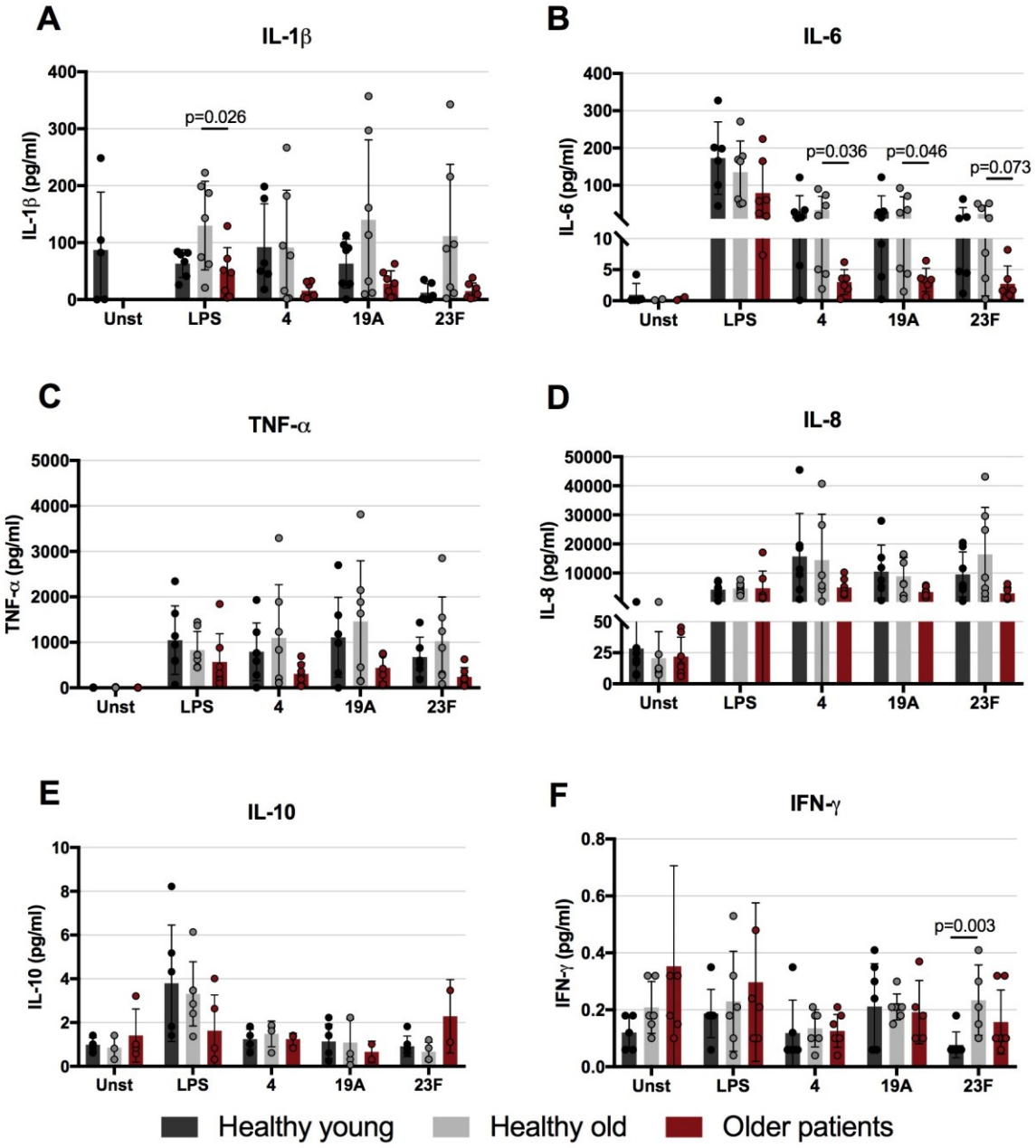

Figure 3.27. Presence of cytokines in supernatants of neutrophils cultured with *S. pneumoniae*. Neutrophils isolated ($2x10^5$) from healthy young (n=7), old (n=7) donors or older patients with pneumonia (n=7) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40). Cytokines were measured in culture supernatants after 18h. Levels of **(A)** IL-1β, **(B)** IL-6, **(C)** TNF-α, **(D)** IL-8, **(E)** IL-10 and **(F)** IFN-γ are shown in pg/ml. Data are mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test.

3.3.13 Effect of *S. pneumoniae* on neutrophil viability

The low levels of neutrophil-produced cytokines in older patients (Figures 3.26 and 3.27) could be associated with neutrophil apoptosis due to the long 18h incubation. Therefore, neutrophil viability was assessed using a dye (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain, Invitrogen, Paisley, UK), that fluoresces upon reacting with amine proteins inside the cells that become accessible via damaged membranes of dead cells. Data suggest that in the presence of pneumococcal strains, neutrophils from healthy controls are more likely to die than neutrophil from older patients (19A, p=0.091; 23F, p=0.091) (Figure 3.28), but this trend could not be confirmed due to the small samples size. Therefore, the low cytokine levels detected in neutrophil supernatants were not due to reduced number of viable neutrophils after an 18h incubation.

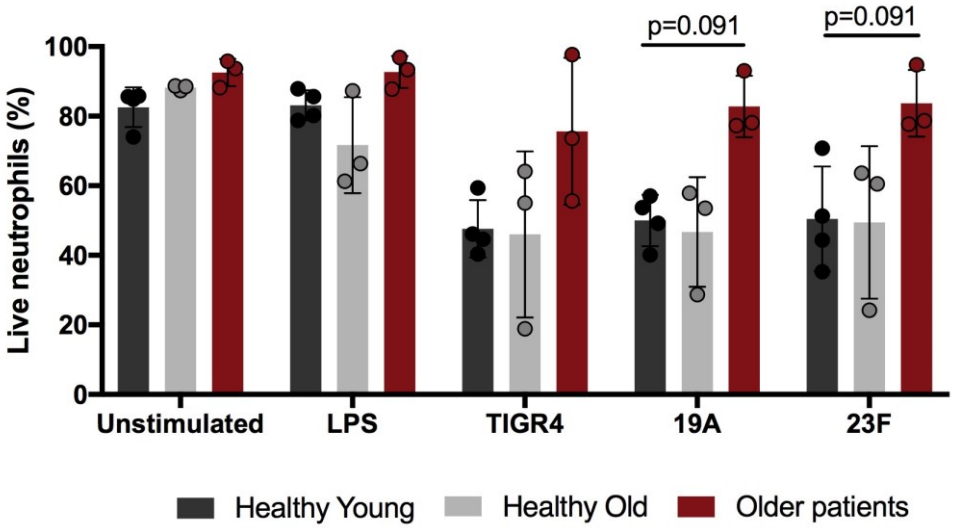

Figure 3.28. Neutrophil viability after 18h incubation. Neutrophils isolated ($2x10^5$) from healthy young (n=4), old (n=3) donors or older patients with pneumonia (n=3) were stimulated with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40) for 18h. Cells were then stained with viability dye and anti-human CD15 ahead of flow cytometry analysis. Cells negative for viability dye were deemed as viable. Data are mean ± SD. Differences between cohorts were analysed by Kruskal-Wallis test with Dunn's post hoc test.

3.3.14 Monocyte activation following stimulation by *S. pneumoniae* in healthy older adults and pneumonia patients

Monocyte TNF-α [260] and IL-6 [259] can drive neutrophils to alveolar spaces during pneumococcal pneumonia and production of both cytokines was shown to be impaired in monocytes from older adults and mice [89]. Having found that monocyte phenotype (Figure 3.3), population frequency and CCR2 expression (Figure 3.5) were altered with ageing and pneumonia infection in the old, monocyte TNF-α and IL-6 intracellular cytokine production and CD11b and CCR2 surface expression following *in vitro* stimulation with *S. pneumoniae* serotypes was investigated.

A time course experiment was performed to determine the optimal time for production of TNF-α and IL-6 by monocytes using flow cytometry analysis. Isolated PBMCs ($2x10^5$) of healthy young donors (n=3) were incubated with LPS (50 ng/ml) or live *S. pneumoniae* TIGR4 ($8x10^6$ CFU) for 4h and 6h. Both cytokines were detected at 4h and 6h (Figures 3.29 A and B). Thus, hereafter monocytes were stimulated for 4h, as cytokine production was detectable at this time point.

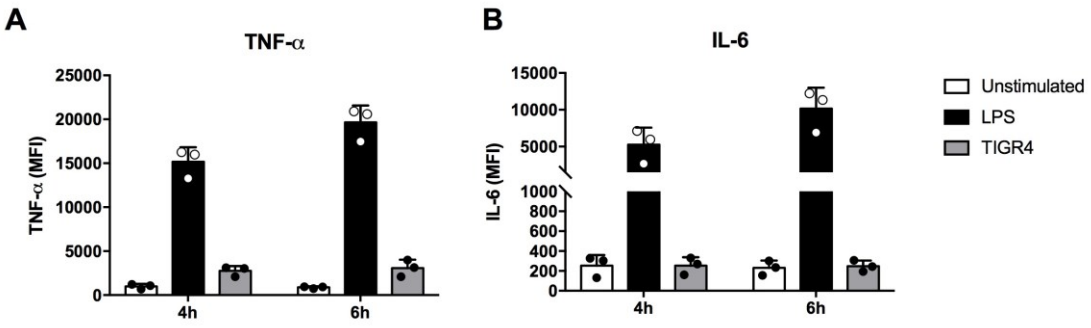

Figure 3.29. Time course experiments showing intracellular cytokine production by monocytes. Isolated ($2x10^5$) PBMCs were incubated with or without LPS (50 ng/ml) or *S. pneumoniae* (MOI 40) for 4h or 6h and stained with antibodies anti-human CD14, TNF-α and IL-6 for flow cytometry analysis of intracellular **(A)** TNF-α and **(B)** IL-6 production by monocytes. Healthy young, n=3. MFI values are expressed as mean ± SD.

Results in Figure 3.30 show the level of expression of CD11b and CCR2 in total $CD14^+$ monocytes from healthy young, older adults, and older pneumonia patients. Different from neutrophils (Figure 3.21), monocytes from healthy old donors increased CD11b expression following LPS incubation, compared to unstimulated control (p=0.002) (Figure 3.30 A, Table A.2). Following incubation with pneumococcal strains, CD11b was diminished in monocytes from both older adults' groups (HO, TIGR4 p=0.002, 19A p=0.005, 23F p=0.005; OP, TIGR4 p=0.001, 19A p=0.002, 23F p=0.001) (Figure 3.30 A, Table A.2), but no difference was found between cohorts. CCR2 expression on resting monocytes was not changed between the cohorts, but LPS-stimulated monocytes from healthy older adults showed increased surface expression of CCR2, compared to young (p=0.048) (Figure 3.30 B). Monocytes from both older groups showed decreased CCR2 expression following stimulation with pneumococcal strains (HO, TIGR4 p=0.002, 19A p=0.005, 23F p=0.002; OP, TIGR4 p=0.001, 19A p=0.001) and patients had higher CCR2 expression than healthy older adults (TIGR4, p=0.003; 23F, p=0.003) (Figure 3.30 B).

Monocyte cytokine production was not changed with age (Figure 3.31), but intracellular levels of IL-6 were increased in older patients, compared to healthy, in the absence of stimulation (p=0.018) (Figure 3.31 B). Incubation with pneumococcal strains did not lead to TNF-a or IL-6 production by monocytes of young but did in both older cohorts. TNF-a was produced following TIGR4 (p=0.002) and 23F treatment (p=0.002) in healthy old, while IL-6 was produced following TIGR4 incubation in this group (p=0.002) and following 19A treatment (p=0.001) in monocytes from pneumonia patients (Table A.2).

Overall, there were no significant age-related changes in monocyte CD11b and CCR2 expression, TNF-α and IL-6 production following stimulation with *S. pneumoniae.*

Analysis of monocytes by immunostaining following 4h-stimulation was difficult, due to the low numbers of cells acquired. This was due to reduced monocyte viability after 4h-incubation with live *S. pneumoniae* (Figure 3.32 A), not observed with LPS or heat killed bacteria. Although neutrophils were incubated under the same conditions, their viability remained high after incubation with live pneumococcus (Figure 3.32 B). This could indicate that monocytes are more susceptible to apoptosis following contact with *S. pneumoniae*, while neutrophils are more resistant, but this observation requires further investigation.

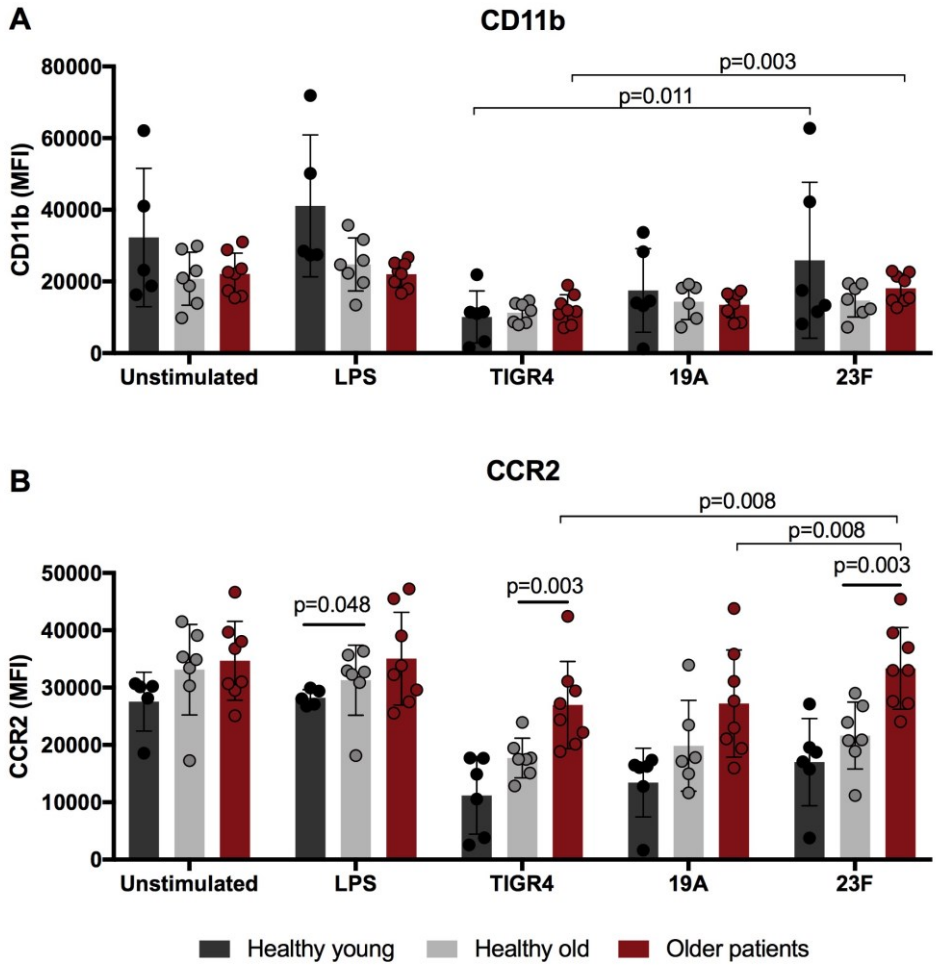

Figure 3.30. CD11b and CCR2 expression on monocytes from healthy subjects and older patients with pneumonia. PBMCs ($2x10^5$) from healthy young (n=6), old (n=7) donors or older patients with pneumonia (n=8) were stimulated with LPS (50ng/ml) or with *S. pneumoniae* serotypes (MOI 40) for 4h ahead of flow cytometry analysis. Surface density (MFI) of **(A)** CD11b and **(B)** CCR2 are expressed as mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test. Comparison between *S. pneumoniae* serotypes was analysed by Friedman test with Dunn's post hoc test.

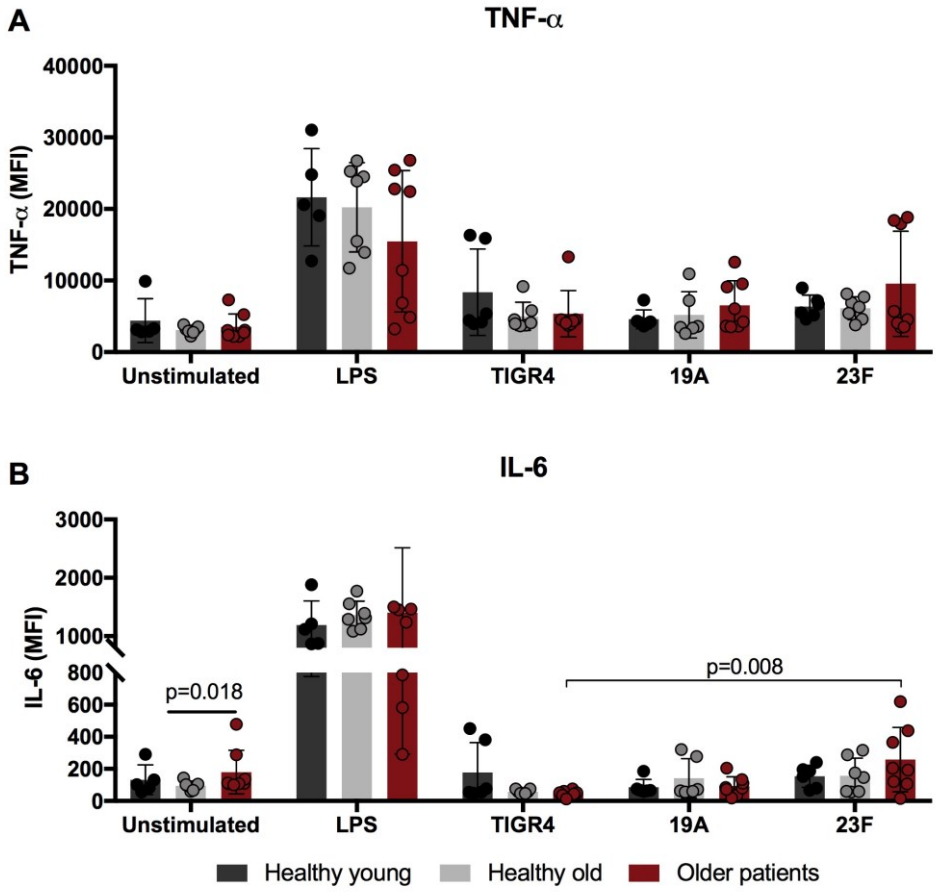

Figure 3.31. Monocyte intracellular cytokine production in healthy subjects and older patients with pneumonia. PBMCs ($2x10^5$) from healthy young (n=6), old (n=7) donors or older patients with pneumonia (n=8) were stimulated with LPS (50ng/ml) or with *S. pneumoniae* serotypes (MOI 40) for 4h ahead of flow cytometry analysis. Intercellular levels (MFI) of **(A)** TNF-α and **(B)** IL-6 in $CD14^+$ monocytes are expressed as mean ± SD. Differences between healthy cohorts or healthy old and patients were analysed by Mann Whitney test. Comparison between *S. pneumoniae* serotypes was analysed by Friedman test with Dunn's post hoc test.

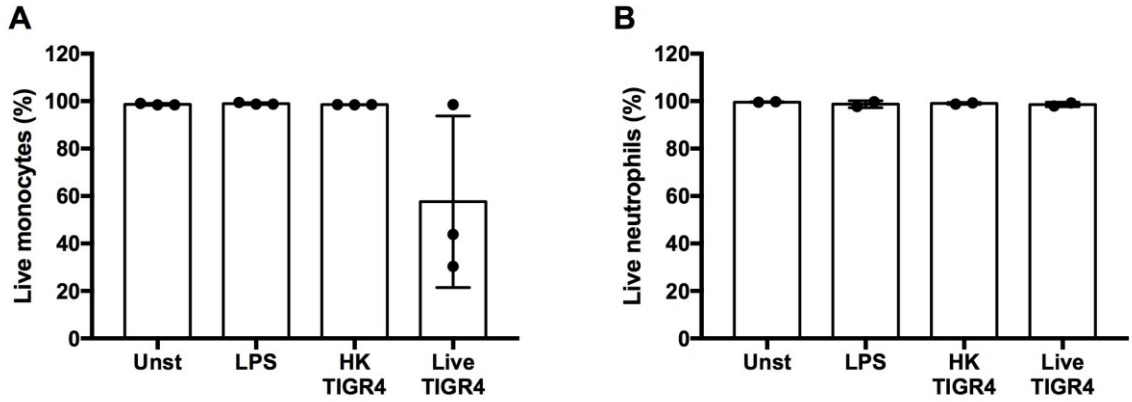

Figure 3.32. Monocyte and neutrophil viability after incubation with *S. pneumoniae*. Isolated ($2x10^5$) neutrophils or PBMCs from healthy older adults (n=3) were incubated with or without LPS (50 ng/ml) or live or heat-killed (HK) *S. pneumoniae* TIGR4 (MOI 40) for 4h and stained with anti-human CD15 or CD14 antibody and viability dye for flow cytometry analysis. Frequency of live, viable **(A)** monocytes and **(B)** neutrophils are expressed as mean ± SD. Comparison between groups was analysed by Kruskall-Wallis test with Dunn's post hoc test.

3.4. Summary of key findings

This chapter aimed to analyse the effect of age on neutrophil and monocyte populations, neutrophil activation, NET generation and ROS production following incubation with three different *S. pneumoniae* serotypes in healthy older individuals and in older adults with pneumonia. Thus, the main findings of this chapter were:

- Older adults had higher frequencies of the neutrophil $CD16^{high}CD62L^{dim}$ subset. This subset was also present in older patients with pneumonia, alongside mature neutrophils and immature granulocytes.
- Neutrophil ROS production in response to *S. pneumoniae* serotypes 4 and 19A was unaltered with ageing, but was reduced in response to 23F, while neutrophils of older pneumonia patients generated higher levels of ROS in response to all *S. pneumoniae* serotypes, compared to healthy controls.

- There were no age-related changes in NET generation in response to serotypes 4 and 19, but this function was increase towards 23F, while older patients with pneumonia had reduced NET generation in response to TIGR4 and 23F, compared to healthy older subjects.
- Neutrophil TLR2 expression was increased in older patients with pneumonia in the presence or absence of stimulation, while intracellular IL-8 production was reduced, compared to healthy elderly.
- The frequency of monocytes subsets was not altered with ageing, whilst the frequency of immature monocytes was increased in older adults with pneumonia. Monocyte TNF-α and IL-6 production following *in vitro* stimulation with *S. pneumoniae* serotypes was not changed with ageing or during pneumonia infection.

3.5 Discussion

Considered as one of the main mediators of immunity against bacteria, neutrophils, together with monocytes, have a central role in combatting pneumonia infection. Neutrophils are key mediators of bacterial killing during pneumococcal infection, as bacterial clearance is severely impaired in neutrophil-depleted mice [256–258]. Due to immunosenescence, neutrophils show reduced chemotaxis [124,126,127] and phagocytic activity [62,134], altered free radical production [127,129] and impaired NET generation [141] in older adults. *S. pneumoniae,* the main causative pathogen of pneumonia, can inhabit the nasopharynx as a commensal species, an event that often precedes disease [193]. The distribution of *S. pneumoniae* serotypes causing invasive or non-invasive pneumonia in older adults varies around the globe, and among the most common are serotypes 19A [27,29,30,326] and 23F [27,28]. Thus, for the experiments in this thesis serotypes 19A and 23F were used, both isolated from older patients with bacteraemia, alongside a genome-sequenced serotype 4, strain TIGR4, originally isolated from an adult male [321,322].

Although neutrophil and monocyte functions have been investigated previously in the context of ageing, the aim of the work described in this chapter was to verify if the immune response of these cells to different strains of *S. pneumoniae* was altered with age in the context of health and pneumonia.

3.5.1 Age-related changes in the innate immune response to *S. pneumoniae*

In line with other studies, the frequency of neutrophils [38,85,113], eosinophils and basophils [113] was unaltered with advanced age, but monocyte frequency increased

[64,66,84]. Lymphocyte frequency has been reported to decrease with age [113], but in our healthy older cohort there was no difference in lymphocyte frequency compared to young subjects. This may be because our older adults were very healthy and possibly also younger overall than in some of the previous studies, even though they show signs of inflammaging, such as increased levels of IL-8 chemokine and a trend toward higher IL-1β in the circulation in agreement with other studies of older adults [37,41,352,353].

Although the total number of neutrophils was unaltered with age, there was a higher frequency of $CD16^{high}CD62L^{dim}$ neutrophils in the circulation of older participants, which has recently been reported by others [121]. This subset was also found in patients with severe injury and in an acute human model of inflammation of LPS-injected volunteers [118,119], but it is still unclear what drives neutrophil differentiation into this subset. We found a positive correlation between serum IL-8 and frequency of this subset, suggesting that increased levels of IL-8 caused by inflammaging could be driving $CD16^{high}CD62L^{dim}$ differentiation or entry in to the bloodstream. These cells were reported as having functional migration towards IL-8 but poor adhesion to endothelium [119], and may be regulatory as they suppress human T-cell proliferation *in vitro* [118]. A single study reported that $CD16^{high}CD62L^{dim}$ neutrophils of older individuals showed reduced phagocytosis and ROS generation [121]. Although more studies are required to confirm these reports in older cohorts, the presence of such a subset with functional impairments and suppressive activity could contribute to the susceptibility of older adults to infection.

Total numbers of monocytes were not altered with increasing age, in agreement with some published studies [85,354]. Further investigation of monocyte subsets in our older cohort showed that frequency of intermediate and non-classical monocytes remained unaltered,

though other groups have reported that these populations increased with age [85,89]. These subsets had different levels of CCR2 expression, however only non-classical monocytes showed increased CCR2 expression compared to cells of young donors. A higher expression of CCR2 in this subset could be driving an early egress from the bone marrow [336], and potentially be involved in functional alterations, as reported by Ong *et al*. (2018), in which non-classical monocytes exhibited more features of cellular senescence than classical and intermediate monocytes, which led to secretion of higher levels of TNF-α, CCL3 and CCL4 [355]. Similarly, increased CCR2 expression in intermediate monocytes led to premature egress from the bone marrow and impaired TNF-α production in old mice [89]. In this thesis, stimulation of total monocytes with *S. pneumoniae* serotypes decreased CCR2, as well as CD11b expression, hence shedding of both receptors could inhibit migration. Considering that monocytes are mostly likely to interact with the pneumococcus in the tissue, shedding of both receptors would allow monocytes to remain in the tissue to aid clearance of the pneumococcus or differentiate into activated macrophages. In addition, monocyte TNF-α and IL-6 production showed no age-related effect. It is possible that age-related changes were not detected due to the small sample size of only 7 donors or because whole PBMCs were used for stimulation and monocytes stained with anti-human CD14 subsequently. Using isolated monocytes in the future could be a better method.

Continuing our investigation on the effect of ageing on neutrophils, neutrophil phagocytosis of fixed *S. pneumoniae* TIGR4 was not impaired by ageing. Reduced phagocytosis of *E. coli* [122,129], *S. aureus* [129] and phagocytic killing of Group B *Streptococcus* and opsonised *S. pneumoniae* [62] have been reported in neutrophils of older adults previously. Here, data generated by the phagocytosis assay was inconclusive, due to the high variability

experienced in our dataset. One limiting factor was the fluorescence degradation of labelled bacteria used in the test. FITC-labelling of bacteria was chosen because the stock could be stored and staining new batches of bacteria every time was not a feasible strategy, as the assay had to be performed in a large sample size on separate days. It was also used a high bacterial CFU per test ($4x10^7$ CFU, estimated MOI of 400), the same used in the PhagoTest™ kit. Although high bacterial concentrations have been used previously [272], it is not a physiological stimulus. Another limitation of this assay was the use of whole blood instead of isolated neutrophils, which made impossible to accurately assess MOI, adding more variability to data acquisition, as the number of circulating neutrophils vary between individuals. Thus, all these factors contributed to the variation observed in our dataset.

Next, reactive oxygen species (ROS) production was investigated, after incubation of isolated neutrophils with *S. pneumoniae*. Generation of ROS occurs via the NADPH oxidase system, which can be triggered by phagocytosis or by activation of pattern recognition receptors (PRRs) [130]. Electron transfer by NADPH oxidase converts molecular O_2 into O_2^- (superoxide). In this thesis, ROS generation was measured using DHR123, a chemical that fluoresces upon reaction with superoxide. Here neutrophils from healthy older donors successfully generated ROS in response to PMA, at slightly higher levels than young participants. This is not in full accordance with the literature, as ROS generation by PMA stimulation in TNF-α-primed neutrophils was equal to that of young [141] or diminished [136]. Of note, the older cohort of this last study was from an outpatient clinical service, therefore not all participants could be in the "healthy" scope or condition. There are also reports of increased ROS generation by neutrophils of older individuals with periodontitis isolated from the oral cavity [356]. Overall, neutrophil ROS production can be altered with

increasing age but this appears to depend on the stimuli given [127,129] with response to PMA less affected than microbial stimuli.

Because PMA is a soluble stimulus that bypasses the cellular membrane to activate protein kinase C PKC directly, then activating NADPH oxidase [357], neutrophil ROS generation by non-opsonised pneumococcal strains was also tested, as this production is dependent on PRRs such as TLR2, which binds surface lipoproteins or lipoteichoic acid from the pneumococcus [229], and TLR4, that binds pneumolysin (PLY) [235], or phagocytosis [130]. Although in this thesis the polysaccharide capsule is referred to as the main component that distinguishes one serotype from another, other factors of the biology of the pneumococcus have to be taken into account in a more detailed study, such as genotype and levels of expression of virulence factors; however, these are beyond the scope of this thesis.

Capsular pneumococcal serotypes were found to promote different levels of ROS generation in neutrophils, with the highest levels of ROS being produced by 19A, followed by TIGR4 and 23F. Such an effect was expected, as serotypes have different resistance to neutrophil phagocytosis [270] and induce variable levels of ROS by neutrophils of young donors [283]. Although this pattern of ROS production was observed in both healthy and patient cohorts, generation of ROS in response to 19A and 23F were lower in healthy older adults. Capsule has been shown to be the main component determining resistance to neutrophil killing [270] and indeed, 23F is a heavily encapsulated serotype [270]. Therefore, it is possible that this serotype was more resistant to neutrophil-mediated phagocytosis in the old, thus leading to lower ROS generation. Levels of PLY can also modulate ROS generation [283], and although 19A has been shown to express PLY with high haemolytic activity compared to non-pathogenic serotypes 19B and 19C [358] and 23F to have low levels of PLY within its capsule

[359], PLY expression varies depending on strains and can have different levels of haemolytic activity [360]. A more detailed investigation of the serotypes used in this thesis is necessary to confirm the role of this toxin in the bactericidal response of neutrophils from older adults.

The TIGR4 capsule-negative mutant did not induce ROS generation in neutrophils of both healthy and patient cohorts. Conversely, Barbuti *et al*. (2010) reported that the non-capsulated R6 (serotype 2) strain induced greater ROS generation than the wild type capsulated D39 strain [272]. This result was achieved by using a MOI of 250, whilst in this thesis a MOI of 40 was used, indicating that ROS generation by a non-capsulated strain may require a higher MOI.

Opsonisation of the serotypes with commercial anti-capsular antibody was able to enhance ROS generation of neutrophils of older donors to TIGR4 and 19A, but not by 23F. It is possible that this is related to the quality of the commercial antibodies used. For instance, the commercial antibody used for TIGR4 opsonisation was against anti-type 4 only, whilst for 19A and 23F the company only provided antibody pools against “Group 19” or “Group 23”. Therefore, levels of anti-19A or anti-23F could be diluted in the commercial antibody mix. Pooled sera from both age groups had detectable levels of anti-capsular 19A and 23F, which could explain the higher ROS generation by sera-opsonised serotypes compared to commercial antibodies.

Independent of pre-opsonisation, neutrophils from older volunteers generated lower ROS in response to 23F. Whether reduced neutrophil ROS generation to 23F in older participants also correlates with reduced killing remains to be tested. However, lack of a strong

inflammatory response to this serotype could lead to reduced control of bacterial CFU thus leading to infection in older adults.

ROS generation is also an integral part of the formation of neutrophil extracellular traps. Here NET generation by PMA was not impaired in healthy older volunteers, in contrast to a previous report of our group [141], in which PMA-induced NETs were reduced in older participants after IL-8 priming. One explanation for this divergence could be that for the work in this thesis neutrophils were not primed ahead of incubation with stimuli. In regard to NET generation in response to *S. pneumoniae*, NET release did not vary in response to different serotypes in young adults, while neutrophils of older donors generated more NETs in response to 23F. These observations are interesting, however, the mechanisms underlying NET generation by *S. pneumoniae* are still unclear. Branzk and colleagues (2014) demonstrated that neutrophils can selectively release NETs depending on size of the microbe, as demonstrated by selective release of NETs to *Candida albicans* hyphae, but not to single yeast, which was dependent on dectin-1 receptor and Syk/ERK signalling [361]. Another group demonstrated that pneumococcal capsular polysaccharide can induce dose-dependent NET generation [362]. As mentioned earlier, 23F is a heavily encapsulated serotype [270], therefore capsule thickness and bacterial size could be associated with elevated NET release in response to 23F in older individuals.

Additionally, the mechanisms underlying ROS interaction with signalling pathways that lead to NET generation and whether these are impaired with ageing is still unknown. A recent study showed that pneumococci can induce autophagy in neutrophils via PI3kinase class III, which also requires activation of autophagy gene Atg5, an event required by NET formation [277]. In neutrophils of older donors, compromised PI3K class I signalling is associated with

loss of chemotaxis accuracy [126], but no study to date has investigated the effects of age on PI3K class III. Therefore, it also remains to be further investigated if augmented NET generation to 23F by neutrophils of older adults is a consequence of bacteria size or of impaired phagocytosis and ROS production in response to this serotype.

As neutrophil ROS generation in response to non-opsonised *S. pneumoniae* serotypes was diminished but PMA-induced NETs and ROS was achieved in older subjects, expression of TLR2 and TLR4 was assessed in neutrophils, as well as IL-8 intracellular production and release. TLR2 is the main activator of IL-8 production in neutrophils [363], although TLR4 also mediates the production of this cytokine [364]. Confirming previous observations, TLR4 [127,141] and TLR2 expression were not altered with age in freshly isolated neutrophils [94]. LPS stimulation successfully activated neutrophils of healthy older volunteers, as observed by the diminished expression of surface TLR4 and high IL-8 production. An interesting finding is that pneumococcal serotypes did not modulate TLR2 expression in neutrophils of both age groups, whilst TLR4 expression were distinct in neutrophils of young and old. Stimulation with 19A and 23F, but not TIGR4, increased TLR4 expression in neutrophils from young, while expression decreased in older adults. Although IL-8 production via TLR1/TLR2 heterodimer activation was shown to be reduced with age previously [94], following *S. pneumoniae* or LPS stimulation intracellular levels of neutrophil IL-8 were comparable between young and old. Higher neutrophil IL-8 production and CD11b/CD62L activation was induced by *S. pneumoniae* TIGR4, followed by 19A and 23F, a different pattern than observed in ROS generation. However, levels of secreted IL-8 following an 18-hour incubation of neutrophils showed that production of this cytokine was comparable between serotypes at this later time point and was not changed with age. Surprisingly, after 18-hour

incubation, neutrophils of older adults produced high levels of IFN-γ in response to 23F. Production of IFN-γ during pneumococcal pneumonia has been found in mice models [365,366] and it is suggested that this cytokine is produced by neutrophils early in the course of bacterial pneumonia [367].

3.5.2. Changes in innate immunity against *S. pneumoniae* during pneumonia infection in older adults

In contrast to the few age-related effects observed in neutrophil and monocyte populations and functions in our healthy cohort, older patients with pneumonia infection demonstrated the expected increase in circulating neutrophils and immature granulocytes. Recently, a high neutrophil-to-lymphocyte ratio has been used as a predictor of mortality for older patients with community-acquired pneumonia [368]. Additionally, mortality between centenarians has been associated with increased frequency of neutrophils [38], thus indicating that elevated frequency of neutrophils in the bloodstream is not always beneficial and this may depend on the specific population of neutrophils seen with infections that has not been extensively studied previously.

As observed with increasing age, a high frequency of $CD16^{high}CD62^{dim}$ neutrophils was also present during pneumonia infection. As previously mentioned, it is possible that increased levels of IL-8 found in serum contributed to maintain this population in the bloodstream during infection. Moreover, compared to the healthy condition, during pneumonia infection older adults had immature granulocytes as a third neutrophil phenotype present in blood, alongside increased frequency of $CD16^{high}CD62^{dim}$ and of mature neutrophils. Compared to

mature neutrophils ($CD16^{bright}CD62L^{bright}$), the $CD16^{high}CD62^{dim}$ subset has normal phagocytic function, [120], superoxide production and levels of MPO [118] and migration towards IL-8 [119,120] in LPS-injected volunteers, but has reduced migration towards fMLF and poor adhesion to activated endothelium, due to its low expression of L-selectin [119]. Furthermore, a recent study showed that $CD16^{high}CD62L^{dim}$ neutrophils fail to contain intracellular growth of methicillin-resistant *Staphylococcus aureus* (MRSA), compared to mature neutrophils [120]. This impaired bacterial containment was associated with decreased acidification of the phagolysosome [120]. A single study reported that $CD16^{high}CD62L^{dim}$ neutrophils of older adults have reduced phagocytosis and ROS generation [121]. Conversely, immature ($CD16^{dim}$) neutrophils are reported as successfully containing MRSA in LPS-injected volunteers [120], but show reduced migration, phagocytosis and ROS production, and prolonged lifespan during sepsis [115]. Taking these findings into the context of pneumococcal infection in older adults, the presence of immature granulocytes in these patients may be a strategy to compensate for the functionally impaired $CD16^{high}CD62^{dim}$ subset [120] and, if immature neutrophils have reduced functional capacity during pneumonia, as observed in sepsis, that could lead to accumulation of non-functional neutrophils in the lungs of the patients [109], thus worsening patient outcomes. However, no study to date has addressed the function of $CD16^{high}CD62^{dim}$ and immature neutrophils during pneumonia infection.

The monocyte population was also altered in older patients with pneumonia. Patients had increased frequency of intermediate monocytes compared to healthy, and elevated CCR2 expression in this subset. Intermediate monocytes from healthy older adults were the major monocyte subset to produce TNF-α and IL-6 following *in vitro* stimulation with *S.*

pneumoniae, 6B serotype [89]. The same study reported that in mice, CCR2 expression in Ly6C^{high} (intermediate) monocytes increased with age. The group proposes that in old mice, TNF-α drives the increase in CCR2 expression by intermediate monocytes, which then enter the circulation, and release higher levels of TNF-α upon challenge with *S. pneumoniae*. Whether these findings are replicated in humans remains to be confirmed, but one could hypothesize that age-related changes in monocyte populations could be aggravated during pneumonia infection, in which high levels of pro-inflammatory cytokines could elevate CCR2 expression on intermediate monocytes and promote premature egress from the bone marrow. Supporting this hypothesis, here there was a positive correlation between CCR2 expression of intermediate monocytes and levels of serum IL-6. When stimulated with *S. pneumoniae*, monocytes from older patients showed similar decreases in CD11b and CCR2 surface expression and intracellular TNF-a or IL-6 levels to healthy older participants. As mentioned earlier, it is possible that no changes were observed because of the small sample size (n=7) or the time point the cells were collected and stimulated [369].

On testing neutrophil function, PMA stimulation promoted ROS generation in both pneumonia cohorts, however in the older subjects, levels of ROS were not as high as in healthy older volunteers. One possible explanation is that pro-inflammatory cytokines TNF-α, IL-6 and/or IL-8 found in patients' serum primed peripheral neutrophils, thus inducing higher ROS generation at baseline, but lower levels following PMA treatment. A similar hypothesis has been tested by Sauce *et al.* (2017), who reported lower PMA-induced ROS generation by neutrophils from older donors, following priming with TLR agonists or TNF-α [121]. Additionally, TNF-α priming ahead of *S. pneumoniae* incubation generated higher levels of ROS than *S. pneumoniae* incubation alone [370], and here, data show that

pneumonia patients produced higher ROS in response to *S. pneumoniae* serotypes than healthy participants. Yet, in patients, pneumococcal-induced ROS generation was lower than PMA-induced indicating that the response to a maximal stimulus such as PMA could be compromised in patients.

Although neutrophil ROS generation is involved in clearance of the pneumococcus, overproduction is detrimental and can cause tissue damage. In a pneumonia model using knockout mice missing the $gp91^{phox}$ subunit of NADPH oxidase, disease outcome was improved by absence of ROS generation [371]. KO mice had increased neutrophil recruitment to the lungs and reduced neutrophil apoptosis, while neutrophil activation, elastase release and bacteria clearance in the lungs were comparable to control mice [371]. This indicates that targeting overproduction of ROS during pneumonia infection could be a strategy to improve the outcome of patients, but also that pneumococci clearance depends on other mechanisms apart from ROS generation. During pneumonia infection in older adults, if other strategies to contain the pneumococcus are impaired, such as discussed below, targeting ROS generation alone would not be sufficient.

During pneumonia infection, neutrophils from the older cohort had reduced NET generation in response to PMA, TIGR4 and 23F, but not 19A. This finding raises two hypotheses: (1) the elevated ROS production by neutrophil of patients could activate a pathway other than autophagy/NETs, and therefore be sufficient for killing the pneumococci or, (2) high levels of ROS induced during a short incubation could not be sustained for the longer period required for NET generation, leading to functional exhaustion of neutrophils. To address the first hypothesis, neutrophil killing function could be tested in the future. Considering the second hypothesis, neutrophil ROS production could be tested over time. Additionally, a study from

our group demonstrated that TNF-α-primed neutrophils of healthy older adults generated lower levels of NETs following IL-8 or LPS stimulation, but not PMA, suggesting that the lack of response to secondary stimuli was related to receptor signalling upstream PKC [141]. However, during pneumonia infection, both young and older volunteers generated lower NETs upon incubation with PMA or pneumococcal serotypes, suggesting signalling both upstream and downstream of PKC were altered during pneumonia. Lower *ex vivo* NET generation by PMA was also detected in patients with sepsis [372] and burn injury [373]. Considering pneumococcal activation of NETs, which can be induced by the whole bacterium [277,279,280] or PLY alone [278], impaired NET generation in pneumonia patients could facilitate pneumococci evasion, of neutrophil-mediated clearance.

Pneumonia patients also had increased Mac-1 complex (CD11b/CD18) and loss of L-selectin (CD62L) in unstimulated neutrophils, as well as increased TLR2 and TLR4 expression. As suggested before, priming of neutrophils by increased serum levels TNF-α, IL-6 and/or IL-8 could have contributed to elevated expression TLR2 and TLR4 in neutrophils of older patients, a phenotype also observed in neutrophils and monocytes of patients with sepsis [374]. Overall, the pattern of neutrophil IL-8 production after 4-hour incubation with *S. pneumoniae* serotypes was the same as in healthy participants and older patients, i.e., TIGR4 induced the highest IL-8 production by neutrophils, followed by 19A and 23F. However, while neutrophil TLR2 and TLR4 were not modulated and remained elevated in patients following stimulation with pneumococcal serotypes, IL-8 production had a trend toward lower intracellular levels in patients and was significantly reduced by 23F stimulation. To confirm whether this is an effect of the systemic inflammation observed in older patients or a consequence of age-related impairment in TLR signalling, as mentioned before, further

study is required. However, a study in mice supports this last hypothesis, having shown that alveolar macrophages of aged mice had reduced phosphorylation of p65, JNK and p38 MAPK following *S. pneumoniae* stimulation, whereas ERK phosphorylation increased, all which contributed to diminished TNF-α and IL-6 production by these cells [97]. No study to date has investigated if neutrophils from humans undergo the same decline in phosphorylation of these kinases following *S. pneumoniae* stimulation.

The clinical outcome of pneumonia infection is not associated with pathogen clearance only but also with the right balance of pro- and anti-inflammatory cytokines [375,376]. Here, neutrophil cytokine production after 18-hour incubation with *S. pneumoniae* showed a trend toward reduced IL-1β, IL-6 and TNF-α in older patients, but no change in IL-10 levels, compared to healthy. This last cytokine was not detected in the serum either. It is possible that IL-10 could have been found in BAL fluid, as usually higher levels of cytokines are found in BAL than serum of patients with community-acquired pneumonia, such as that IL-1β, IL-6, IL-8 and IL-22. [376]. Still, our patients had elevated levels of IL-6 and IL-8 in serum; IL-6 levels, for instance have been reported to be higher in older patients with CAP than in younger patients [377], and high levels of circulating IL-6 and TNF-α were associated with earlier mortality during CAP [378]. In this thesis cohort, older patients also had increased serum levels of IL-33. This cytokine has been associated with protection of the mucosal-barrier [379], and epithelial lungs are the major source of this cytokine in COPD [380,381], so this site could also be the source of IL-33 in our patients. IL-33 has been detected during eosinophilic pneumonia [382], reported to recruit neutrophils during sepsis [383] and to the lungs during influenza A and MRSA infection [379]. The role of IL-33 on neutrophil recruitment will be further discussed in Chapter 5 (General Discussion).

In summary, data presented in this chapter indicate that neutrophils from healthy older donors show some altered bactericidal responses to pneumococcal serotypes, in particular to 23F. However, overall no major differences were found between young and old for a range of neutrophil and monocyte functions, indicating that our older adult cohort could be relatively healthy and not particularly susceptible to pneumonia. Here is also show for the first time the differences in neutrophil subpopulations with age and during pneumonia, and that not all neutrophil functions are reduced in older patients. These results still need further investigation in order to assess the influence of age-related changes on neutrophil responses during pneumonia. Thus, the next chapter addressed the presence of CD4+ T-helper subsets during pneumococcal colonisation and pneumonia infection, as these subsets can control neutrophil activity, in particular Th17, $CD4^+$ T cells that control neutrophil recruitment via IL-17.

Chapter 4

The effect of age on $CD4^+$ T lymphocyte polarisation during Experimental Human Pneumococcal Carriage (EHPC) and pneumonia infection

4.1 Introduction

Nasopharyngeal colonisation by the pneumococcus is considered as a primary reservoir of transmission of this bacteria and also a precursor of disease [193]. However, frequency of commensal carriage is high in children, as it was detected in 50% of children aged 0-2 years and in 45% of children 3-4 years, while in adults, pneumococcal carriage is present in 1% to 10% of individuals [195]. In contrast, older individuals have lower colonisation rates of 2.3% [197] but are the age group with a high disease incidence [5]. Whereas elevated pneumococcal density in the nasopharynx has been associated with a risk of developing pneumonia in an adult cohort [384], colonisation is virtually absent in hospitalized older adults with CAP, found in only 0.3% of patients [385]. These data suggest a role for the aged immune system in the high pneumonia incidence relative to bacterial colonisation in older adults. Interestingly, in aged mice colonisation was prolonged compared to young mice, due to delayed upregulation of proinflammatory mediators in the nasal-associated lymphoid tissue (NALT) [386].

In humans, the decrease in carriage rates with increasing age is associated with the development of specific immunity [194], represented by antigen-specific memory $CD4^+$ T cells [228,261] and anti-protein and capsular antibodies [195,297]. Differentiation of cells to a Th17 phenotype is considered important to mediating the mucosal and systemic antigen-specific response to *S. pneumoniae* [257,261,308], and their production of IL-17A is also central to immunity by regulating neutrophil infiltration in mucosal sites. In addition, in the NALT, dendritic cells can rapidly differentiate into professional antigen-presenting cells for activation of T and B lymphocytes [387].

The experimental human pneumococcal carriage methodology (EHPC) is a well-established model that consists of inoculation of *S. pneumoniae* in the nasal cavity of healthy volunteers [328]. It has proven to be a reproducible method for studying immune mechanisms involved during colonisation and in the development of protective immunity. Experimental colonisation of young volunteers leads to generation of $CD4^+$IL-$17A^+$ cells in BAL and peripheral blood [302] as well as serum anti-IgG PspA antibody [223,314], and mucosal and serum anti-capsular IgG [311,314], but no study to date has investigated whether these responses are changed with age.

As the beneficial effect of carriage remains controversial in older adults, a study of its effect on immunogenicity in older adults is a novel approach for a better understanding of this phenomenon. Thus, the effect of age on dendritic cells as well as on $CD4^+$ T cell polarisation during experimental carriage, and changes in $CD4^+$ T subset frequency during pneumonia in older adults were addressed in this chapter.

4.2 Aims

The aims of this chapter were:

- To analyse the effect of age on the frequency of peripheral blood dendritic cell subsets, their CD40 and HLA-DR expression;
- To investigate the effect of age on peripheral blood $CD4^+$ T lymphocyte subset differentiation during Experimental Human Pneumococcal Carriage (EHPC) in healthy volunteers;

- To analyse changes in the frequency of $CD4^+$ T lymphocytes subsets following *in vitro* stimulation with *S. pneumoniae* serotypes with ageing, during healthy and pneumonia infection in older adults, using PBMCs;
- To investigate changes in Treg activation, IL-10 and TGF-β1 production during pneumonia infection in older individuals.

4.3. Results

4.3.1 Experimental Human Pneumococcal Carriage (EHPC) study participants

PBMCs from 15 healthy young (mean age 20.73 ± 3.7 years; range 18-29 years) and 15 healthy older adults (59.8 ± 7.1 years; range 50-73 years) from the Experimental Human Pneumococcal Carriage (EHPC) study were used in this thesis. The EPHC older adult cohort was younger than the cohort from Chapter 3 which had a mean age of 72.43 ± 5, range 67-83 years. Information on the participants age, gender and carriage are summarized in Table 4.1. To determine the effect of carriage on CD4+ T cell polarisation, samples from pre-inoculation (-day 5) and post inoculation were assessed. Post inoculation samples were collected from the young cohort at day 14 and from the old cohort at day 29. Carriage length between carriage positive volunteers was similar between age groups (Table 4.1). Of the older cohort, only 2 participants had been vaccinated with the pneumonia vaccine. In this chapter healthy young volunteers will be referred to as HY and healthy old as HO.

Table 4.1. Demographics of healthy volunteers from the EHPC study

	Young (HY)	Old (HO)
Number of participants	15	15
Mean age and range (years)	20.73 ± 3.7 (18-29)	59.8 ± 7.1 (50-73)
Male (%)	8/15 (53%)	7/15 (46%)
Carriage positive (%)	7/15 (46%)	7/15 (46%)
Mean carriage length (days)	21.71	20.14

4.3.2. Pneumonia patients

Information about pneumonia patients recruited for this thesis is described in detail in Chapter 3 (section 3.3.2). Briefly, 24 older patients were also enrolled in this thesis and a summary of patients age, gender and vaccination status are shown in table 3.2. As mentioned in Chapter 3, only 3 young patients were enrolled during the recruitment period of this study and because recovery of viable PBMCs following -80°C storage was low in samples from several patients, not all stimulations and immunophenotyping were performed in all patients. Therefore, this chapter focus on data from $CD4^{+}$T cell populations obtained from older patients.

4.3.3 Changes in dendritic cell (DC) frequency and subsets with age

To assess the changes in the frequency of DCs and their subsets with increasing age, thawed PBMCs ($1x10^6$) from pre-colonisation samples from young and old EHPC volunteers were stained and analysed by flow cytometry using the gating strategy shown in Figure 4.1.

There were no age related changes in the frequency of total dendritic cells (Figure 4.2 A), however frequency of subsets was altered with age (Figure 4.2 B). Three subsets of dendritic cells can be identified in blood: the classical myeloid $CD1c^+$ (BDCA-1, mDC1), the plasmacytoid $CD303^+$ (BDCA-2, pDC), and the myeloid $CD141^+$ (BDCA-3, mDC2). In blood and tissues, the frequency of mDC2 subset is 10% of the frequency of mDC1 [79]. Here, older donors had an increased frequency of mDC1 compared to young donors (p=0.014) (Figure 4.3 A), while frequency of pDCs was lower (p=0.013) (Figure 4.3 B), and mDC2 frequency was not affected by age (Figure 4.3 C). The absolute number of these subsets showed that both subsets from the myeloid lineage were increased in the old (mDC1, p=0.002; mDC2, p=0.078), whereas numbers of pDCs were comparable between age groups (Figure 4.3 D, E and F). Surprisingly, these changes in dendritic cells were more pronounced in "younger" older adults, from 50 to 64 years old, as absolute numbers of mDC1 decreased with ageing (R^2=0.29, p=0.037) (Figure 4.4 A). Although pDCs and mDC2 data also suggest a trend towards decrease in number of these populations with ageing, there was no statistical correlation, but a larger sample size could confirm this association (Figure 4.4 B and C).

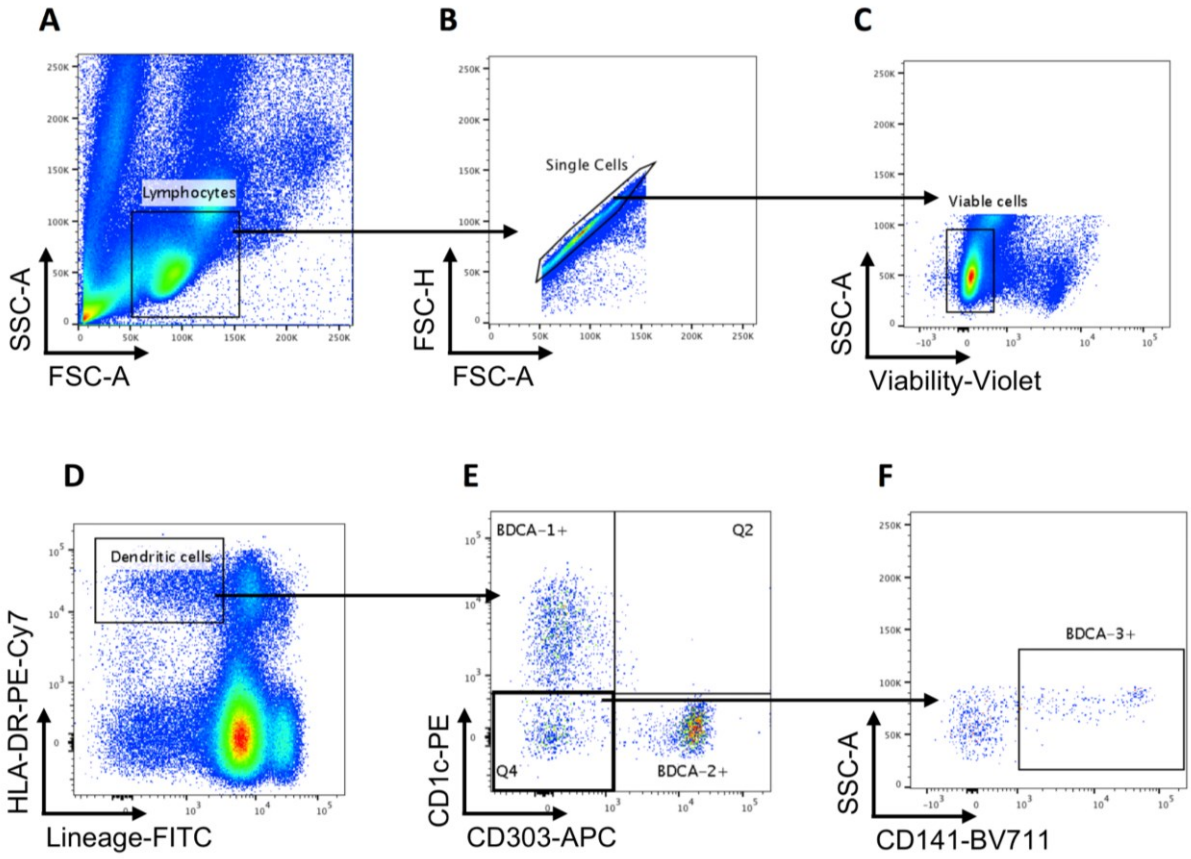

Figure 4.1. Gating strategy for identification of dendritic cells and their subsets. 1x10^6 PBMCs were stained with viability dye followed by antibody mix containing lineage cocktail (anti-human CD3, CD14, CD19, CD20 and CD56), and anti-human CD1c, CD303, CD141, HLA-DR and CD40 ahead of flow cytometry acquisition. **(A)** Lymphocytes were initially identified by SSC-A and FSC-A, **(B)** Debris and doublets were excluded by gating on single cells using FSC-H and FSC-A. **(C)** Live lymphocytes were selected using a viability dye, **(D)** Live cells positive for HLA-DR and lacking lineage cocktail markers were considered dendritic cells. Lineage cocktail allowed exclusion of lymphocytes, monocytes, macrophages, B cells and NK cells. **(E)** Within this DCs population, mDC1 (BDCA-1, $CD1c^+$) and pDCs (BDCA-2, $CD303^+$) were identified. **(F)** Within $CD1c^-CD303^-$ gate, cells positive for CD141 were identified as mDC2 (BDCA-3, $CD141^+$). Surface density of CD40 and HLA-DR was assessed in all subsets by analysis of median fluorescence intensity (MFI).

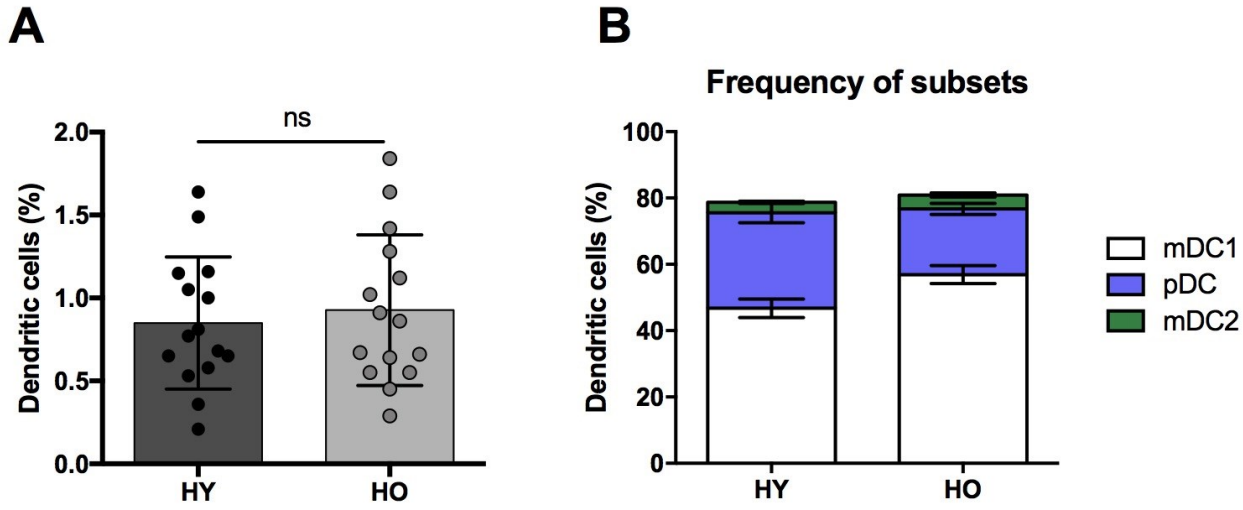

Figure 4.2. Frequency of total dendritic cells and dendritic cell subsets in healthy young and old volunteers. PBMCS ($1x10^6$) from pre-colonisation samples of healthy young (n=15) and old (n=15) volunteers were stained for identification of dendritic cells by flow cytometry. **(A)** Frequency of total dendritic cells, data are expressed as mean ± SD **(B)** Frequency of dendritic cell subsets mDC1, pDC and mDC2 within total dendritic cells, data are expressed as mean ± SEM. Differences between age groups were analysed by unpaired T test. HY = healthy young. HO = healthy old. Ns = non-significant.

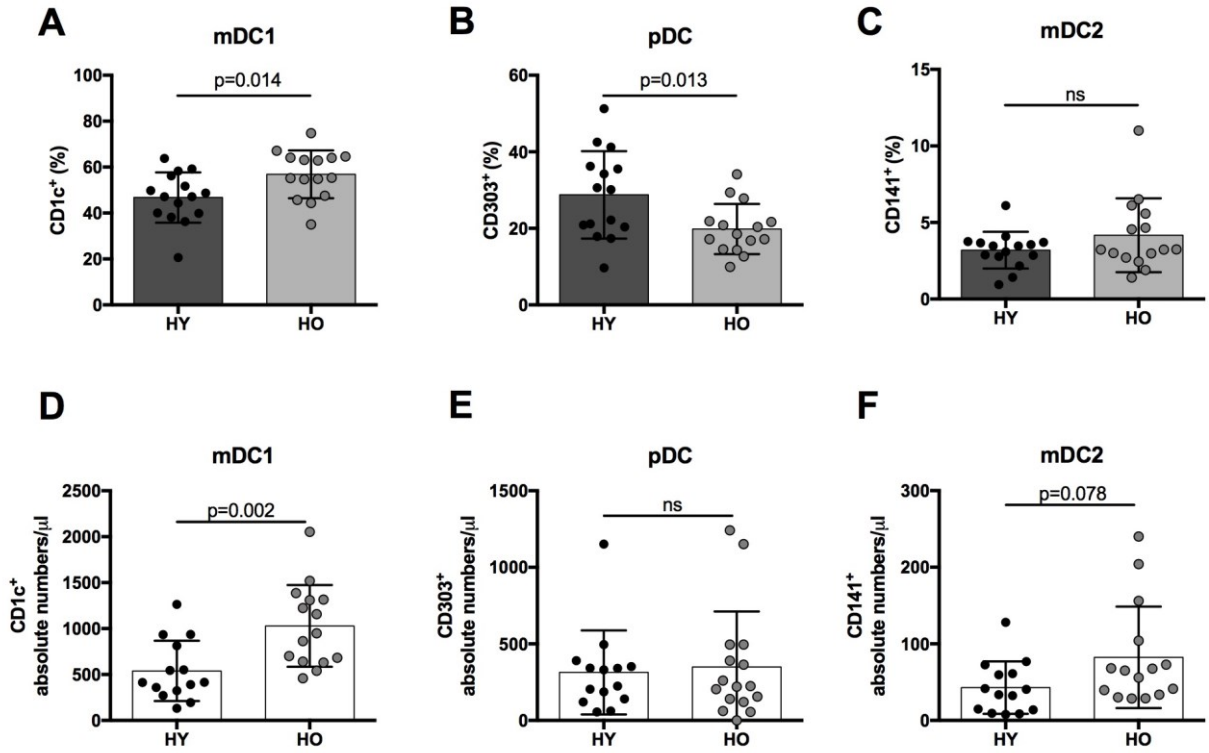

Figure 4.3. Frequency and absolute number of dendritic cell subsets in healthy young and old volunteers. PBMCS ($1x10^6$) from pre-colonisation samples of healthy young (n=15) and old (n=15) volunteers were stained for flow cytometry analysis for identification of dendritic cells. Frequency of subsets **(A)** mDC1 ($CD1^+$) **(B)** pDC ($CD303^+$) and **(C)** mDC2 ($CD141^+$). Absolute number of subsets **(D)** mDC1 **(E)** pDC and **(F)** mDC2. Data are expressed as mean ± SD. Differences between age groups were analysed by unpaired T test. HY = healthy young. HO = healthy old.

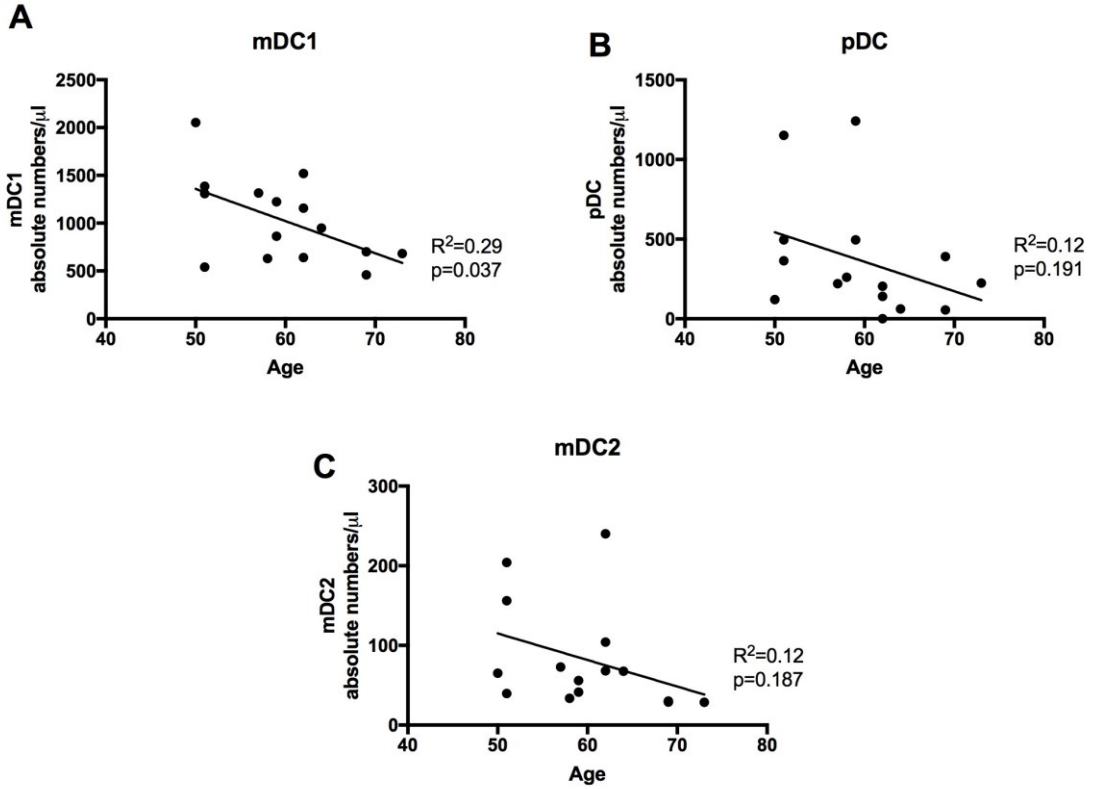

Figure 4.4. Association between numbers of dendritic cell subsets and age. Correlation between absolute numbers of dendritic cell subsets and age in healthy old (n=15). **(A)** mDC1 ($CD1^+$), **(B)** pDC ($CD303^+$) and **(C)** mDC2 ($CD141^+$).

Next, surface density of activation markers CD40 and HLA-DR were analysed in the main DC subsets. Classical mDC1 dendritic cells showed no changes in CD40 expression with older age (Figure 4.4 A), but the expression of this co-receptor was increased in plasmacytoid DCs from older donors (p=0.016) (Figure 4.4 B). Therefore, ageing seems to cause an imbalance in CD40 expression between these subsets, as expression of CD40 was comparable between mDC1 and pDC from young donors (Figure 4.4 C) but was increased in pDCs from older volunteers (p=0.002) (Figure 4.4 D). Such changes were not observed in the expression of HLA-DR, which was unaltered with age in both subsets (Figure 4.5 A and B). Moreover, the difference in surface expression of this marker between mDC1 and pDC subsets was conserved with advancing age (HY, p=0.003; HO, p=0.005) in our cohort (Figure 4.5 C and D).

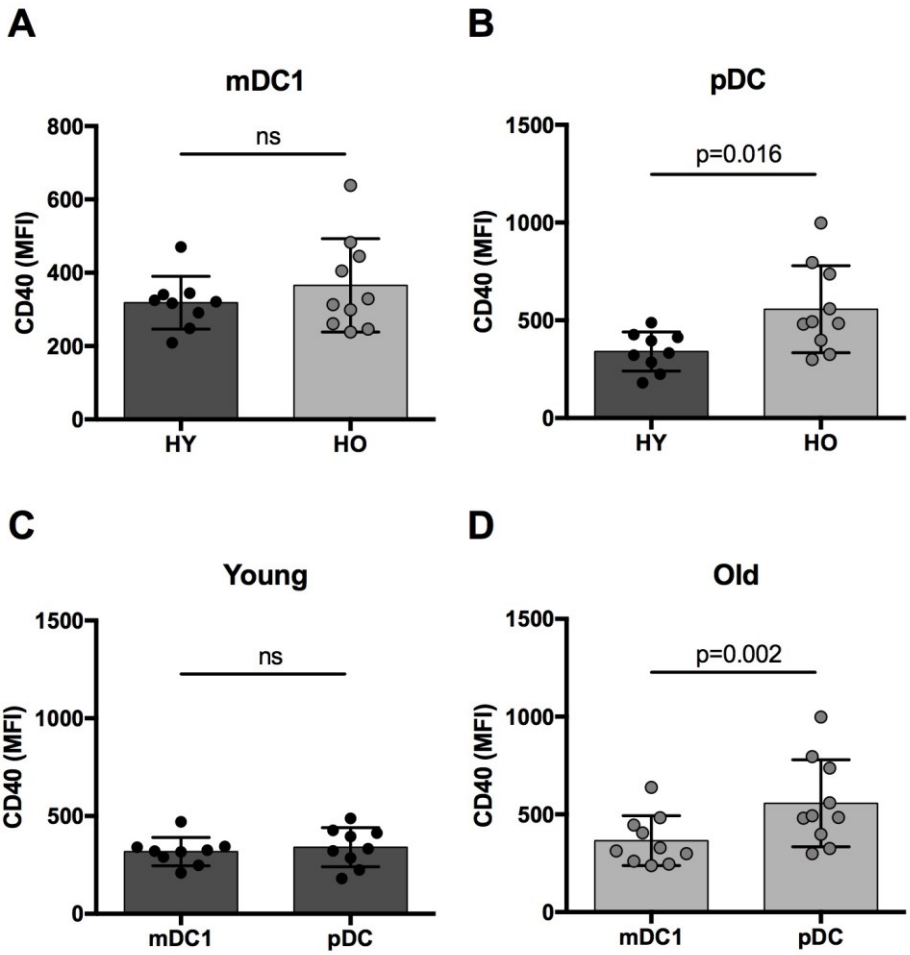

Figure 4.5. Surface CD40 expression in dendritic cell subsets of healthy young and old volunteers. PBMCS ($1x10^6$) from pre-colonisation samples of healthy young (n=15) and old (n=15) volunteers were stained for flow cytometry analysis for identification of dendritic cells. Surface density (MFI) of CD40 between age groups of subsets **(A)** mDC1 ($CD1^+$) and **(B)** pDC ($CD303^+$). Comparison of surface density of CD40 between subsets in **(C)** young and **(D)** old. Data are expressed as mean ± SD. Differences between age groups were analysed by unpaired T test and differences between subsets were analysed by paired T test. HY = healthy young. HO = healthy old.

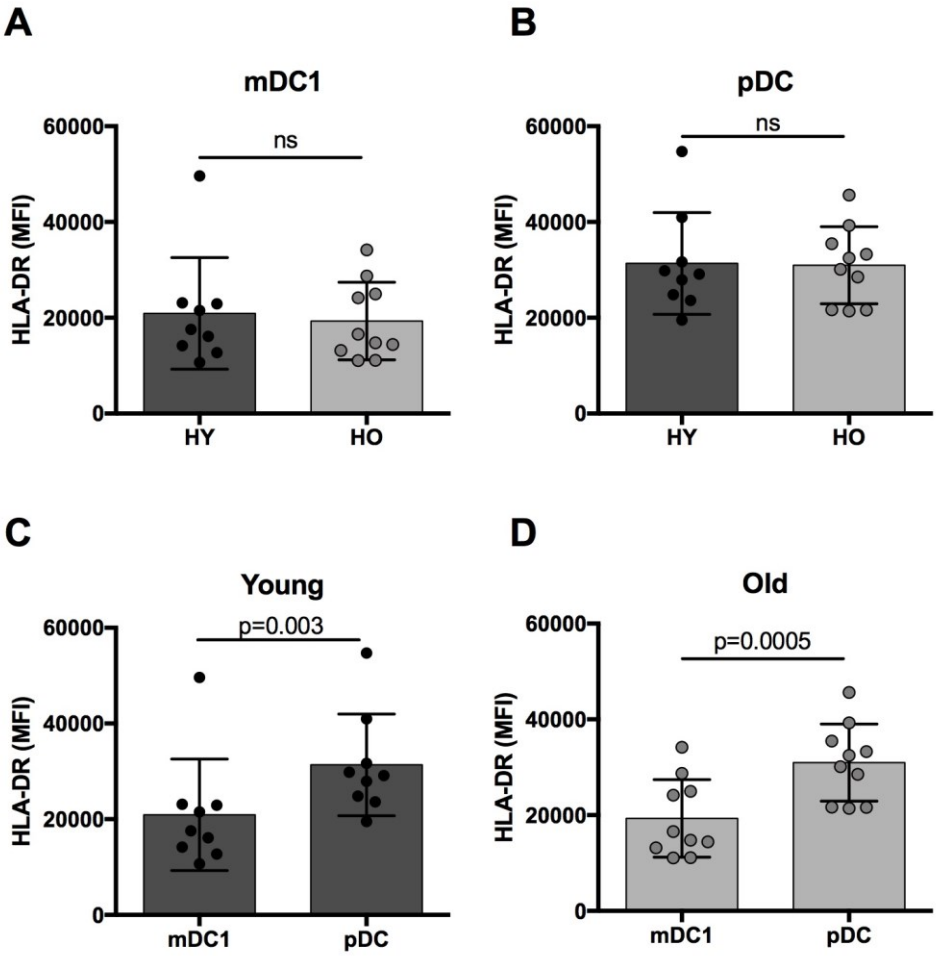

Figure 4.6. Surface HLA-DR expression in dendritic cell subsets of healthy young and old volunteers. PBMCS ($1x10^6$) from pre-colonisation samples of healthy young (n=15) and old (n=15) volunteers were stained for flow cytometry analysis for identification of dendritic cells. Surface density (MFI) of HLA-DR between age groups of subsets **(A)** mDC1 ($CD1^+$) and **(B)** pDC ($CD303^+$). Comparison of surface density of HLA-DR between subsets in **(C)** young and **(D)** old. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test and differences between subsets were analysed by Wilcoxon test. HY = healthy young. HO = healthy old.

4.3.4 Effect of age on $CD4^+$ T cell polarization during experimental human pneumococcal carriage

In order to determine the best experimental condition for detection of Th17 cells ($CD3^+CD4^+IL\text{-}17A^+$), PBMCs from HY were incubated in 96-flat-bottom cell plates under three different concentrations per well: $5x10^4$, $1x10^5$, $2x10^5$ (Figure 4.7 A-F). The cells were stimulated with PMA (10 ng/ml) and ionomycin (50 ng/ml) for over 120 hours. 6 hours prior to the 24-hour time points, protein transport inhibitor Brefeldin A was added to cells to stop cytokine secretion. Every 24 hours, cells were collected, counted and immunostained for detection of $CD3^+CD4^+IL\text{-}17A^+$ lymphocytes. Based on these pilot assays, a concentration of $2x10^5$ cells per well and 72h incubation were chosen, as at these conditions offered both better cell viability and frequency of Th17 (Figure 4.7 C and F).

Next, to enhance detection of intracellular cytokines, incubation with different protein transport inhibitors was tested. Cells were stimulated with PMA (10 ng/ml) and ionomycin (50 ng/ml) at 48h and, 2 hours after, protein transport inhibitors GolgiPlug was added to cells, followed by 16-hour incubation; or stimulated with PMA/ionomycin together with Brefeldin A at 66h, for 6 hours. A viability dye was also incorporated in to the staining panel to exclude dead cells. Higher frequency of intracellular IFN-γ in $CD3^+CD4^+$T cells was detected following longer incubation with GolgiPlug than Brefeldin A (Figure 4.8). Thus, the final protocol incorporated the re-stimulation of cells at 48h of incubation, and addition of GolgiPlug 2 hours after re-stimulation for 16h (total of 66h hours of incubation).

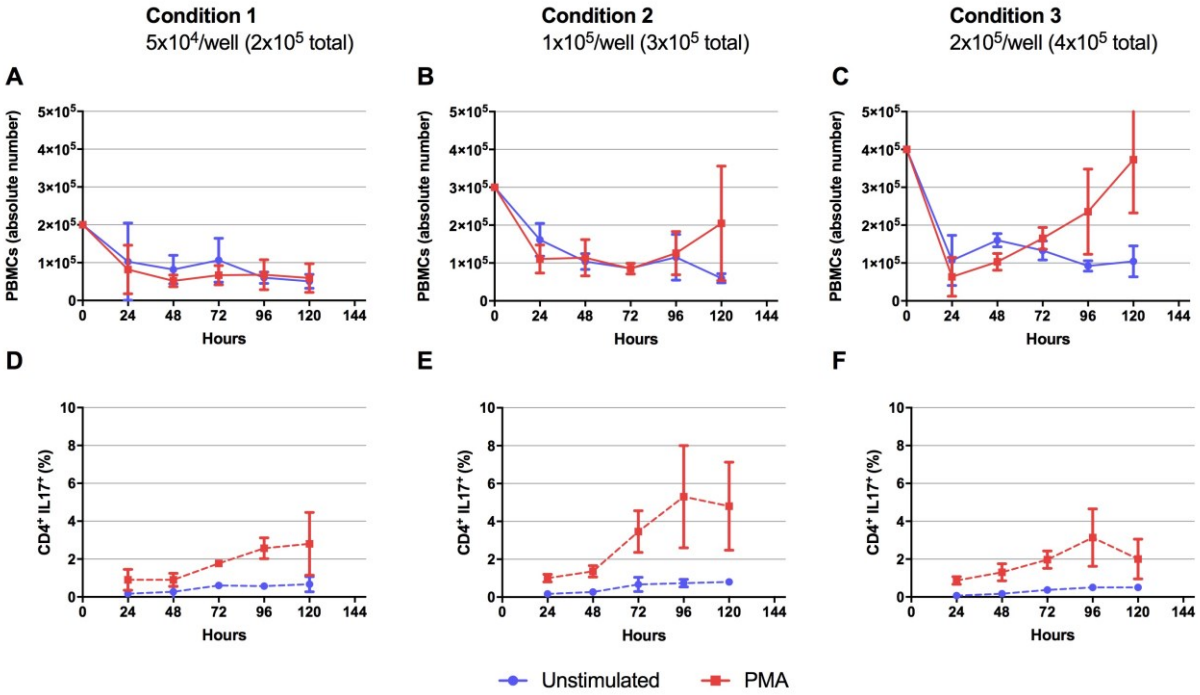

Figure 4.7. Absolute number of PBMCs and $CD3^+CD4^+IL\text{-}17A^+$ over time. PBMCS of healthy young donors (n=3) were stimulated with PMA (10 ng/ml) and ionomycin (50 ng/ml) over 120 hours at different cell concentrations per well. 6 hour-prior to 24-hour time points, Brefeldin A was added to cells to stop cytokine secretion, ahead of flow cytometry analysis. Absolute number of PBMCs overtime at **(A)** $5x10^4$ cells/well **(B)** $1x10^5$ cells/well **(C)** $2x10^5$ cells/well and frequency of $CD3^+CD4^+IL\text{-}17A^+$ at **(D)** $5x10^4$ cells/well **(E)** $1x10^5$ cells/well **(F)** $2x10^5$ cells/well. Data are expressed as mean ± SD.

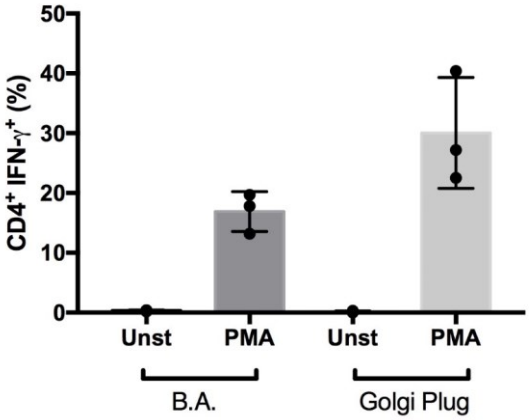

Figure 4.8. Frequency of $CD3^+CD4^+IFN\text{-}\gamma^+$ cells following incubation with protein transport inhibitors. PBMCS healthy young (n=3) were stimulated with PMA (10 ng/ml) and ionomycin (50 ng/ml) for 6 hours with Brefeldin A (B.A.) or 16 hours with GolgiPlug ahead of flow cytometry staining and analysis. Data are expressed as mean ± SD. Differences between PMA-treated groups were analysed by Mann-Whitney test.

Using this methodology, PBMCs from young and older adults experimentally colonised with *S. pneumoniae* serotype 6B in the nasal cavity were stimulated *in vitro* with a combination of PMA (10 ng/ml) and ionomycin (50 ng/ml) or *S. pneumoniae* 6B (5 µg/ml). Following *in vitro* 66 hours of stimulation, samples were immunostained for flow cytometry analysis for identification of the main $CD4^+$T cells subsets. PMA and ionomycin (referred to as PMA in the subsequent text) were not used to stimulate PBMCs ahead of Treg staining as the treatment significantly reduced CD25 expression, preventing identification of this subset by flow cytometry.

As shown by the gating strategies in Figure 4.9, the $CD4^+$T cell subsets identified in the EHPC samples were Th1 ($CD3^+CD4^+$IFN-γ^+), Th2 ($CD3^+CD4^+$IL-4^+), Th17 ($CD3^+CD4^+$IL-$17A^+$) and Treg ($CD3^+CD4^+CD127^-CD25^+Foxp3^+$) as well as $CD4^+$ T naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and effector memory cells re-expressing CD45RA (EMRA, $CD45RA^+CCR7^+$). For Th1, Th2 and Th17, their respective transcriptor factors T-bet, GATA3 and RORγt were used as markers of induction to these phenotypes, whereas membrane protein GARP (glycoprotein A repetitions predominant) was used as a marker of activation of Tregs [388].

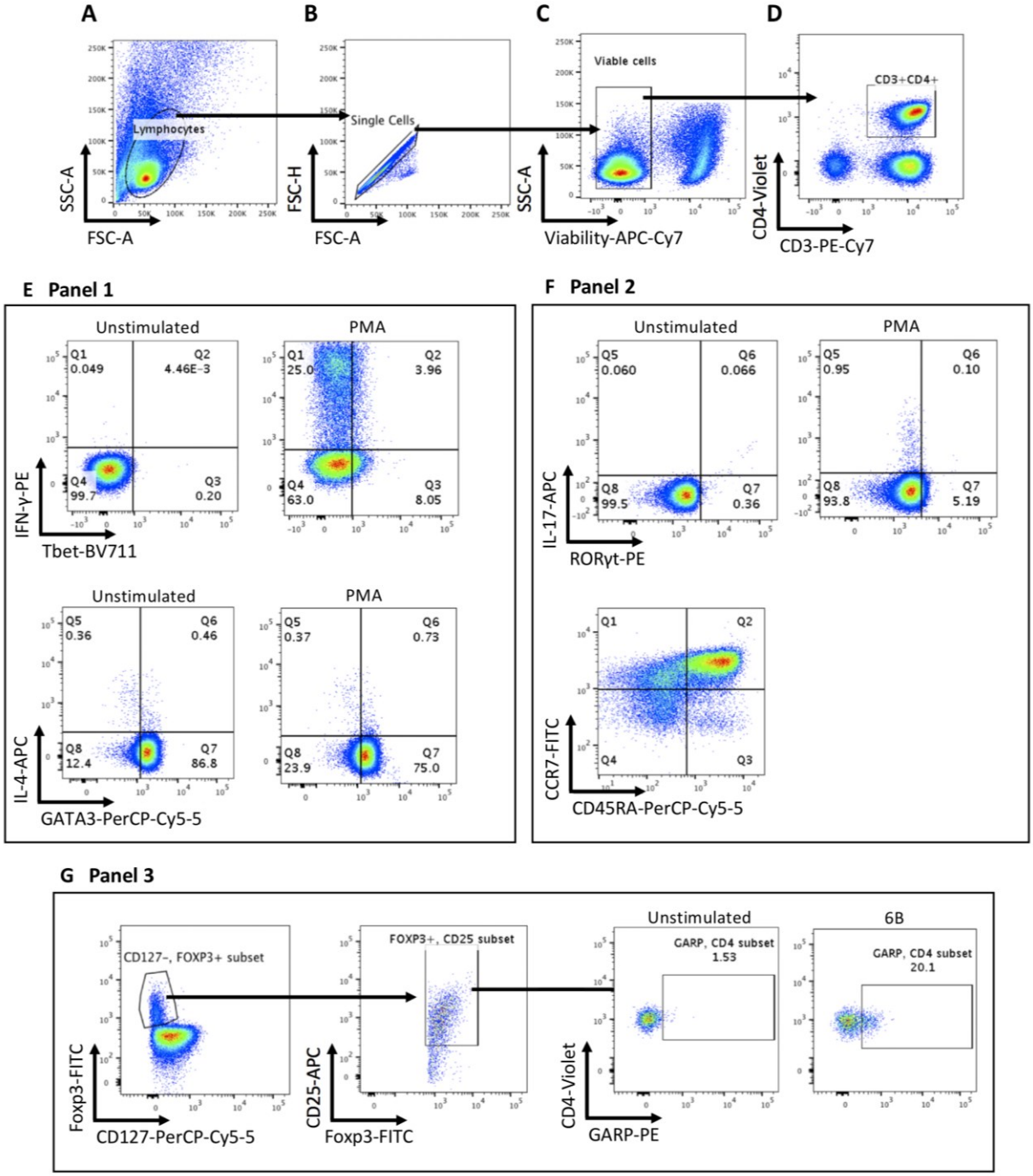

Figure 4.9. Gating strategy for identification of $CD3^+CD4^+$ T cell subsets. PMBCs from pre and post colonisation samples were stained with viability dye followed by antibody mix containing antibodies for identification of $CD3^+CD4^+$ subsets. **(A)** Lymphocytes were initially identified by SSC-A and FSC-A, **(B)** Debris and doublets were excluded by gating on single cells using FSC-H and FSC-A. **(C)** Live lymphocytes were selected using a viability dye, **(D)** Lymphocytes were defined as live cells positive for $CD3^+CD4^+$. **(E)** Panel 1 used for identification of $CD3^+CD4^+$IFN-γ^+T-bet$^+$ and $CD3^+CD4^+$IL-4^+GATA3^+ cells. **(F)** Panel 2 used for identification of $CD3^+CD4^+$IL-17A$^+$ RORγt$^+$ and naïve and memory $CD3^+CD4^+$ T cells by expression of CD45RA and CCR7. **(G)** Panel 3 used for identification of $CD3^+CD4^+CD127^-$ $CD25^+Foxp3^+$ (Tregs) and $GARP^+$Tregs.

4.3.4.1 Age-related changes in $CD4^+$ T cell polarisation pre and post EHPC

Although there were age-related differences in both pre- and post-colonisation samples, frequency of $CD4^+$ T cell subsets between time points in samples of young and old volunteers showed no statistical differences, with a couple of exceptions as described in the next paragraphs.

There were age-related differences in the frequency $CD4^+$ T cell subsets before and after nasal inoculation of 6B in volunteers. Older donors showed a higher frequency of both $CD4^+IFN\text{-}\gamma^+$ (Figure 4.10 B) and $CD4^+IFN\text{-}\gamma^+T\text{-}bet^+$ cells (Figure 4.10 D) than young donors, following PMA stimulation in both pre ($IFN\text{-}\gamma^+$, $p=0.0002$; $IFN\text{-}\gamma^+T\text{-}bet^+$, $p=0.002$) and post ($IFN\text{-}\gamma^+$, $p<0.0001$; $IFN\text{-}\gamma^+T\text{-}bet^+$, $p=0.024$) colonisation samples. Neither unstimulated or 6B (Figure 4.10 A and C) *in vitro* stimulation altered the frequency of $IFN\text{-}\gamma^+$ cells in both age groups and at both time points. The frequency of $CD4^+IL\text{-}4^+$ (Figure 4.11 A) and activated $CD4^+IL\text{-}4^+GATA3^+$ (Figure 4.11 C) was unaltered with age in both time points analysed, and PMA (Figures 4.11 B and D) or 6B (Figures 4.11 A and C) stimulations did not change the frequency of this subset.

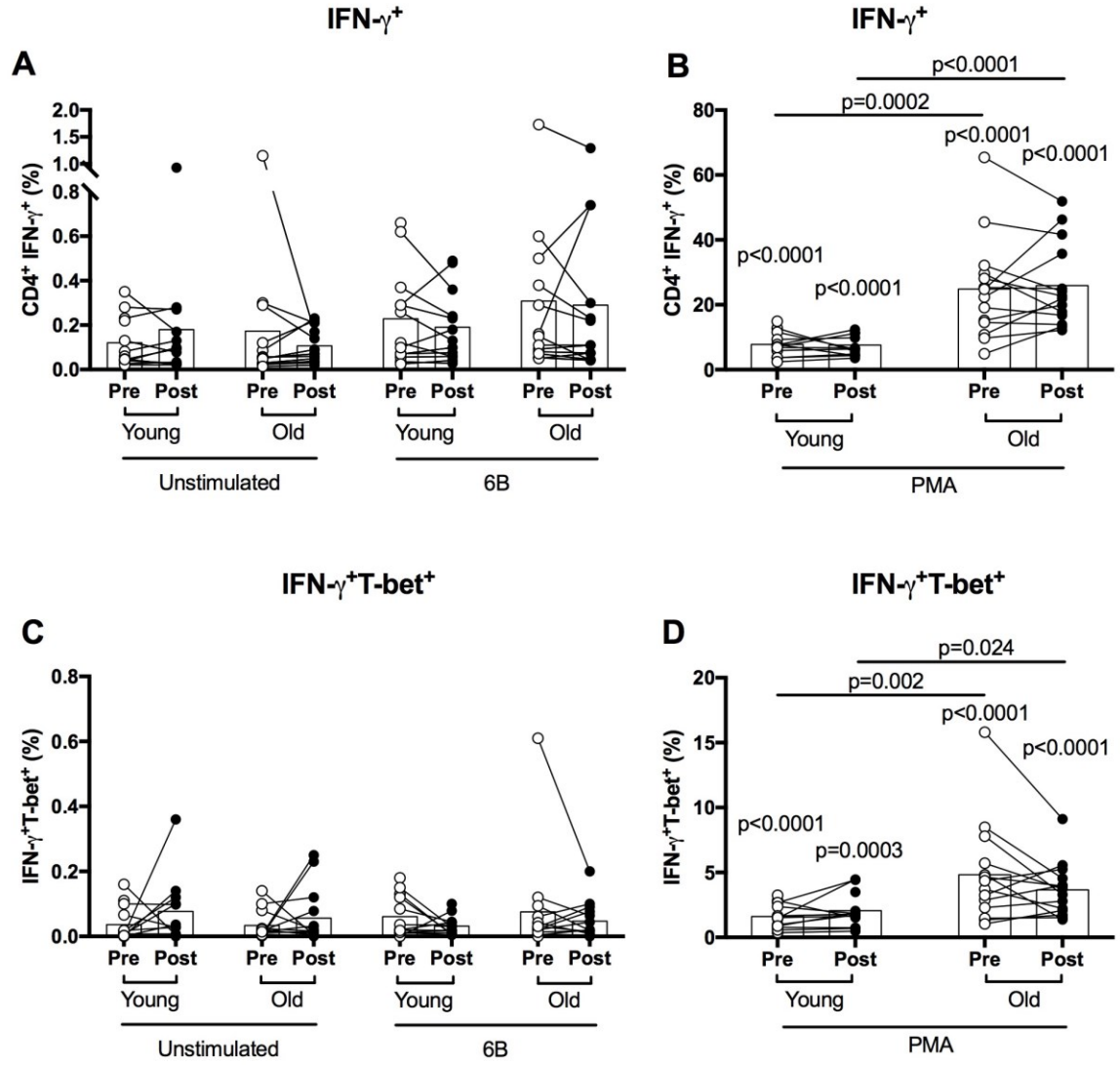

Figure 4.10. Frequency of IFN-γ$^+$ and IFN-γ$^+$T-bet$^+$ following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** IFN-γ$^+$ frequency and **(C)** IFN-γ$^+$T-bet$^+$ frequency in unstimulated and in *S. pneumoniae* 6B (5 µg/ml) treated cells. **(B)** IFN-γ$^+$ frequency and **(D)** IFN-γ$^+$T-bet$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test and differences between pre- and post-colonisation were analysed by Wilcoxon test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test and statistical differences with unstimulated control are shown on top of the bars.

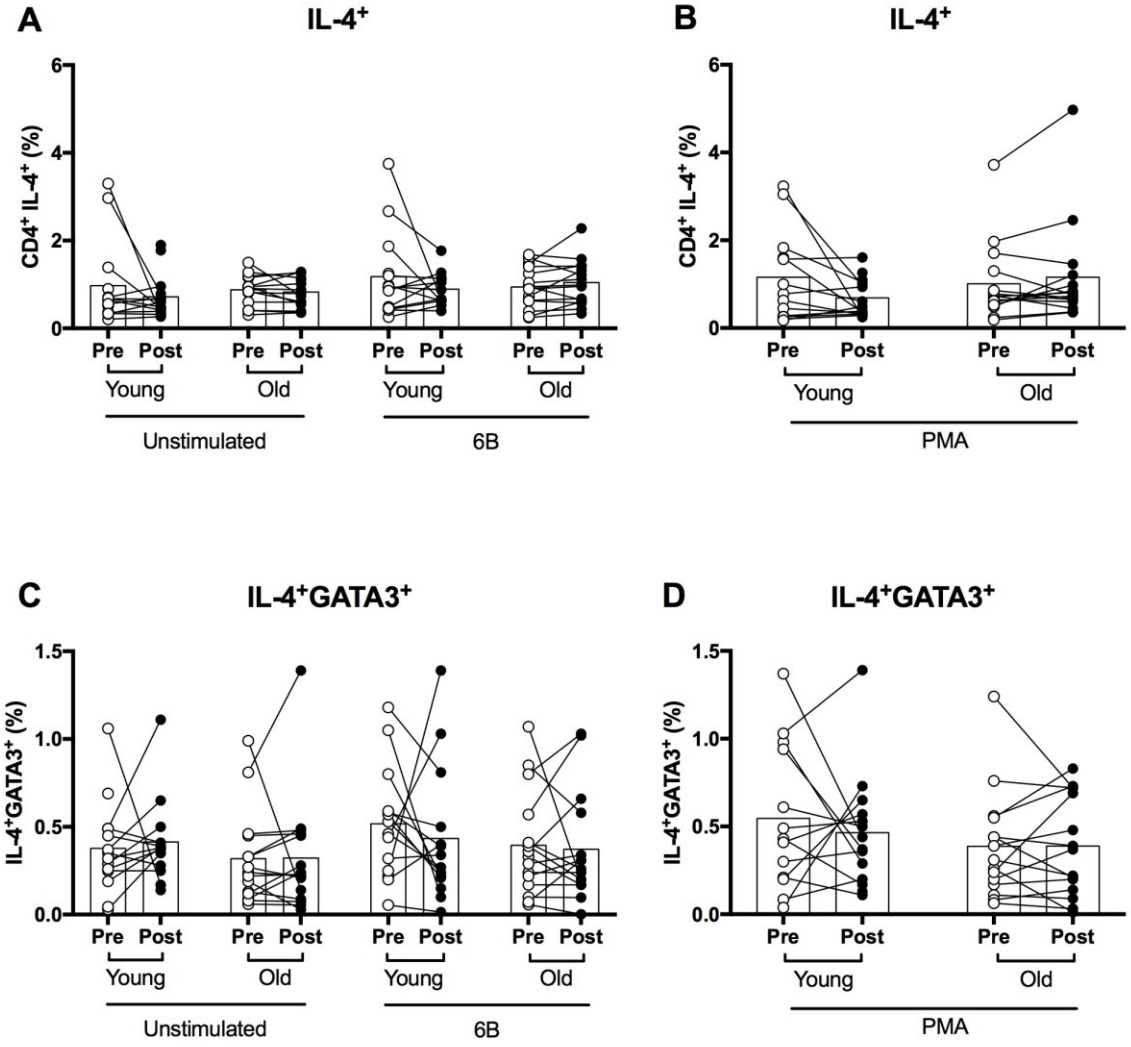

Figure 4.11. Frequency of IL-4$^+$ and IL-4$^+$GATA3$^+$ following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** IL-4$^+$ frequency and **(C)** IL-4$^+$GATA3$^+$ frequency in unstimulated cells and in *S. pneumoniae* 6B (5 µg/ml) treated cells. **(B)** IL-4$^+$ frequency and **(D)** IL-4$^+$GATA3$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test and differences between pre- and post-colonisation were analysed by Wilcoxon test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test.

Although the frequency of $CD3^+CD4^+IL\text{-}17A^+$ cells in unstimulated pre-colonisation samples from older adults was slightly higher than in the young, the difference was not statistically significant (Pre, HY = 0.065±0.049 vs HO = 0.087±0.048, p=0.204) (Figure 4.12 A). PMA stimulation successfully increased the frequency of both $IL\text{-}17A^+$ and $L\text{-}17A^+ROR\gamma t^+$ phenotypes in samples pre and post colonisation, compared to unstimulated controls of both age groups ($IL\text{-}17A^+$: pre, HY, p=0.0007; HO, p=0.051. Post, HY, p=0.0001; HO, p=0.011. $IL\text{-}17A^+ROR\gamma t^+$: pre, HY, p=0.013; HO, p=0.001. Post, HY, p<0.0001; HO, p=0.002) (Figures 4.12 B and D). PMA also increased the frequency of $IL\text{-}17A^+ROR\gamma t^+$ in post-colonisation samples from young donors but failed to do so in older adults (p=0.018) (Figure 4.12 D). As a consequence, young volunteers had a trend towards an increase in frequency of $IL\text{-}17A^+ROR\gamma t^+$ cells post colonisation, a trend not observed in older participants (HY: pre = 0.181±0.218 vs post = 0.306±0.24, p=0.064. HO: pre = 0.168±0.099 vs post = 0.15±0.114, p=0.534) (Figure 4.12 D). While 6B stimulation did not change the frequency of $IL\text{-}17A^+$ or $IL\text{-}17A^+ROR\gamma t^+$ cells (Figures 4.12 A and C), it significantly increased the frequency of activated Tregs ($CD127^-CD25^+Foxp3^+GARP^+$) in pre and post colonisation samples from both age groups, compared to unstimulated controls (pre, HY, p=0.0002; HO, p<0.0001. Post, HY, p<0.0001; HO, p<0.0001) (Figures 4.13 B), whereas the frequency of Tregs ($CD127^-CD25^+Foxp3^+$) remained unaltered in both age groups in unstimulated and 6B-stimulated samples (Figures 4.13 A).

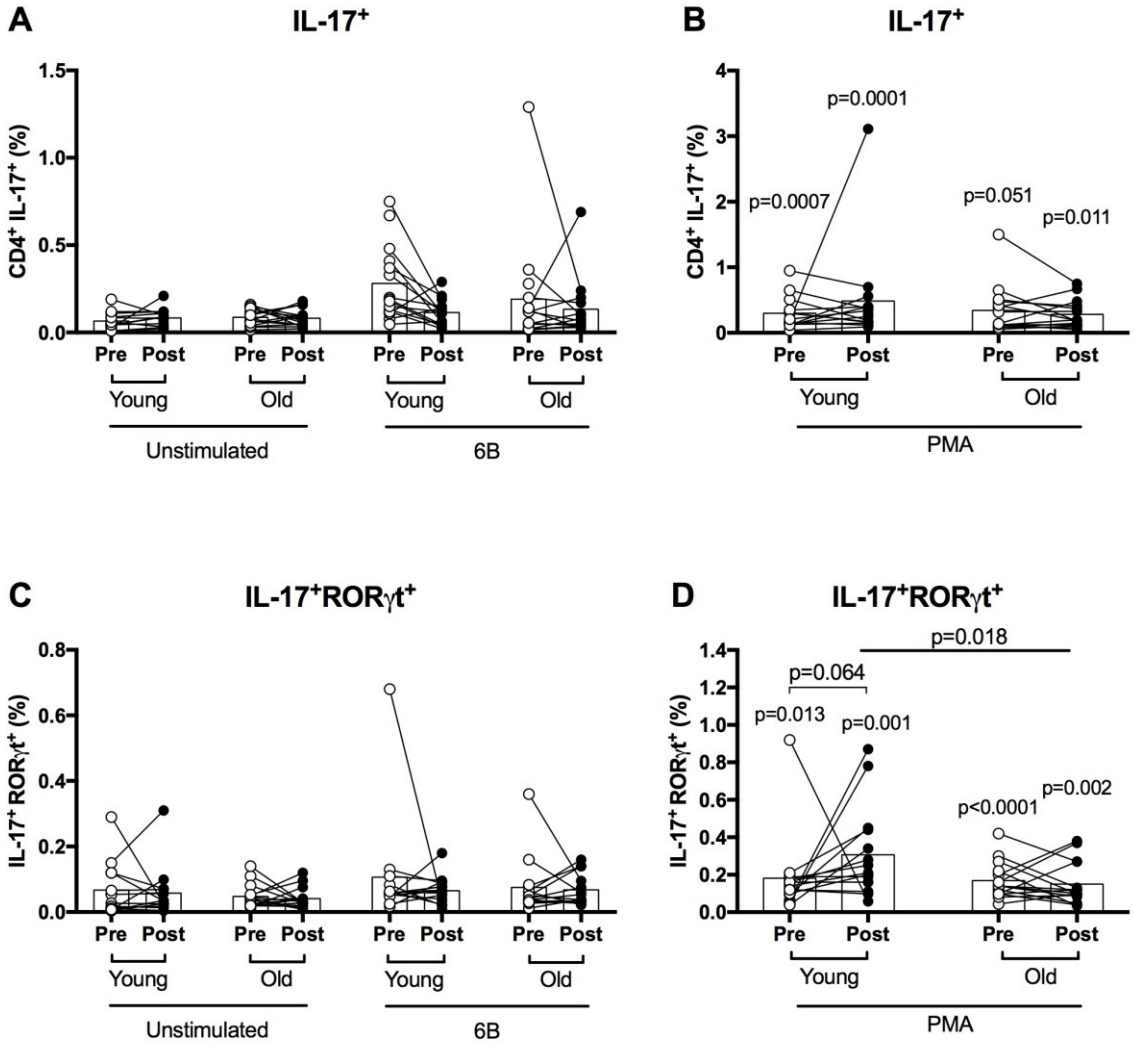

Figure 4.12. Frequency of IL-17A$^+$ and IL-17A$^+$RORγt$^+$ following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** IL-17A$^+$ frequency and **(C)** IL-17A$^+$RORγt$^+$ frequency in unstimulated and in *S. pneumoniae* 6B (5 µg/ml) treated cells. **(B)** IL-17A$^+$ frequency and **(D)** IL-17A$^+$RORγt$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Differences between age groups were analysed by Mann-Whitney test and differences between pre- and post-colonisation were analysed by Wilcoxon test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test and statistical differences with unstimulated control are shown on top of the bars.

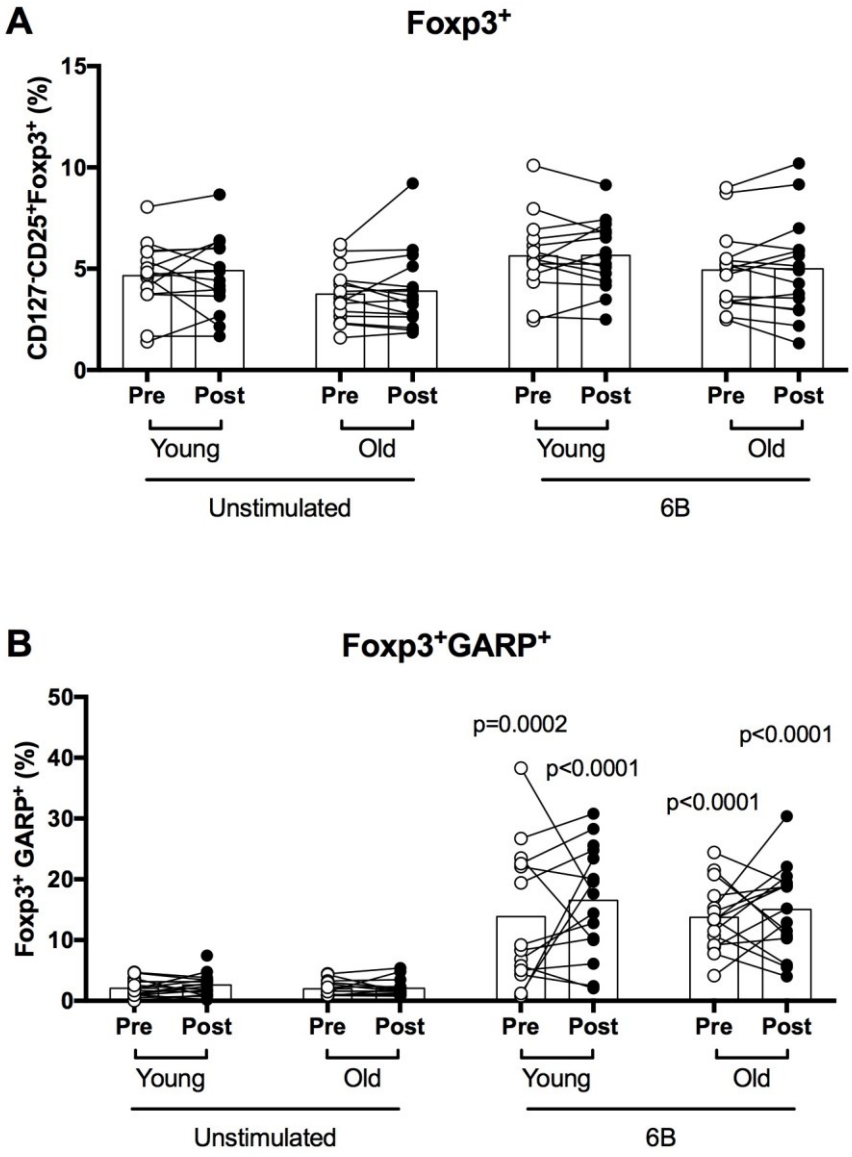

Figure 4.13. Frequency of Foxp3$^+$ and Foxp3$^+$GARP$^+$ following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** Foxp3$^+$ frequency and **(B)** Foxp3$^+$GARP$^+$ frequency in unstimulated cells and in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Unpaired T test and differences between pre- and post-colonisation were analysed by Paired T test. Differences between treatments were analysed by paired T test and statistical differences with unstimulated control are shown on top of the bars.

Age-related changes were also observed in the naïve and memory $CD4^+$ T cell compartments. As expected, older volunteers showed lower frequency of naïve $CD4^+$ T ($CD45RA^+CCR7^+$) cells in both unstimulated (Pre, p=0.037; post, p=0.0003), PMA (Pre, p<0.0001; post, p=0.0001) and 6B-stimulated samples (Pre, p=0.023; post, p=0.004) (Figures 4.14 A). PMA-treatment also significantly expanded the naïve subset in young participants, in samples from both time points, compared to unstimulated controls (Pre, p=0.021; post, p=0.030) (Figure 4.14 A). Conversely, older volunteers had an increased frequency of highly differentiated $CD4^+$T memory cells. Frequency of $CD4^+$ T_{CM} cells (central memory, $CD45RA^-CCR7^+$) was increased in pre and post samples of older participants in unstimulated (Pre, p=0.004; post, p=0.009), PMA (Pre, p=0.0003; post, p=0.0005) and 6B-stimulated samples (Pre, p=0.018; post, p=0.018) (Figures 4.14 B). $CD4^+$ T_{EM} cells (effector memory, $CD45RA^-CCR7^-$) were only higher in older donors following PMA treatment (Pre, p=0.003; post, p=0.0004) (Figure 4.15 A). Also, young volunteers showed reduction of $CD4^+$ T_{EM} frequency at post colonisation (p=0.49), following 6B stimulation, whereas this change was not found in older donors (HY: pre = 18.36±10.2 vs post = 15.32±7.673, p=0.049. HO: pre = 19.95±13.65 vs post = 19.78±13.33, p=0.625) (Figure 4.15 A). This could be associated with the increased frequency of naïve $CD4^+$T found in young subjects (Figure 4.14 A). The frequency of $CD4^+$ T_{EMRA} cells ($CD45RA^+CCR7^+$), such as observed with $CD4^+$ T_{CM}, was elevated in older participants at both time points, compared to young subjects, in unstimulated (Pre, p=0.013; post, p=0.005), treatments with 6B (Pre, p=0.012; post, p=0.005) or PMA (Pre, p=0.005; post, p=0.0002) (Figures 4.16 B). PMA treatment also significantly increased frequency of $CD4^+$ T_{EMRA} cells compared to unstimulated controls (pre, HY, p=0.0001; HO, p=0.002. Post, HY, p<0.0001; HO, p=0.0003) (Figures 4.16 B).

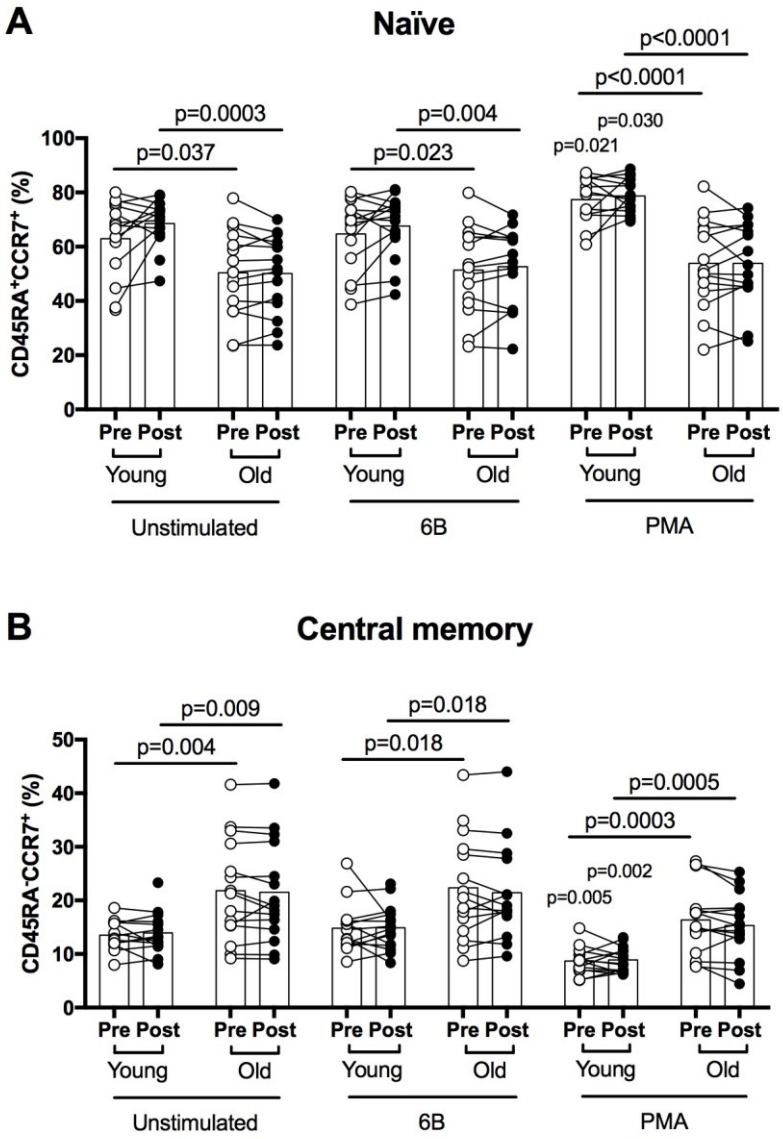

Figure 4.14. Frequency of naïve and central memory CD4$^+$ T cell following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs ($4x10^5$ cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** naïve cell frequency and **(B)** central memory cell frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) treated cells or PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Unpaired T test and differences between pre- and post-colonisation were analysed by Paired T test. Differences between treatments were analysed by One-Way ANOVA test with Tuckey's post hoc test and statistical differences with unstimulated control are shown on top of the bars.

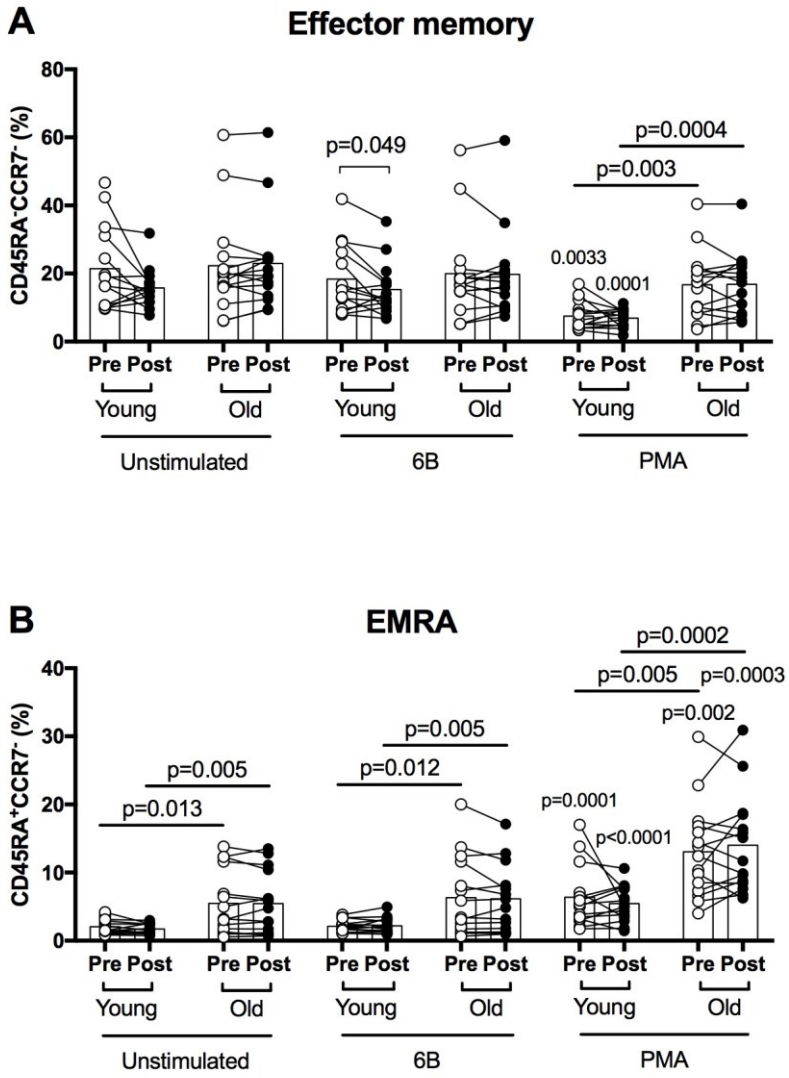

Figure 4.15. Frequency of effector memory and EMRA CD4$^+$ T cell following *in vitro* stimulation before and after experimental pneumococcal colonisation in young and old volunteers. PBMCs ($4x10^5$ cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stained for flow cytometry analysis of **(A)** effector memory cell frequency and **(B)** EMRA cell frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) treated cells or PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Data are expressed as mean ± SD. Differences between age groups were analysed by Unpaired T test and differences between pre- and post-colonisation were analysed by paired T test. Differences between treatments were analysed by One-Way ANOVA test with Tuckey's post hoc test and statistical differences with unstimulated control are shown on top of the bars.

Overall, ageing promoted changes in pro-inflammatory subsets, with increased frequencies of IFN$^+$ cells, but a decline in IL-17A$^+$RORγt$^+$ cells after experimental colonisation, while frequency of 6B-responding GARP$^+$Tregs remained elevated with ageing.

4.3.4.2 Effect of age on CD4$^+$ T cell polarisation in carriage-positive and negative volunteers

To further investigate the effect of ageing on CD4$^+$ T cell polarisation following experimental pneumococcal carriage, data from donors was divided into carriage-positive and carriage-negative, given by detection of 6B serotype in nasal samples collected at day 7 post inoculation.

As described above, no statistical differences were found between pre and post colonisation samples in the majority of CD4$^+$T cell subsets. Here are shown data from PMA and 6B-stimulated PBMCs from post-colonisation samples. Figures 4.16 A and B show that the frequency of IFN-γ$^+$ and IFN-γ$^+$T-bet$^+$ in PMA-treated PBMCs remained higher in older volunteers than in young subjects independent of carriage status (IFN-γ$^+$: carriage-Neg, p=0.0006; carriage-Pos, p=0.001. IFN-γ$^+$T-bet$^+$: carriage-Pos, p=0.022). Possibly due to the smal sample size, no statistical differences in frequency of IFN-γ$^+$ subsets were detected in 6B-stimulated PBMCs (Figures 4.16 C and D). Cell frequency of IL-4$^+$ and IL-4$^+$GATA3$^+$ populations were not altered by carriage status in our cohort following stimulation of cells with PMA or 6B (Figure 4.17).

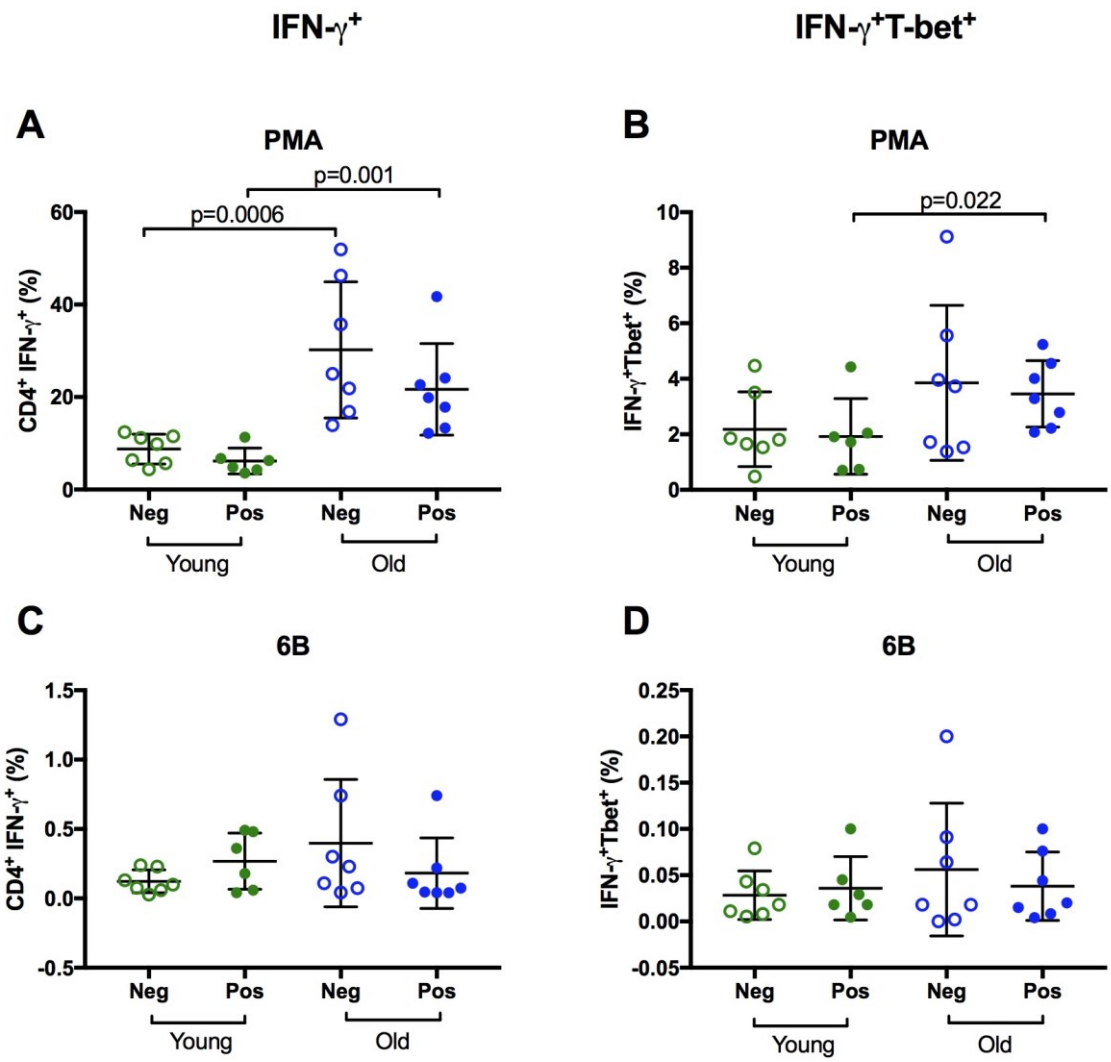

Figure 4.16. Frequency of IFN-γ$^+$ and IFN-γ$^+$T-bet$^+$ following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from post-colonisation were stimulated and analysed by flow cytometry. Data were divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** IFN-γ$^+$ frequency and **(B)** IFN-γ$^+$T-bet$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells; **(C)** IFN-γ$^+$ frequency and **(D)** IFN-γ$^+$T-bet$^+$ frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Neg = negative. Pos = positive.

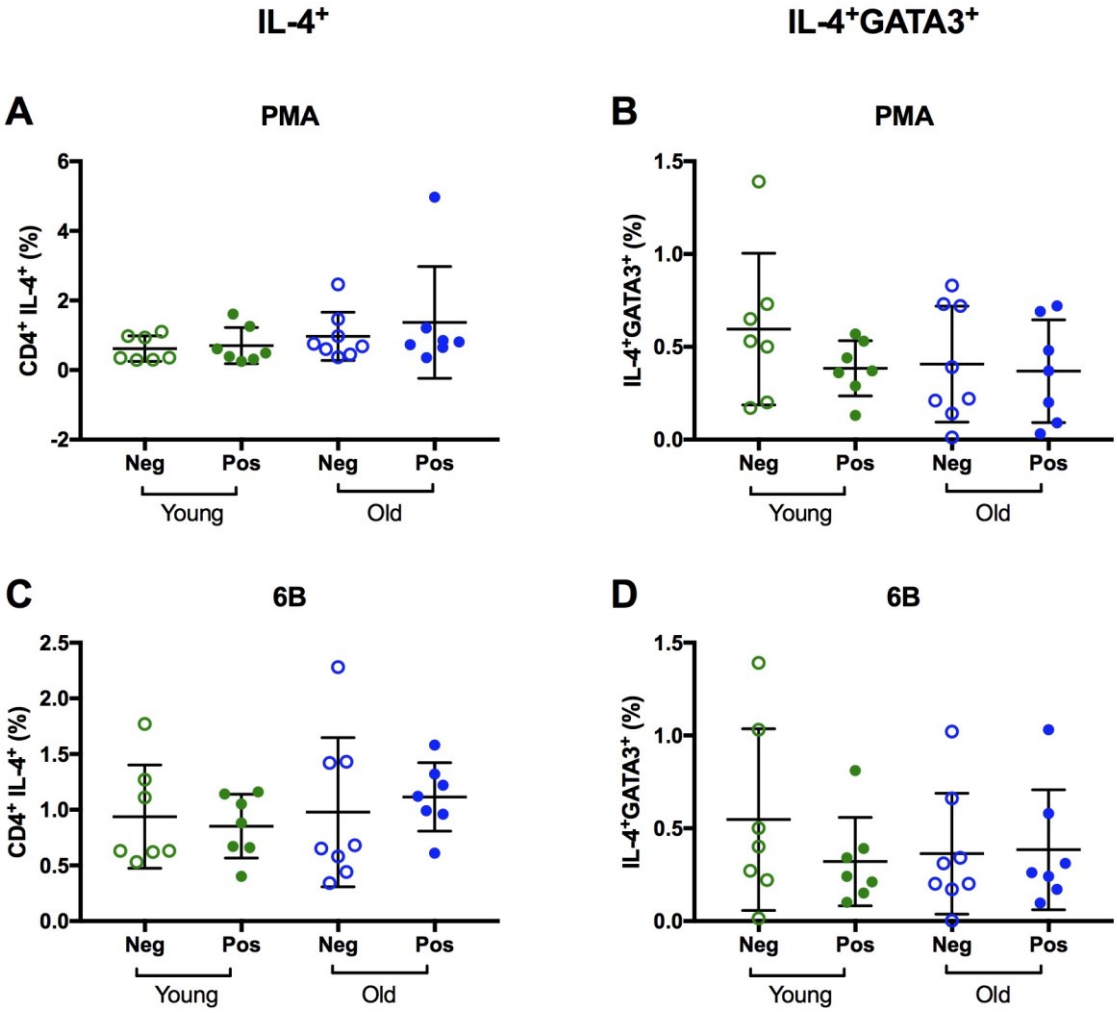

Figure 4.17. Frequency of IL-4$^+$ and IL-4$^+$GATA3$^+$ following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from post-colonisation were stimulated and analysed by flow cytometry. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** IL-4$^+$ frequency and **(B)** IL-4$^+$GATA3$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells; **(C)** IL-4$^+$ frequency and **(D)** IL-4$^+$GATA3$^+$ frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Neg = negative. Pos = positive.

Having found that young volunteers had higher frequency of IL-17A$^+$RORγt$^+$ post-colonisation upon *in vitro* PMA stimulation and that this trend was not found in the old (Figure 4.12 D), data from both time points were divided according to carriage status of the volunteer. In young and old, presence of carriage was not associated with changes in the frequency of IL-17A$^+$ cells following *in vitro* stimulation with PMA or 6B in our cohorts (Figures 4.18 A and C). However, in PMA-treated PBMCs IL-17A$^+$RORγt$^+$ frequency increased post-colonisation in carriage-negative young volunteers, compared to frequency at pre-colonisation (p=0.001) (Figure 4.18 B), and upon 6B re-stimulation, these volunteers had higher IL-17A$^+$RORγt$^+$ frequency than carriage-positive young post-colonisation (p=0.009) (Figure 4.18 D). Comparing carriage-negative volunteers, IL-17A$^+$RORγt$^+$ frequency was higher in young than in old donors (p=0.009) in PMA-stimulated cells, where frequency of this subset was not altered at post-colonisation (Figure 4.18 B), suggesting age-related changes in induction of IL-17A$^+$ cells during pneumococcal carriage.

To find out if these higher frequency of IL-17A$^+$RORγt$^+$ cells in carriage-negative participants were newly differentiated cells or memory cells generated by the colonisation event, based on the gating shown in Figure 4.9, IL-17A$^+$RORγt$^+$ cells from post-colonisation were analysed based on their expression of CD45RA and CCR7. Figure 4.19A shows that in PMA-stimulated cells, most IL-17A$^+$RORγt$^+$ of young donors are central memory cells, while there was no difference between 6B-stimulated cells (Figure 4.19 B). Moreover, carriage-negative young donors presented more IL-17A$^+$RORγt$^+$ in memory subsets than carriage-positive (EMRA, p=0.030; EF, p=0.014), although this difference was not significant for central memory IL-17A$^+$RORγt$^+$ cells (p=0.189). A larger experimental group could confirm this observation. In contrast, older individuals showed no such trends, as carriage-negative and positive older

donors showed no difference between frequency of IL-17A^{+}RORγt^{+} cells in any subset. Furthermore, between carriage-negative individuals, the young had higher frequency of central memory IL-17A^{+}RORγt^{+} cells than the old ($p=0.0003$), showing that experimental colonisation successfully generated immune memory in young individuals, and these cells could be associated with clearance of colonisation. Most importantly, in the old, this suggests that no induction of IL-17A^{+}RORγt^{+} memory was promoted during experimental colonisation, which could indicate that another cell type is mediating clearance of carriage. Furthermore, presence of lower frequency of IL-17A^{+}RORγt^{+} memory cells following colonisation in old adults contrasts with the higher frequency of total CD4^{+} T central, effector and EMRA memory cells found in both carriage-negative and positive older volunteers, compared to young (Figures 4.21 and 4.22).

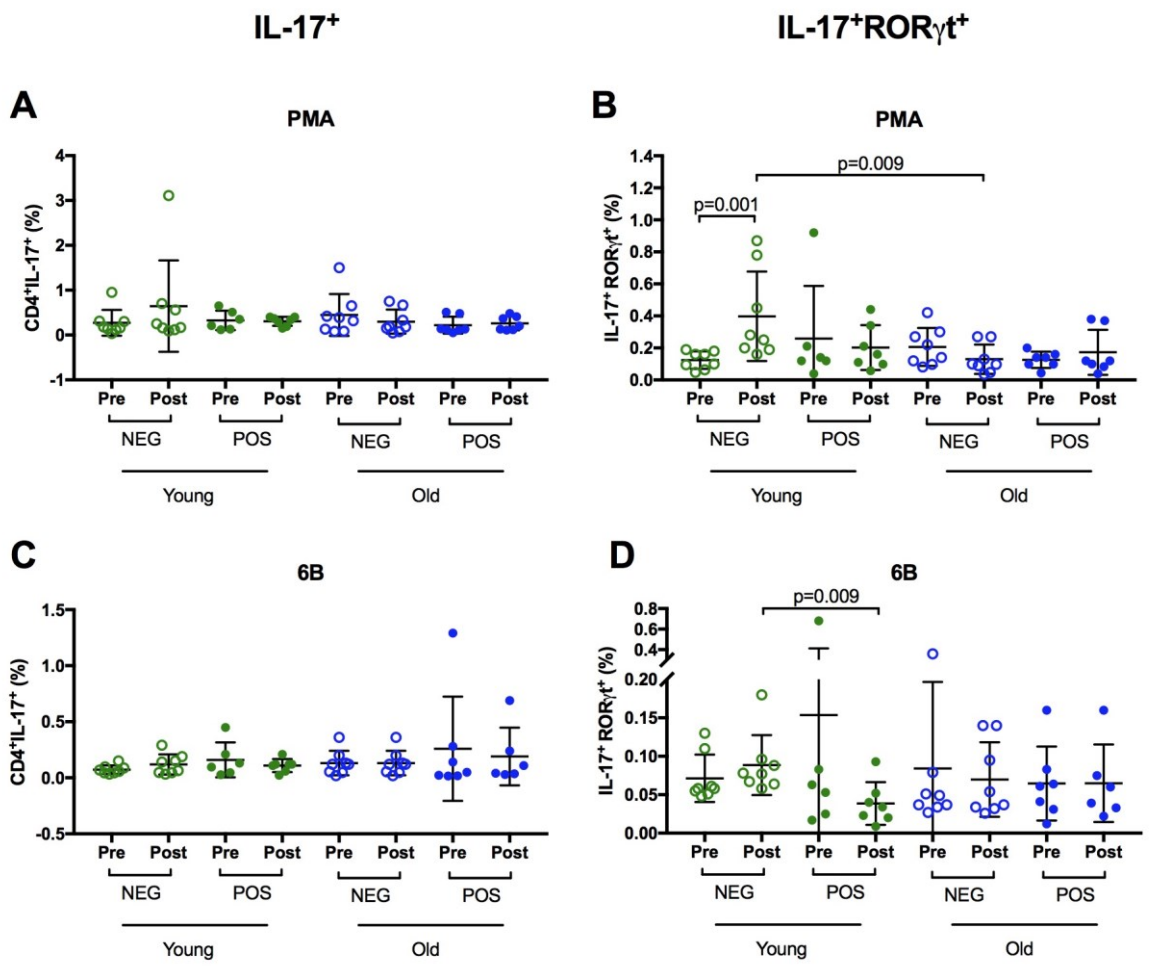

Figure 4.18. Frequency of IL-17A$^+$ and IL-17A$^+$RORγt$^+$ following *in vitro* stimulation in carriage-negative and carriage-positive volunteers before and after experimental pneumococcal colonisation. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from pre- and post-colonisation were stimulated and analysed by flow cytometry. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** IL-17A$^+$ frequency and **(B)** IL-17A$^+$RORγt$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells; **(C)** L-17A$^+$ frequency and **(D)** IL-17A$^+$RORγt$^+$ frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Differences between pre- and post-colonisation were analysed by Wilcoxon test. Neg = negative. Pos = positive

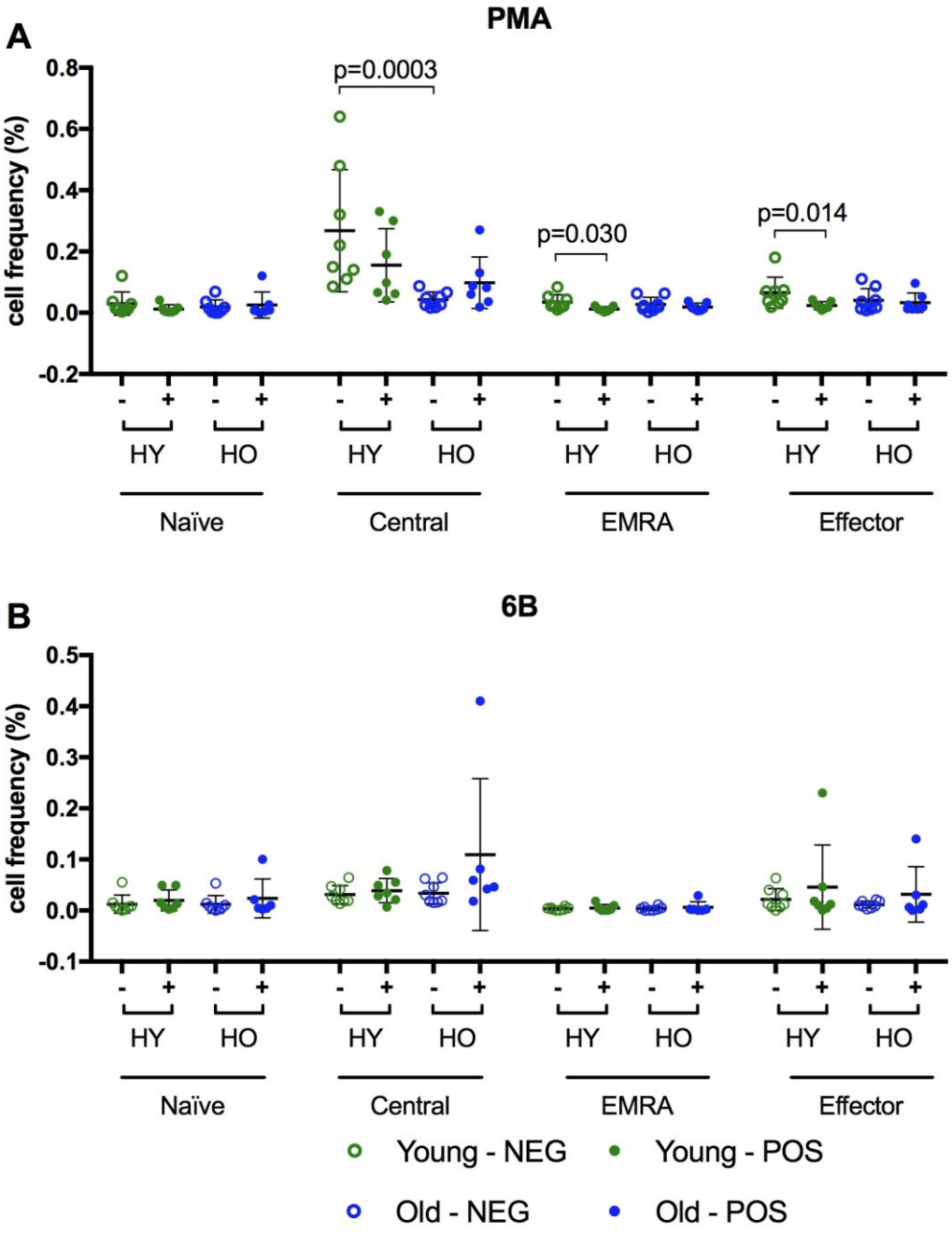

Figure 4.19. Frequency of naïve and memory IL-17A$^+$RORγt$^+$ cells following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. Frequency of IL-17A$^+$RORγt$^+$ cells were divided based on the expression of CD45RA and CCR7, for identification of naïve and memory cells. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). Frequency of IL-17A$^+$RORγt$^+$ stimulated with **(A)** PMA or **(B)** *S. pneumoniae* 6B. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. (**–**) = carriage-negative. (**+**) = carriage-positive, HY = healthy young. HO = healthy old.

When analysing the Treg population, there were no statistical differences between frequency of Tregs between carriage status in both age groups nor $GARP^{+}$Tregs (Figure 4.20 A and B). There was a trend that carriage-positive young donors had higher frequency of $GARP^{+}$Tregs, while this was not observed in carriage-positive older adults. A larger sample size could confirm these observations.

A study has reported that the ratio of Th17/Treg frequencies found in adenotonsillar tissue is increased with age [304] and ratios of Th17/Treg are reported to be lower in carriage-positive children compared to carriage-negative [304]. Here, there was no correlation between advancing age and Th17 frequency (in unstimulated cells R^2=0.002, p=0.80) or Treg frequency (R^2=0.024, p=0.40) in post colonisation samples and also no effect of carriage in older donors on the Th17/Treg ratio (carriage-NEG, R^2=0.23, p=0.21; carriage-POS R^2=0.008, p=0.86).

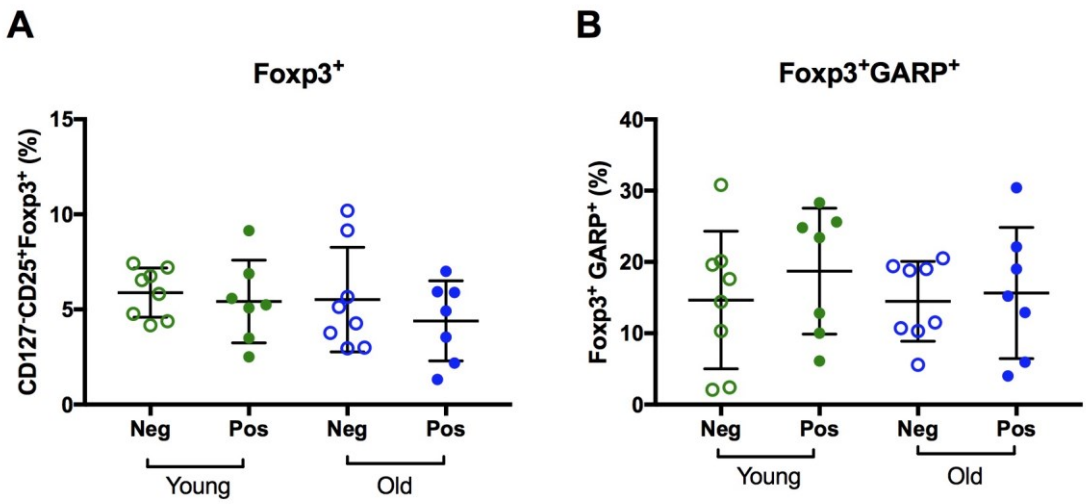

Figure 4.20. Frequency of Foxp3$^+$ and Foxp3$^+$GARP$^+$ following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. PBMCs ($4x10^5$ cells) of young (n=15) and old (n=15) volunteers from post-colonisation were stimulated and analysed by flow cytometry. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** Foxp3$^+$ frequency and **(B)** Foxp3$^+$GARP$^+$ frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Neg = negative. Pos = positive

In the naïve and central memory $CD4^+$ T cell compartments, independent of carriage status and stimulation, the frequency of naïve cells was reduced in old donors, compared to young, (PMA: carriage-NEG, p=0.002; carriage-POS, p=0.001. 6B: carriage-NEG, p=0.065; carriage-POS, p=0.022) (Figure 4.21 A and C), while frequency of $CD4^+$ central memory cell was increased in older donors, compared to young (PMA: carriage-NEG, p=0.015; carriage-POS, p=0.026. 6B: carriage-NEG, p=0.060) (Figure 4.21 B and D).

The frequency of effector memory cells and EMRA were also elevated in the old, EMRA cells were reduced in carriage-positive young, compared to donors in which carriage was absent (PMA, p=0.040) (Figure 4.22 A and C, B and D). While the same trend was observed in the old, it was not statistically significant. This suggests that establishment of immune memory is present in carriage-negative older adults but may be reduced in carriage-positive.

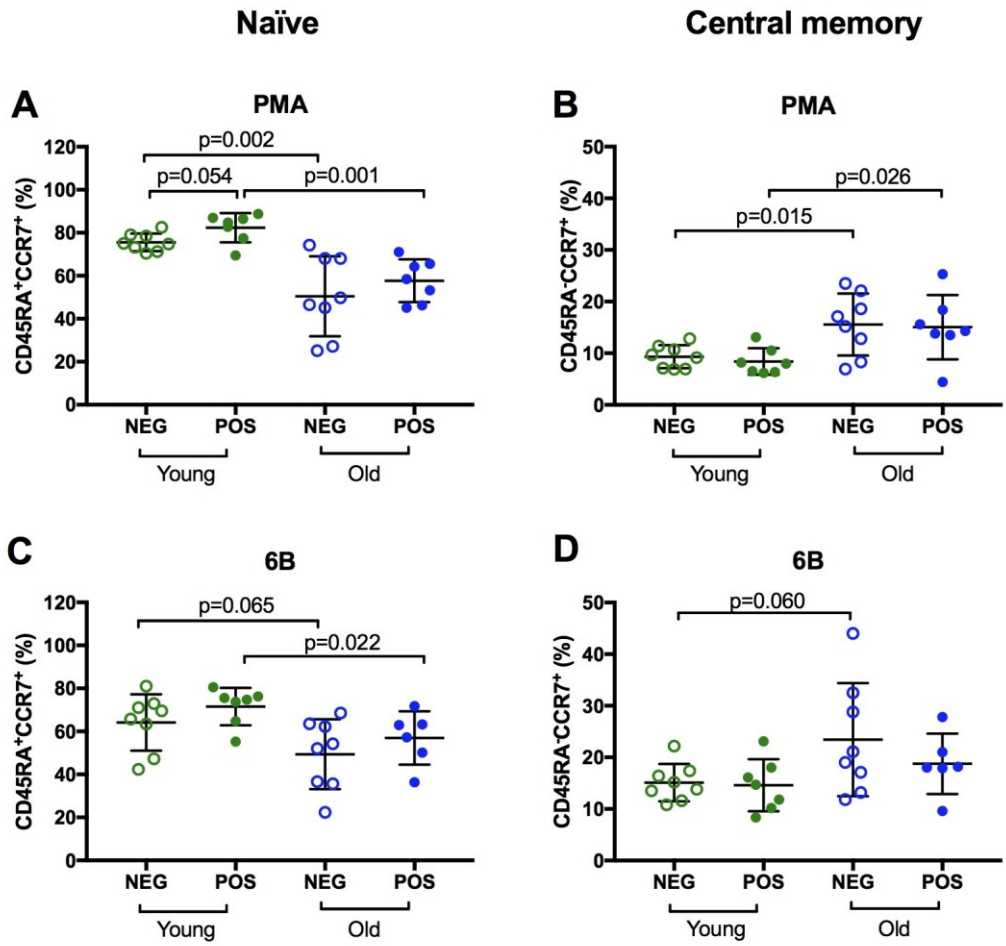

Figure 4.21. Frequency of naïve and central memory CD4$^+$ T cells following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from post-colonisation were stimulated and analysed by flow cytometry. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** naïve cell frequency and **(B)** central memory cell frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells; **(C)** naïve cell frequency and **(D)** central memory cell frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Neg = negative. Pos = positive.

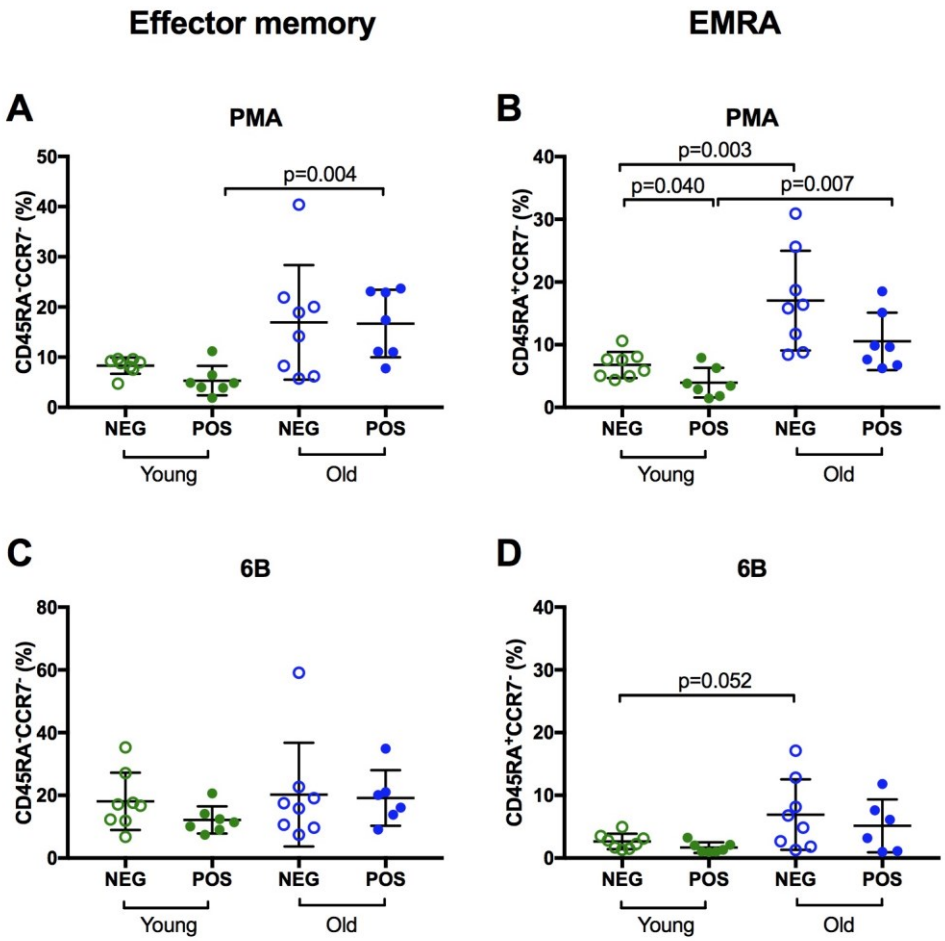

Figure 4.22. Frequency of effector memory and EMRA CD4$^+$ T cells following *in vitro* stimulation in carriage-negative and carriage-positive volunteers after experimental pneumococcal colonisation. PBMCs (4x10^5 cells) of young (n=15) and old (n=15) volunteers from post-colonisation were stimulated and analysed by flow cytometry. Data was divided based on carriage status of the volunteers (carriage-NEG, n=8; carriage-POS, n=7). **(A)** effector memory cell frequency and **(B)** EMRA cell frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells; **(C)** effector memory cell frequency and **(D)** EMRA cell frequency in *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups and carriage status were analysed by Mann-Whitney test. Neg = negative. Pos = positive.

4.3.5 $CD4^+$ T cell polarisation during pneumonia infection in older adults

In order to determine if older subjects had changes in $CD4^+$ T cell polarisation during pneumonia infection, PBMCs from patients were stimulated *in vitro* with PMA and ionomycin or with *S. pneumoniae* serotypes 4 (TIGR4) or 6B, aiming to induce serotype specific responses. Because no detailed investigation of the causative pathogen of pneumonia was performed in patients in this study, two pneumococcal serotypes were used. As controls, $CD4^+$ T cell data from pre-colonisation samples of healthy young and old participants of the EHPC cohort were used for comparison with data from pneumonia patients, as PBMCs were stimulated under the same conditions. Data from healthy young are plotted together on the same graphs to show the age-related differences that were described in section 4.3.4.1.

In contrast to the few age-related changes found in pre-colonisation PBMCS from healthy old donors (section 4.3.4.1), during pneumonia infection older patients had increased frequencies of Th1 and Th17 cells in the absence of *in vitro* stimulation. In older patients, frequency of IFN-γ^+ or IFN-γ^+T-bet$^+$ cells did not change following *in vitro* stimulation with TIGR4 or 6B, compared to unstimulated controls (Figures 4.23 A and C), but frequency of both phenotypes was elevated following PMA stimulation (IFN-γ^+, $p<0.0001$; IFN-γ^+T-bet$^+$, $p=0.0002$) (Figures 4.23 B and D). Moreover, following PMA stimulation, older patients had lower IFN-γ^+ frequency than healthy ($p=0.005$) (Figure 4.23 B), and the frequency of T-bet-expressing IFN-γ^+ cells showed a trend towards being increased in patients, in unstimulated ($p=0.096$) or PMA-treated, cells ($p=0.076$) (Figure 4.23 D). These data suggest that during disease, activation of T-bet increases in IFN-γ^+ cells and more $CD4^+$T cells commit to the Th1 subset.

Similar to these findings, during pneumonia IL-17A$^+$ frequency was also elevated in young and older patients, in both unstimulated (HO vs OP, p=0.004; HY vs YP, p=0.036) and PMA-stimulated PBMCs (HO vs OP, p=0.008; HY vs YP, p=0.003), compared to healthy controls (Figures 4.24 A and B). In young patients, IL-17A$^+$ frequency was increased following *in vitro* incubation with TIGR4 (p=0.009), and possibly 6B, but the difference was not statistically significant (Figure 4.24 A). Although no statistical difference was found between young and older patients, both patient groups had elevated frequency of IL-17A$^+$RORγt$^+$ cells upon PMA stimulation, compared to their healthy counterparts (HO vs OP, p=0.019; HY vs YP, p=0.063) (Figure 4.24 D). However, this subset seems to be increased in older patients, even in the absence of stimulation and following *S. pneumoniae* stimulation (Figure 4.24 C). Possibly due to CD4$^+$ T cell polarisation towards Th1 and Th17 subsets, during pneumonia in the old no changes were observed in the frequency of IL-4$^+$ and IL-4$^+$GATA3$^+$ cells (Figure 4.25).

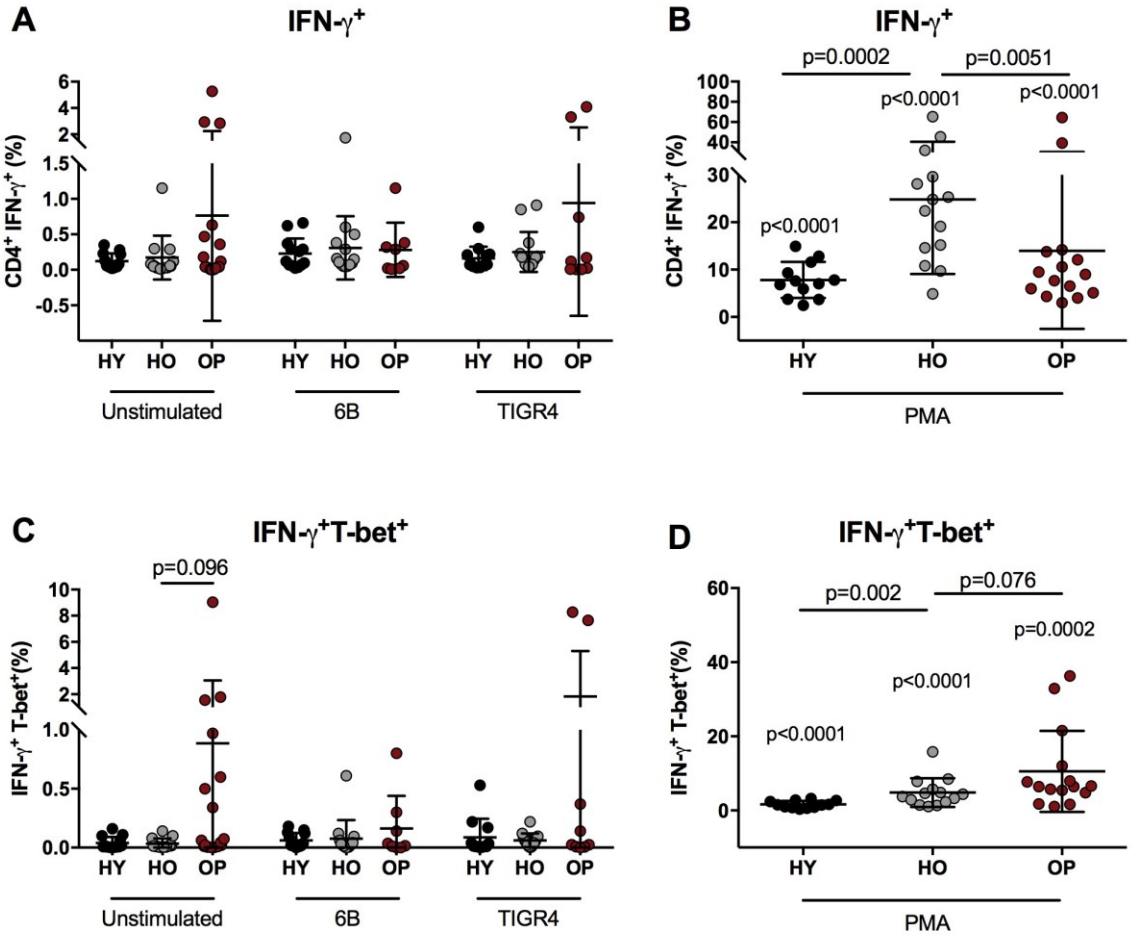

Figure 4.23. Frequency of IFN-γ$^+$ and IFN-γ$^+$T-bet$^+$ following *in vitro* stimulation of PMBCs from older adults, during healthy and pneumonia. PBMCs (4x10^5 cells) of healthy young (n=15), old (n=15) volunteers and old with pneumonia (n=17) were stimulated and analysed by flow cytometry. **(A)** IFN-γ$^+$ frequency and **(C)** IFN-γ$^+$T-bet$^+$ frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) or TIGR4 (MOI 10) treated cells. **(B)** IFN-γ$^+$ frequency and **(D)** IFN-γ$^+$T-bet$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test and statistical differences with unstimulated control are shown on top of the bars. HY = healthy young. HO = healthy old. OP = old with pneumonia.

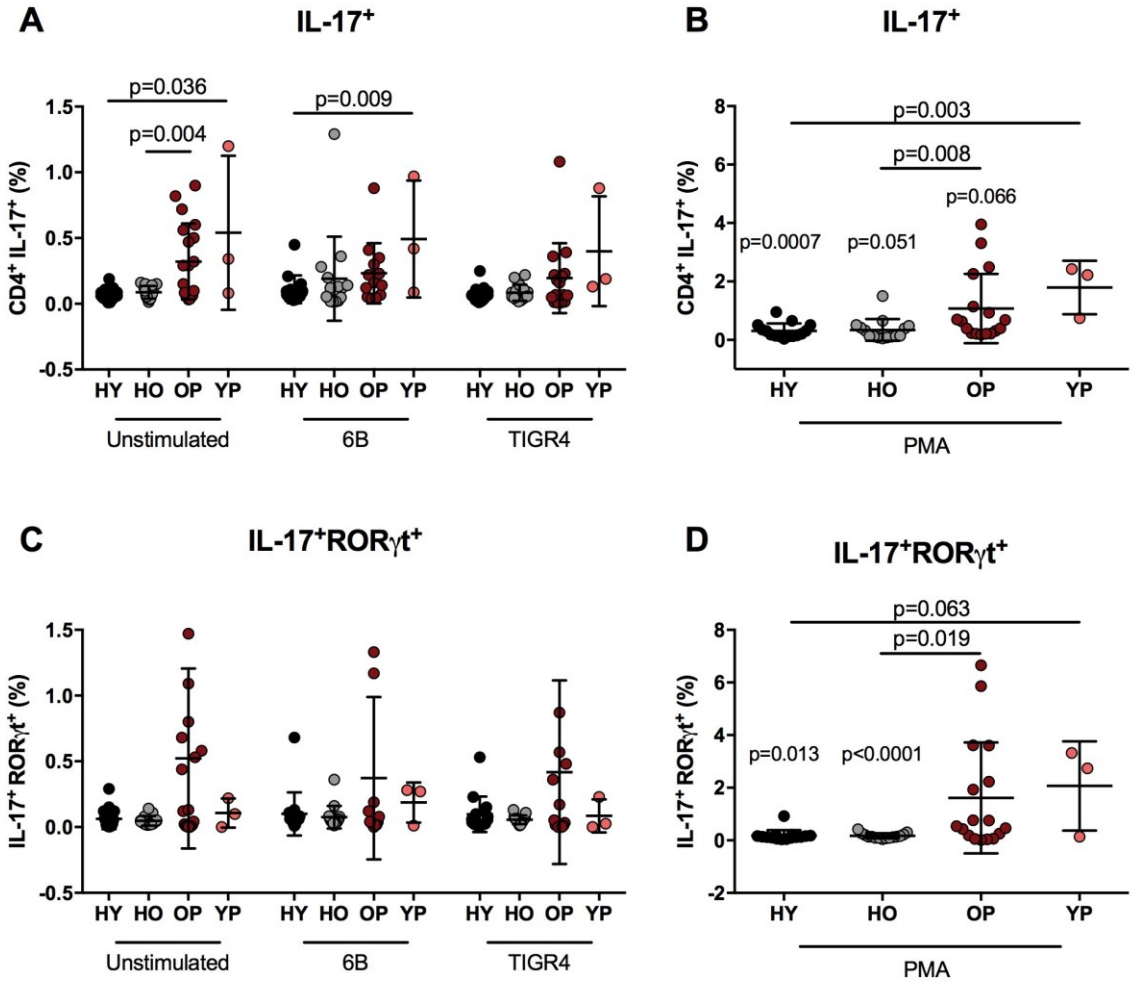

Figure 4.24. Frequency of IL-17A$^+$ and IL-17A$^+$RORγt$^+$ following *in vitro* stimulation in PBMCs from healthy volunteers and pneumonia patients. PBMCs (4x10^5 cells) of healthy young (n=15) and old (n=15) volunteers and young (n=3) and older adults (n=19) with pneumonia were stimulated and analysed by flow cytometry. **(A)** IL-17A$^+$ frequency and **(C)** IL-17A$^+$RORγt$^+$ frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) or TIGR4 (MOI 10) treated cells. **(B)** IL-17A$^+$ frequency and **(D)** IL-17A$^+$RORγt$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test and statistical differences with unstimulated control are shown on top of the bars. HY = healthy young, HO = healthy old. YP = young with pneumonia, OP = old with pneumonia.

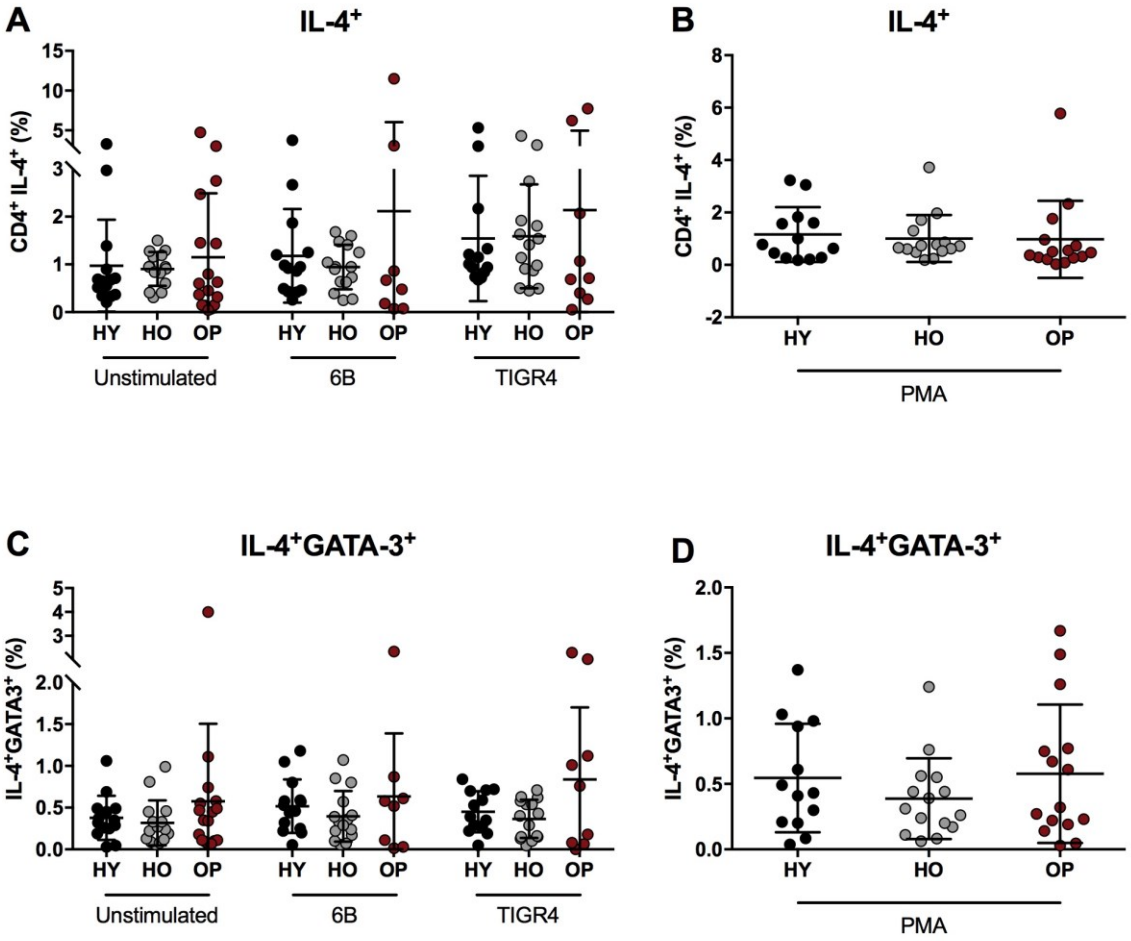

Figure 4.25. Frequency of IL-4$^+$ and IL-4$^+$GATA3$^+$ following *in vitro* stimulation of PMBCs from older adults, during healthy and pneumonia. PBMCs (4x10^5 cells) of healthy young (n=15), old (n=15) volunteers and old with pneumonia (n=17) were stimulated and analysed by flow cytometry. **(A)** IL-4$^+$ frequency and **(C)** IL-4$^+$GATA3$^+$ frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) or TIGR4 (MOI 10) treated cells. **(B)** IL-4$^+$ frequency and **(D)** IL-4$^+$GATA3$^+$ frequency in PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test. HY = healthy young. HO = healthy old. OP = old with pneumonia.

There were also no changes in the frequency of Tregs in pneumonia patients in unstimulated and TIGR4-stimulated PBMCs, but older patients had a trend towards reduced Treg frequency following 6B stimulation (p=0.054) (Figure 4.26 A). Frequency of activated Tregs ($GARP^+$) was increased in both patient cohorts at baseline, unstimulated cells (HO vs OP p=0.033; HY vs YP, p=0.002) (Figure 4.26 B). Stimulation with TIGR4 did not changes in frequency of activated Tregs from both healthy volunteers and patient, however, upon 6B stimulation, $GARP^+$Tregs frequency was reduced in older patients (p=0.040) but increased in young (p=0.006), compared to their healthy counterparts (Figure 4.26 B). Moreover, young patients had a higher $GARP^+$Tregs frequency than old (p=0.022) (Figure 4.26 B), suggesting that activation of Tregs could be altered in older individuals during pneumonia infection. Using data from the small sub-group of older patients that had IL-33 measured in serum, a correlation analysis showed a positive association between high levels of serum IL-33 (section 3.3.6) and increased frequency of $GARP^+$Tregs in old patients (R^2=0.84, p=0.003) (Figure 4.26 C).

The frequency of naïve $CD4^+$ T cells remained unaltered in PBMCs from pneumonia patients following in *in vitro* stimulation (Figure 4.27 A). However, the frequency of $CD4^+T_{CM}$ cells in older patients was increased compared to healthy subjects, in all conditions tested (unstimulated, p=0.030; PMA, p=0.027; TIGR4, p=0.041; 6B, p=0.025) (Figure 4.27 B). The same was observed in young patients, compared to healthy, but only for two conditions (unstimulated, p=0.045; PMA, p=0.011) (Figure 4.27 B). There were also no changes in the frequency of $CD4^+T_{EM}$ cells during pneumonia infection, as frequencies remained comparable in the presence or absence of *in vitro* stimulation between the cohorts (Figure

4.28 A). The only exception was that young patients had higher frequency of $CD4^+T_{EM}$ cells than healthy controls, following PMA treatment (p=0.048) (Figure 4.28 A). Also, during pneumonia, patients had a lower frequency of $CD4^+T_{EMRA}$ cells compared to healthy individuals (HO vs OP, unstimulated: p=0.0008; TIGR4, p=0.010; 6B, p<0.0001. HY vs YP, 6B: p=0.026) (Figure 4.28 B), likely due to the expansion of the $CD4^+T_{CM}$ subset, as observed in Figure 4.27 B.

Having detected higher frequencies of $CD4^+$IL-$17A^+$ cells in patients, the distribution of these cells in the naïve and memory phenotypes was checked. Although there were no statistical differences between the subsets, Figure 4.29 shows that in the presence and absence of stimulations, most of the IL-$17A^+$ cells were naïve cells. However, young patients showed a trend towards higher frequency of memory IL-$17A^+$ cells following stimulation with both *S. pneumoniae* serotypes than older adults (Figure 4.29 C and D), suggesting that vaccination or previous carriage episodes in the old could have failed to generate immune memory in the older cohort.

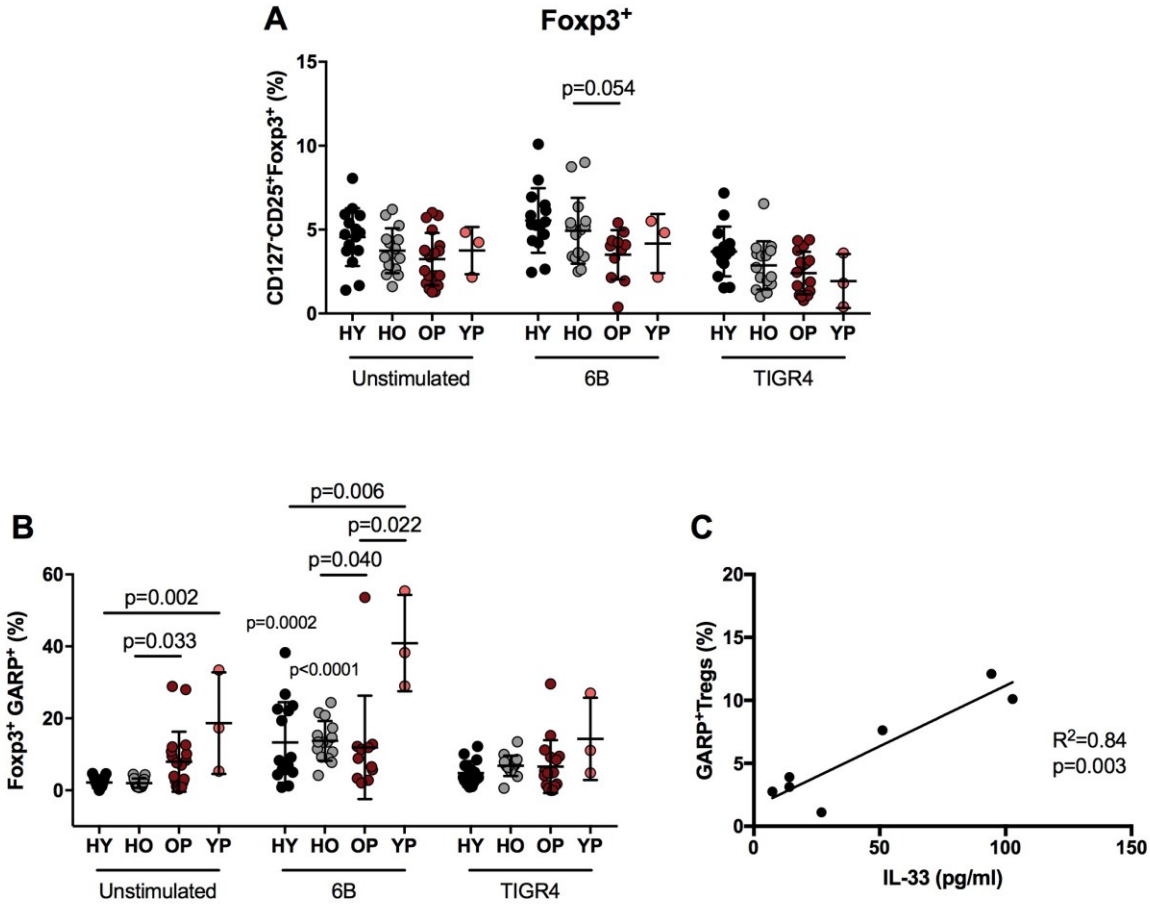

Figure 4.26. Frequency of Foxp3$^+$ and Foxp3$^+$GARP$^+$ following *in vitro* stimulation in PBMCs from healthy volunteers and pneumonia patients. PBMCs (4x10^5 cells) of healthy young (n=15) and old (n=15) volunteers and young (n=3) or older adults (n=19) with pneumonia were stimulated and analysed by flow cytometry. **(A)** Foxp3$^+$ frequency and **(B)** Foxp3$^+$GARP$^+$ frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml) or TIGR4 (MOI 10) treated cells. **(C)** Correlation between GARP$^+$Treg frequency and serum IL-33 in older patients (n=7). Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc test. HY = healthy young, HO = healthy old. YP = young with pneumonia, OP = old with pneumonia, YP = young with pneumonia.

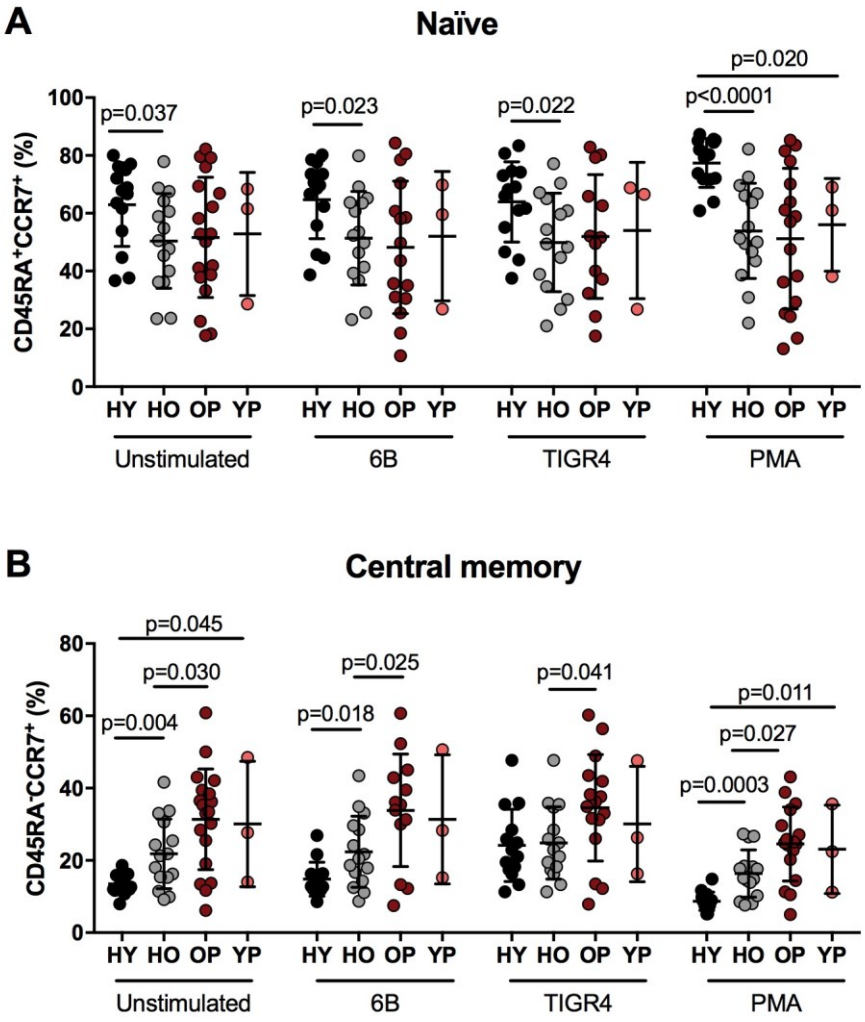

Figure 4.27. Frequency of naïve and central memory CD4$^+$ T cells following *in vitro* stimulation in PBMCs from healthy volunteers and pneumonia patients. PBMCs (4x10^5 cells) of healthy young (n=15) and older (n=15) volunteers and young (n=3) or older adults (n=19) with pneumonia were stimulated and analysed by flow cytometry. **(A)** naïve cell frequency and **(B)** central memory cell frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml), TIGR4 (MOI 10) treated cells or PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Unpaired T test and differences between pre- and post-colonisation were analysed by Paired T test. Differences between treatments were analysed by One-Way ANOVA test with Tuckey's post hoc test and statistical differences with unstimulated control are shown on top of the bars. HY = healthy young, HO = healthy old. YP = young with pneumonia, OP = old with pneumonia, YP = young with pneumonia.

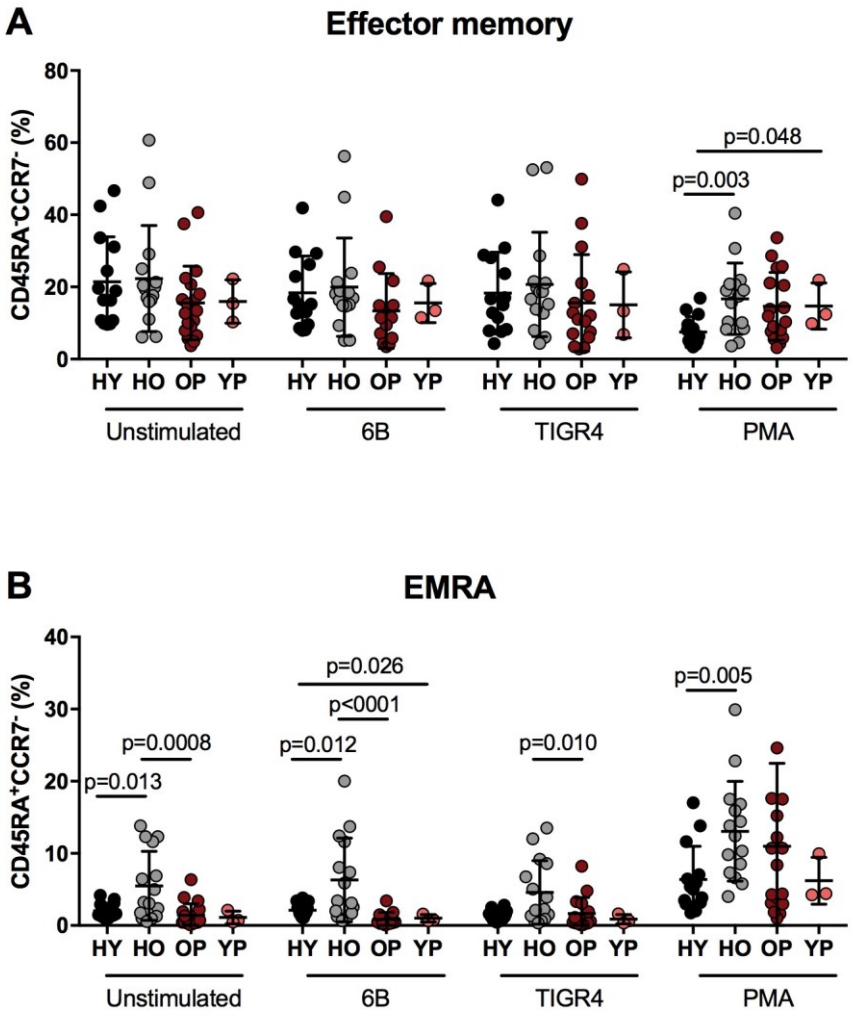

Figure 4.28. Frequency of effector memory and EMRA CD4$^+$ T cells following *in vitro* stimulation in PBMCs from healthy volunteers and pneumonia patients. PBMCs (4x10^5 cells) of healthy young (n=15) and old (n=15) volunteers and young (n=3) or older adults (n=19) with pneumonia were stimulated and analysed by flow cytometry. **(A)** effector memory cell frequency and **(B)** EMRA cell frequency in unstimulated, *S. pneumoniae* 6B (5 µg/ml), TIGR4 (MOI 10) treated cells or PMA (10 ng/ml) and ionomycin (50 ng/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Unpaired T test and differences between pre- and post-colonisation were analysed by Paired T test. Differences between treatments were analysed by One-Way ANOVA test with Tuckey's post hoc test and statistical differences with unstimulated control are shown on top of the bars. HY = healthy young, HO = healthy old. YP = young with pneumonia, OP = old with pneumonia, YP = young with pneumonia.

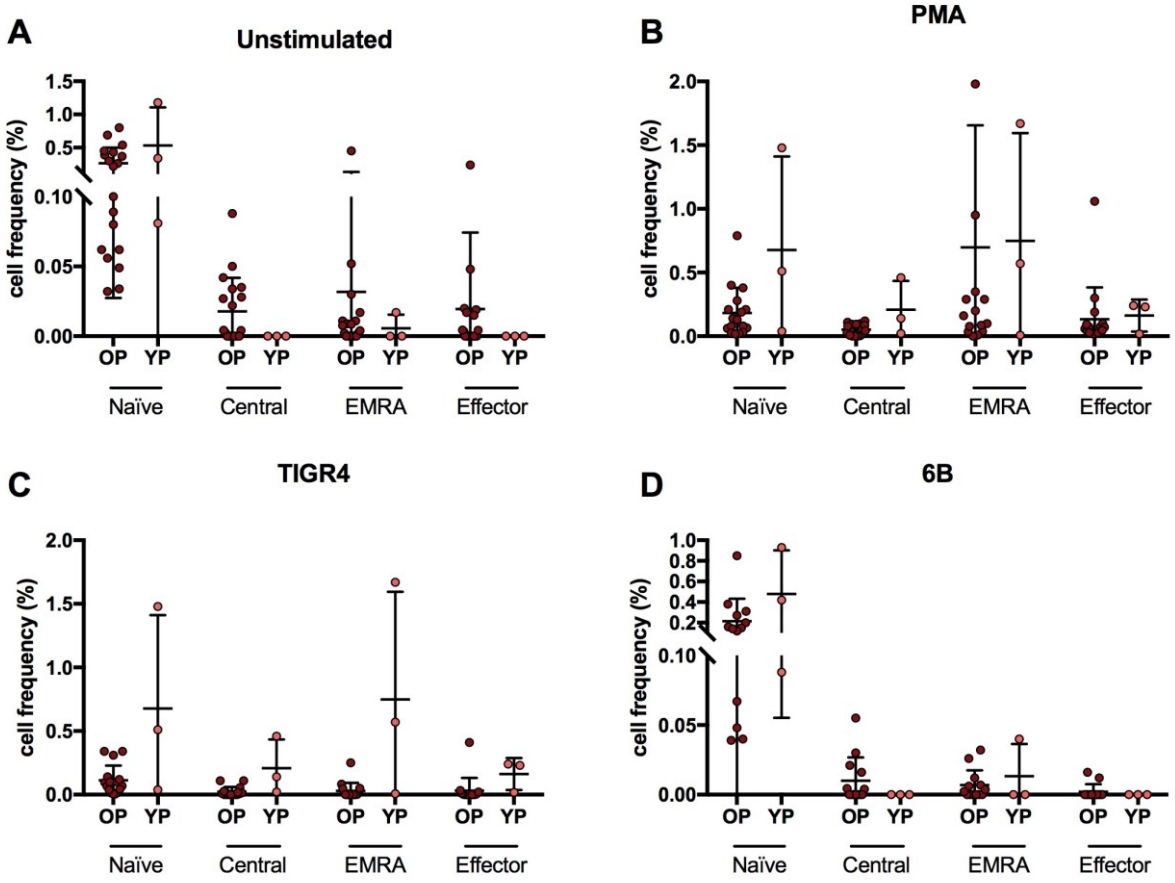

Figure 4.29. Frequency of CD4$^+$IL-17A$^+$ naïve and memory and cells following *in vitro* stimulation in PBMCs from pneumonia patients. PBMCs (4x10^5 cells) of young (n=3) or older adults (n=19) with pneumonia were stimulated and analysed by flow cytometry for identification of naïve, central memory, effector memory and EMRA CD4$^+$IL-17A$^+$ cells. **(A)** Unstimulated, **(B)** PMA (10 ng/ml) and ionomycin (50 ng/ml), **(C)** *S. pneumoniae* TIGR4 (MOI 10), **(D)** *S. pneumoniae* 6B (5 µg/ml) treated cells. Data are expressed as mean ± SD. Differences between age groups were analysed by Mann-Whitney test.

4.3.6. Association between $CD4^+$ T cell subsets with age and disease severity in pneumonia infection in older adults

In the previous section it was demonstrated that during pneumonia in older adults, frequency of IFN-γ^+ and IL-$17A^+$ subsets, as well as of $GARP^+$Tregs are elevated in the absence of *in vitro* stimulation. Thus, to verify if such changes were more pronounced in the very old, correlation analyses of $CD4^+$ T cell frequencies were performed.

While the frequency of Tregs (R^2=0.02, p=0.530) and of $GARP^+$Tregs (R^2=0.02, p=0.561) remained unaltered with advancing age in older patients (Figures 4.30 A and B), frequency of pro-inflammatory subsets increased. There was a trend towards increased frequency of IL-$17A^+$ cells at baseline with increasing age, which reached significance in PMA-stimulated cells (unstimulated: R^2=0.16, p=0.080; PMA: R^2=0.31, p=0.018) Figures 4.30 C and D). The same association was found between increasing age and elevated frequency of IFN-γ^+ cells (R^2=0.34, p=0.013) (Figure 4.30 E). Moreover, there was no change on the ratio of Th17/Treg in older patients (R^2=0.08, p=0.23).

Next, variation in the frequency of these subsets according to the severity of pneumonia infection was checked, using the CURB-65 score (section 3.3.2). Due to small sample sizes in some scores, there was no statistical difference between groups. Still, data suggests that in severe pneumonia (higher scores), there may be an increase of IL-$17A^+$ and IFN-γ^+ subsets (Figures 4.31 A and B), whereas Treg frequency remains unaltered (Figure 4.31 C) and possibly activation of Tregs is impaired in severe pneumonia (Figure 4.31 D).

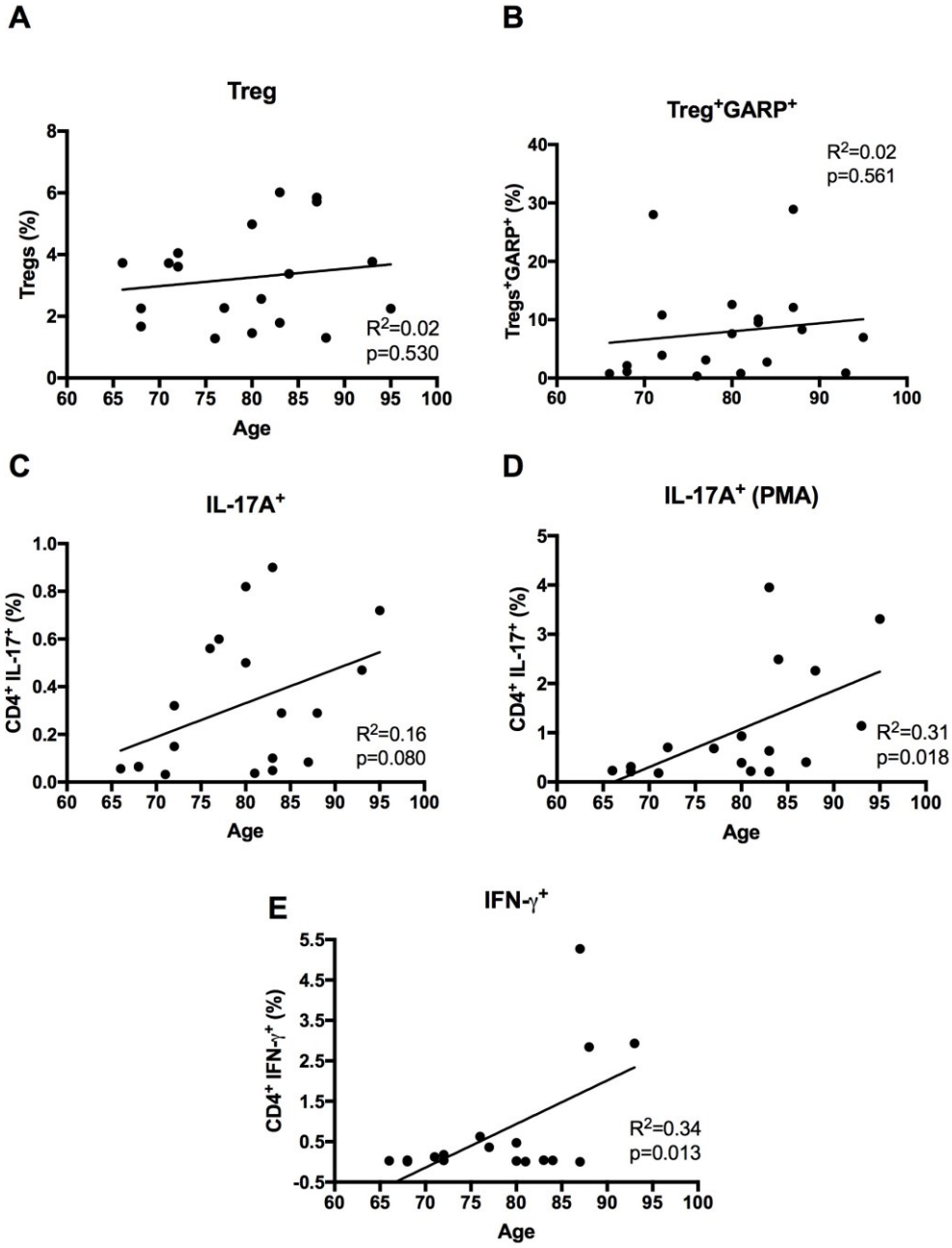

Figure 4.30. Association between frequency of CD4$^+$ T-helper subsets and age. Correlation between frequency of CD4$^+$ cell subsets and age in older adults with pneumonia (n=19) in unstimulated PBMCs or stimulated with PMA/ionomycin. **(A)** Tregs, **(B)** GARP$^+$Tregs cells, **(C)** CD4$^+$IL-17A$^+$ cells, **(D)** CD4$^+$IL-17A$^+$ cells in PMA/ionomycin treated PBMCs, **(E)** CD4$^+$IFN-γ$^+$ cells.

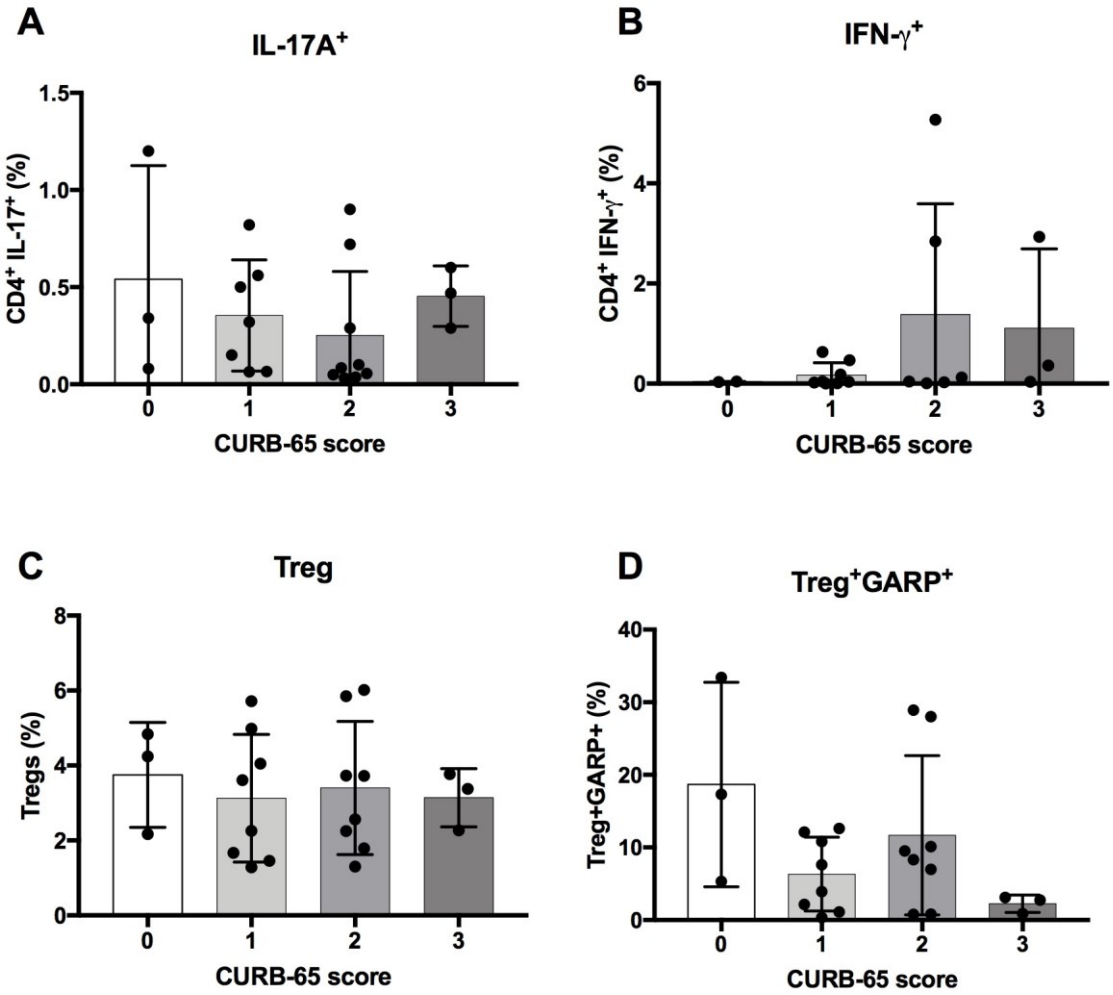

Figure 4.31. Frequency of CD4$^+$ T-helper subsets and according to disease severity. Frequency of CD4$^+$ T cells from patients with pneumonia (young, n=3; old, n=19) were grouped according to patient CURB-65 score. **(A)** CD4$^+$IL-17A$^+$ cells, **(B)** CD4$^+$IFN-γ$^+$ cells, **(C)** Tregs, **(D)** GARP$^+$Tregs.

4.3.7 Treg activation and cytokine production during pneumonia infection in older adults

Having found that the frequency of activated Tregs ($GARP^+$) was increased in young and old healthy volunteers from the EHPC study, following *in vitro* stimulation with 6B in PBMCs from pre and post experimental colonisation with pneumococcus (Figure 4.13 B) and increased in pneumonia patients during pneumonia infection (Figure 4.26 B), Treg function was next examined by testing cytokine production.

As GARP expression in Tregs is involved in TGF-β1 secretion (reviewed by [388]), the hypothesis was that elevated activation of Tregs during disease led to alterations in IL-10 and TGF-β1 production in the old. Briefly, PBMCs from healthy old and older pneumonia patients were stimulated with anti-human CD3 and CD28 for 66 hours ahead of immunostaining for intracellular cytokines. CD3 and CD28 stimulation were chosen for this protocol as they induced a more physiological response. Moreover, the combination of PMA and ionomycin used for stimulations made it difficult to gate on Tregs, as mentioned earlier (section 4.3.4). Stimulation of samples from young patients was not performed because at the time these assays were done no young patients were recruited. Also, it was not possible to have unstimulated controls for every donor, as for some there were not enough cells. As shown in Figure 4.32, concentrations of anti-human CD3 and CD28 were tested to find the best condition for detecting intracellular IL-10 in Tregs. The concentration of 5 μg/ml was chosen, as this treatment resulted in less variation in the percentage of Tregs (Figure 4.32 A) and $GARP^+$Tregs (Figure 4.32 B) between samples following treatment, compared to the concentration of 2 μg/ml.

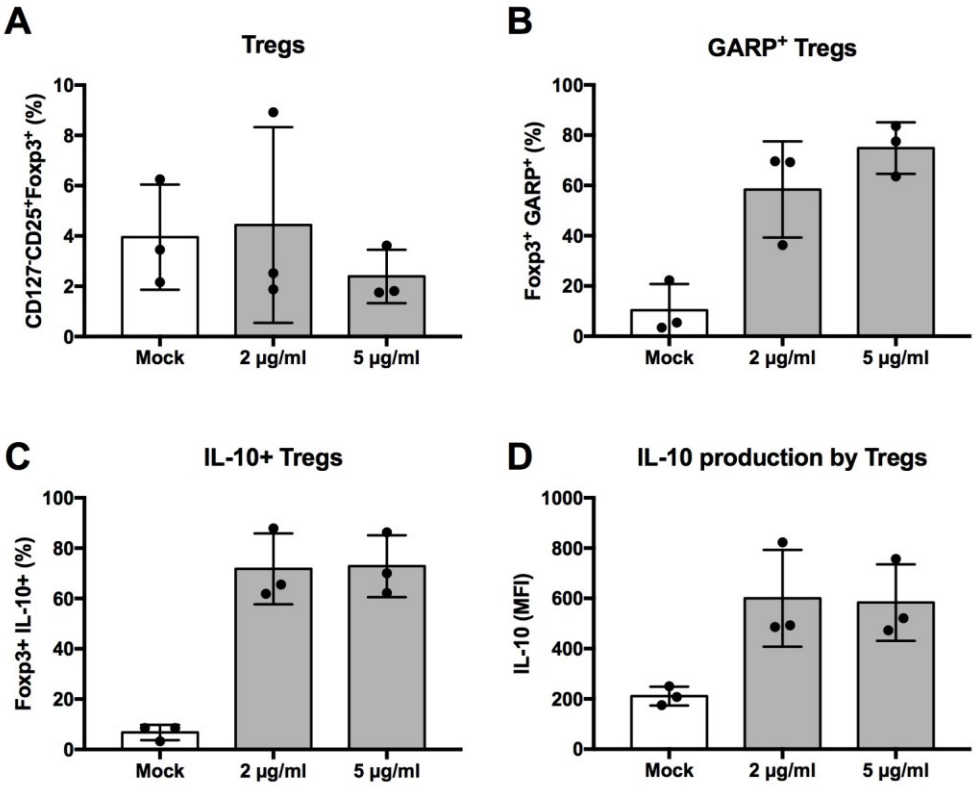

Figure 4.32. Intracellular IL-10 production by Tregs. PBMCs ($4x10^5$ cells) of healthy old (n=3) were stimulated with or without (mock) different concentrations of anti-CD3 and anti-CD28 for 66h and stained for flow cytometry analysis. **(A)** Frequency of Tregs **(B)** Frequency of $GARP^+$Tregs; **(C)** Frequency of IL-10-producing Tregs **(D)** Production of intracellular IL-10 in Tregs given by MFI. Data are expressed as mean ± SD. Differences between treatments were analysed by Kruskal-Wallis test with Dunn's post hoc.

Stimulation with CD3 and CD28 antibodies lowered the frequency of total Tregs in older patients, compared to untreated (mock) controls (p=0.002) and healthy old (p=0.015) (Figure 4.33 A), but significantly increased the frequency of activated Tregs in both groups (HO, p=0.04; OP, p=0.002) (Figure 4.33 B). Also, older patients had a higher frequency of $GARP^{+}$Tregs following CD3 and CD28 stimulation than healthy (p=0.0003) (Figure 4.33 B). This high activation of Tregs was also accompanied by higher frequency of IL-10-producing Tregs in older patients (p=0.021) and a trend toward increase of intracellular IL-10 (p=0.061), compared to healthy donors (Figures 4.33 C and D). Frequency of TGF-β1-producing Tregs was unaltered between groups, but intracellular levels of TGF-β1 were higher in older individuals with pneumonia, compared to healthy (p=0.032) (Figures 4.33 E and F). These results indicate that upon *in vitro* stimulation, Tregs from older patients are highly activated and produce more cytokines than healthy older subjects. Whether these augmented functionality of Tregs is beneficial or detrimental to older patients, still remain to be tested.

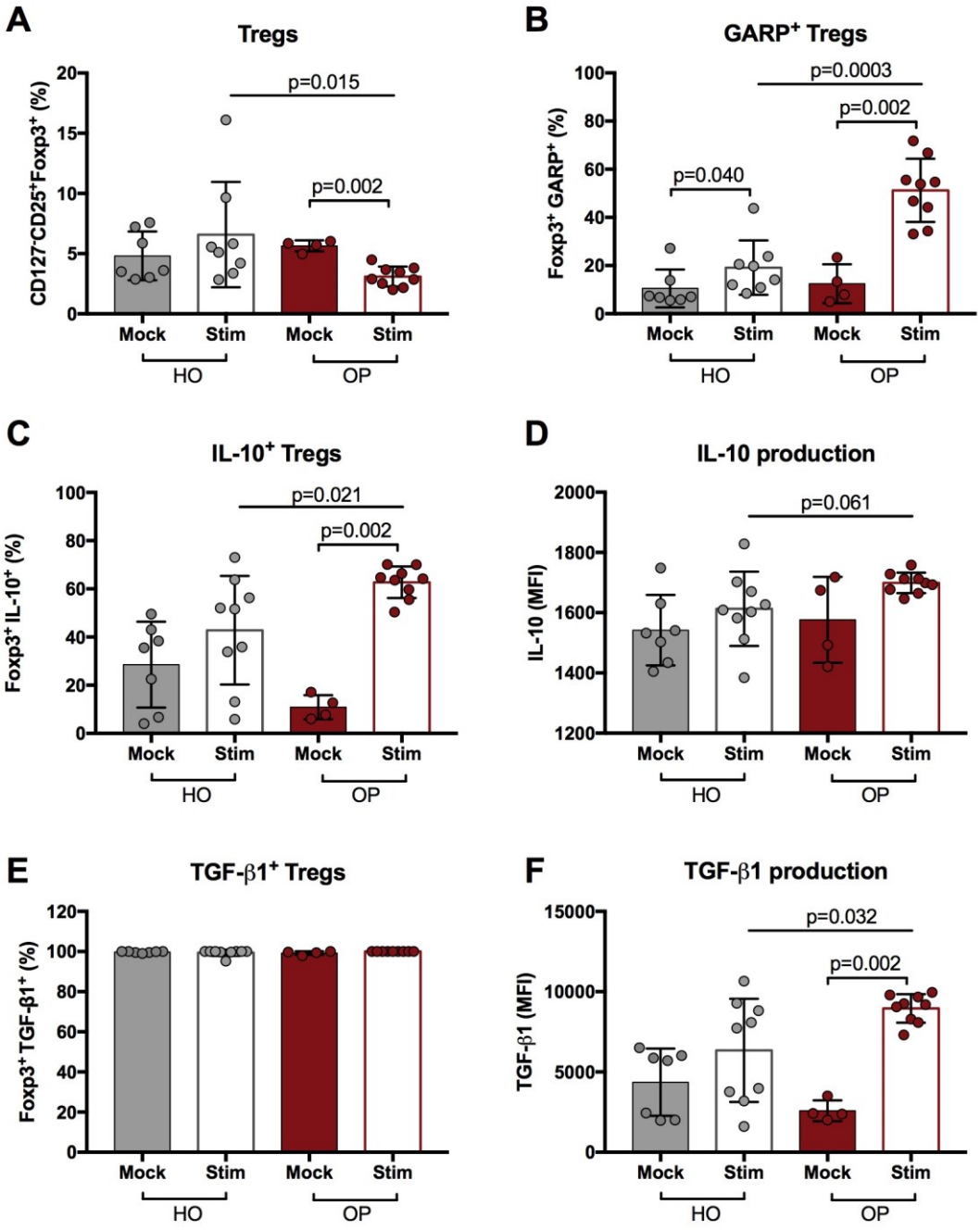

Figure 4.33. Intracellular IL-10 and TGF-β1 production by Tregs of older adults, healthy and with pneumonia. PBMCs ($4x10^5$ cells) of healthy old (n=9) and older pneumonia patients (n=9) were stimulated with or without (mock) anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml) for 66h and stained for flow cytometry analysis. **(A)** Frequency of Tregs **(B)** Frequency of $GARP^+$Tregs; **(C)** Frequency of IL-10-producing Tregs **(D)** Production of intracellular IL-10 in Tregs given by MFI; **(E)** Frequency of TGF-β1-producing Tregs **(F)** Production of intracellular TGF-β1 in Tregs given by MFI. Data are expressed as mean ± SD. Differences between age groups and treatments status were analysed by Mann-Whitney test. HO = healthy old. OP= old with pneumonia.

4.4. Summary of key findings

This chapter aimed to analyse age-related changes in dendritic cell population as well in the frequency of $CD4^+$ T cell subsets following experimental colonisation with the pneumococcus, and to determine the frequencies of $CD4^+$ T cell subsets in older individuals during pneumonia infection. Thus, the main findings of this chapter were:

- The frequency and absolute numbers of mDC1 were elevated in older individuals, but this subset showed expression of CD40 and HLA-DR comparable to those of young subjects, while pDC frequency decreased with ageing.
- Following *in vitro* PMA stimulation of PBMCs, experimentally colonised older adults had high frequencies of IFN-γ^+T-bet^+ and IFN-γ^+ $CD4^+$ T cells, and reduced frequency of IL-$17A^+$RORγt^+ $CD4^+$ T cells at post colonisation, compared to young subjects.
- The frequency of central memory IL-$17A^+$RORγt^+ cells was reduced in older volunteers post-colonisation, while this subset was found in both carriage-negative and positive young participants.
- Both young and older volunteers had a high frequency of 6B-responding $GARP^+$Tregs before and after experimental colonisation, while the frequencies of 6B-responding $CD4^+$ T-helper cells was low in the other subsets analysed.
- Older adults with pneumonia had increased frequencies of IFN-γ^+T-bet^+, IL-$17A^+$ $CD4^+$ and $GARP^+$Tregs in unstimulated PBMCs, compared to healthy older adults.
- Tregs of elderly patients with pneumonia produced higher levels of IL-10 and TGF-β1 following *in vitro* stimulation, compared to healthy older subjects.

4.5 Discussion

Nasopharyngeal colonisation by *S. pneumoniae* is an immunising event that generates both humoral and cellular responses. Studies in mice have highlighted the protective effect of antibody-mediated clearance of pneumococcus and the role of Th17 cells in mediating mucosal and systemic protection [261,297]. In humans, the role of pneumococcal nasopharyngeal colonisation in inducing protective immunity in both sites is still understudied, in particular the effect of colonisation in promoting protective adaptive immunity against *S. pneumoniae* in older adults is poorly understood and was the focus of this chapter. Previous studies using the experimental colonisation model have demonstrated the generation of anti-PspA antibody [223,314], mucosal and serum anti-capsular IgG [311,314], increases in $CD4^+IL\text{-}17A^+$ frequency in BAL and peripheral blood [302] following carriage in healthy young subjects, but no study to date has investigated whether these responses are changed with age.

In this chapter, the human model of pneumococcal colonisation was used to test the effect of experimental nasopharyngeal carriage in older adults on differentiation of $CD4^+$ T-cell helper subsets. Immunophenotyping of dendritic cell subsets was also performed in samples from healthy volunteers from pre-colonisation, thus gathering more evidence of changes in the immune cell compartment that participates in the development of humoral and cellular adaptive immunity against *S. pneumoniae*. Lastly, identification of $CD4^+$ T-helper cell subsets during pneumonia infection in older patients was performed, as our hypothesis was that $CD4^+$ polarisation would be compromised in the old during pneumonia, most likely with reduced polarisation towards the $CD4^+IL\text{-}17^+$ subset and with impaired Tregs function. The combination of PMA and ionomycin was used to promote expansion of $CD4^+$T cells

independently of receptor-depended antigen activation of these cells, which was achieved by stimulation of cells with *S. pneumoniae* serotypes 4 or 6B.

4.5.1. Age-associated changes in dendritic cell subsets

The overall frequency of dendritic cells in PBMCs from our volunteers was not affected by age, but the frequency of myeloid and plasmacytoid subset was. The findings in this chapter were obtained from adults aged between 50-73 years (mean age 59.8 ± 7.1 years), thus younger than several other published reports. Still, the decline in plasmacytoid DC frequency found in our cohort agrees with other studies [64,65,70,71]. However, frequency of mDC1 which was increased in our old adult cohort, has been reported as unaltered [68,69] or reduced [64–66] and further reduced in advanced-age, frail elderly (aged 81-100 years) [64]. A correlation analysis of mDC1 data showed association between decline in cell numbers of this subset with increasing age (Figure 4.4 A; R^2=0.029, p=0.039), whereas this association was not statistically significant for the pDC subset. This suggests that the number of mDC1 declines in more advanced age, whereas the population of pDCs may decline much earlier in the ageing process. Further analysis of these subsets showed that surface density of HLA-DR was not altered with age in both mDC1 and pDC, but CD40 expression was increased pDCs of older adults. Whether this increased CD40 expression in pDCs is associated with alterations in cell function is yet to be confirmed. Plasmacytoid DCs from aged adults are reported to secrete lower levels of IFN-I and IFN-III following *in vitro* stimulation with influenza virus and to induce lower subsequent proliferation of $CD8^+$ and $CD4^+$ T cells [82], but no difference in upregulation of co-stimulatory molecules CD40, CD80 and CD86 and cytokine production

following LPS/IFN-γ treatment [389]. This suggests that the poor response of pDCs to influenza virus could contribute to elderly susceptibility to influenza infection which is often associated with secondary pneumonia infection [390]. Still, immunophenotyping showed no age-related changes mDC1s, which is the major subset responsible for antigen presentation to and differentiation of $CD4^+$ T cells into helper subsets [79], indicating that at the time of the experimental pneumococcal inoculation study, these DCs presented a normal phenotype.

4.5.2. Age-related changes in $CD4^+$ T subset polarisation during experimental human pneumococcal carriage

Data from this chapter demonstrated that experimental nasopharyngeal colonisation with *S. pneumoniae* in older adults does not induce several significant changes in the frequency of specific $CD4^+$ T-helper subsets in peripheral blood. Upon stimulation of cells with PMA/ionomycin older participants showed contrasting frequencies of IFN^+ subsets and $IL\text{-}17^+ROR\gamma t^+$ cells with young participants.

With a few exceptions, there were no statistical differences in cell frequencies between pre and post-colonisation. This finding may be associated with the fact that the presence of 6B-specific $CD4^+$ T lymphocytes, expected to be generated by the colonisation event, were lower in peripheral blood, and that this frequency would have been higher if cells studied had been isolated from the nasal cavity. Also, the time of collection of the post-colonisation samples may also be involved, as post-colonisation samples compared in this thesis were from different time points, *i.e* day 29 for young volunteers, and day 14 for old volunteers.

Perhaps by using samples from young volunteers from an earlier time point (*i.e.* day 14) differences in cell frequency could have been more evident, whilst in older adults, absence of higher cell frequency at day 14 could be an effect of immunosenescence itself.

Possibly due to an adaptive response skewed towards pro-inflammatory subsets, no changes were observed in the $CD4^+IL\text{-}4^+$ and $IL\text{-}4^+GATA3^+$ subsets in the older or young cohorts. In mice, IL-4 was detected following colonisation with *S. pneumoniae* [391] but had no significant effect in clearance of colonisation [230].

Here, the older cohort had increased frequencies of IFN-γ^+ and IFN-γ^+T-bet^+, following stimulation with PMA, but not 6B. Increased IFN-γ-producing $CD4^+$ T cells is known to occur with advancing age [156–158] and during infection, IFN-γ contributes to *S. pneumoniae* containment by promoting neutrophil influx to infected sites [256,392,393]. The increase in these subsets following PMA stimulation is not only associated with expansion of the $CD4^+$IFN-γ^+ subset, but because PMA treatment promotes downregulation of $CD4^+$ in T cells, this subset was mixed with $CD8^+$ T cells, that too produce high levels of IFN-γ with ageing [157,158], thus contributing to the elevated frequencies found in older adults.

Due of the small sample sizes in this thesis, observed differences between carriage positive and negative participants failed to reach statistical significance. However, in older carriage-negative individuals, there was a trend towards increased frequencies of IFN-γ^+ and IFN-γ^+T-bet^+ compared to carriage-positive, but no similar trend was observed in the young cohort. Indeed, in mice, the presence of IFN-γ has been associated with absence of pneumococcal carriage [230], but clearance of colonisation still occurs in mice lacking IFN-γ receptor [261], indicating that clearance is not exclusively dependent on $CD4^+$IFN-γ^+ T cells.

A previous study using the EHPC model showed that experimentally-colonised adult volunteers had higher frequencies of 6B-responding $CD4^+IL\text{-}17A^+$ in BAL and PBMCs than non-colonised, but not $CD4^+IFN\text{-}\gamma^+$ T-cells [302]. Here, while frequencies of $CD4^+IL\text{-}17A^+$ cells were not altered with ageing during experimental colonisation, young participants had an increase in $IL\text{-}17A^+ROR\gamma t^+$ frequency post EHPC following *in vitro* PMA stimulation, compared to old. Young individuals with high $IL\text{-}17A^+ROR\gamma t^+$ frequency were mostly carriage-negative and with 6B *in vitro* stimulation, had higher $IL\text{-}17A^+ROR\gamma t^+$ frequency than carriage-positive. This confirms that in young subjects, 6B-responding $IL\text{-}17A^+$ cells remain detectable in the periphery at day 29 post-colonisation, supporting the finding of Wright *et al*. (2013) in which $CD4^+IL\text{-}17A^+$ cells were detected at day 35 post-colonisation in young participants [302]. A higher frequency of $CD4^+IL\text{-}17A^+$ cells was also found in NALT of carriage-negative children, compared to carriage-positive [304]. Conversely, in older individuals studied in this thesis, frequency of $IL\text{-}17A^+ROR\gamma t^+$ cells were not increase following PMA treatment, and presence of carriage had no effect on $IL\text{-}17A^+ROR\gamma t^+$ frequency, which remained lower than in young, in carriage-negative participants. Previous studies of RORγt expression in $CD4^+IL\text{-}17A^+$ cells of older cohorts show conflicting results, being reported as increased [159,394] or unaltered [147]. Still, the lower frequency in $IL\text{-}17A^+ROR\gamma t^+$ cells found in older adults as demonstrated in this chapter raises the possibility that if RORγt induction in $CD4^+IL\text{-}17A^+$ is impaired in older adults, other $CD4^+$-helper subset, possibly $IFN\text{-}\gamma^+$, could be mediating clearance of colonisation in carriage-negative volunteers, as this cytokine can recruit neutrophils for clearance of *S. pneumoniae* [256,392,393].

Furthermore, $CD4^+IL\text{-}17A^+$ are not the only source of IL-17 during colonisation, as alveolar macrophages were an alternative source of IL-17 in BAL of experimentally-colonised

volunteers and had opsonophagocytic killing enhanced by *in vitro* stimulation with recombinant human IL-17A [302]. The mucosal and epithelial-resident γδ T cells also produce IL-17 [395,396] and can aid pneumococcal clearance in the lungs of mice by contributing to regulation of alveolar macrophages and dendritic cells [397] and neutrophils [323,398], but whether they contribute to clearance of pneumococcal carriage in humans is unknown. Thus, the presence of IL-17 secreted by cell types other than $CD4^+$ could still mediate neutrophil recruitment in sites where *S. pneumoniae* is present.

Surprisingly, re-stimulation with *S. pneumoniae* 6B *in vitro* greatly increased the frequency of activated Tregs, identified by GARP surface expression. GARP is a transmembrane protein expressed on the surface of activated Tregs and is associated with TGF-β secretion and activation, thus contributing to maintenance of Treg homeostasis [388]. GARP is present at low levels in the surface of Tregs and increases following TCR stimulation [399,400]. In young individuals, presence of carriage seems to be accompanied by a trend towards increase frequency of activated Tregs, while in older donors, this trend was not observed. It is possible that these trends would be statistically significant in the young cohort if measured at an earlier time point, as it is known that high frequency of Tregs and elevated levels of TGF-β and contribute to prolong carriage, in mice and adults [401], as well in children [304,402]. Mubarak and colleagues (2016) showed that the ratio Th17/Treg frequencies found in adenotonsillar tissue increased with ageing, from children to adults, as well as the frequency of Th17. Furthermore, carriage-positive children had lower ratios of Th17/Treg than carriage-negative [304]. Here, these associations were absent in older adults, and as frequency of Th17 cells [147,159,160] and Tregs [167–169] increase in adults over 65 years,

data from this chapter could indicate that the ratio reaches a balance with increasing age and may progress to an imbalance in more advanced age.

Pneumococcal carriage is also associated with generation of protective memory $CD4^+$ T cells in mice [298] and humans [302,314]. A closer analysis of IL-$17A^+RORγt^+$ subset at post-colonisation following PMA stimulation, revealed most cells were central memory cells in young donors, whereas older participants had low frequencies of this subset. This contrasts with the elevated frequency of total central memory $CD4^+$ cells, in both carriage-positive and negative, present in older volunteers, compared to young, as accumulation of memory cells occurs with age [146,147]. Hence, absence of IL-$17A^+RORγt^+$ memory cells in older donors following colonisation suggests failure in induction of long-lasting protective immunity during carriage, which could contribute to the susceptibility of this age group to disease. Here, mDC1 from colonised volunteers had no age-related changes in CD40 and HLA-DR expression, suggesting that T-cell activation, rather than impaired antigen presenting cells, may be involved in absence of IL-$17A^+RORγt^+$ memory cells in the old. Also, memory $CD4^+$ T cells from older adults were reported as producing lower levels of IL-17 than young, whereas IL-17 production by naïve cells was unaltered [147]. These data indicate that both induction of immune memory and its effector function may be impaired in the elderly.

One of the limitations of the colonisation study presented in this thesis is that although volunteers selected were not colonised with pneumococcus at the time of the study, it is very likely that participants have had previous colonisation episodes thought life, which could have, potentially, generated protective immunity, which could explain the high frequency of $GARP^+$Tregs following *in vitro* re-stimulation with 6B, in samples from pre and post colonisation. Furthermore, although PMA treatment increased the frequency of

$CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17A^+$ the only strong 6B-specific response found in PBMCs were detected in Tregs, as seen by the elevated frequency of $GARP^+$Tregs. It is possible that pneumococcal-specific $CD4^+$ T memory cells could be found in the nasopharynx or BAL and at an earlier time point, as previously reported in young colonized volunteers [302].

Other elements from the nasopharynx microenvironment are potential contributors to generation of imbalanced $CD4^+$ T-helper cell populations. For instance, elevated gene expression of TLR1, CCL2 and IL-1β genes, such as found in nasal mucosa-associated lymphoid tissue in aged mice [386], reduced nasal mucociliary clearance with ageing [53] or disruption of nasal epithelium homeostasis by influenza virus, which facilitates pneumococcal adherence [403,404].

Still, having detected $IFN\text{-}\gamma^+$ and $IL\text{-}17A^+$ $CD4^+$ T cells in post colonisation samples, it is possible that in young subjects carriage clearance is mediated by mixed activation of Th1 and Th17 cells and that in carriage positive donors, these subsets together with activation of Tregs contribute to control bacteria density in the nasopharynx [303]. With ageing, reduced frequency of $IL\text{-}17A^+ROR\gamma t^+$ could indicate that clearance is being mediated mostly by $CD4^+IFN\text{-}\gamma^+$ cells, and that unchanged proportion of Th17 and Tregs could cause an imbalance in the nasopharynx microenvironment, allowing prolonged pneumococcal carriage, which could eventually lead to disease. This hypothesis will be further discussed in chapter 5.

4.5.3. $CD4^+$ T-helper subset polarisation during pneumonia infection in older adults

An investigation of the presence of pneumococcal carriage in patients with pneumonia was not conducted in this study, but it has been reported that in hospitalized unvaccinated older

individuals, *S. pneumoniae* colonisation was absent (found in 0.3% of participants using classical culture method) [385], very low in adults with lower respiratory tract infections (5% of participants, using culture method) [405] but detectable in 21% of older patients with confirmed influenza in the intensive care unit (using quantitative PCR) [406]. Whether clearance of nasopharyngeal carriage occurs before or during pneumonia infection remains to be further studied.

Here, among older patients, 33% (7 out of 21) had been vaccinated with the pneumococcal vaccine. In comparison with healthy older volunteers, of which 13% (2 out of 15) had been vaccinated, patients had a higher frequency of central memory $CD4^+$T cells but reduced frequency of $CD4^+$ T_{EMRA}. Whether this increased frequency in memory cells was promoted by previous vaccination or colonisation events it is not possible to confirm, although as discussed in the previous section, experimental colonisation of older volunteers did not enhance the frequency of memory $CD4^+$ T cells subsets in the peripheral blood at day 14. As $CD4^+$IL-17^+ cells and naïve and memory $CD4^+$ T cell were stained under the same colour panel (Figure 4.9 F), further analysis showed that older patients had central, EMRA and effector memory $CD4^+$IL-17^+ lymphocytes, and in young patients, frequency of $CD4^+$IL-17^+ EMRA cells increased following stimulation with *S. pneumoniae* serotypes. As young patients had not been vaccinated against pneumococcus, it is possible that immune memory was acquired by natural colonisation events throughout life, even though during experimental colonisation young participants did not show increase in the frequency of memory $CD4^+$T subsets, at day 29. Although these frequencies of memory $CD4^+$IL-$17A^+$ T cells found in peripheral blood were low overall, it is possible that higher frequencies can be found it the

lung, as previously demonstrated for old COPD patients, who had higher frequencies of influenza-specific memory $CD4^+$T cells in the lung than in the periphery [407].

Also, the frequency of total $CD4^+$IL-$17A^+$ T cells was increased in both young and older patients. The role of the $CD4^+$IL-17^+ subset as a mediator of adaptive immunity against *S. pneumoniae* is well demonstrated in mice [257,261,308]. In humans, the frequency of $CD4^+$IL-$17A^+$ cells were reduced in patients with ventilator associated pneumonia [408], but patients with non-severe and severe CAP had higher frequency of $CD4^+$IL-$17A^+$ and $CD4^+$IL-22^+ than healthy controls in PBMCs and even higher frequencies in BAL fluid at the time of admission [409]. Both cohorts of this last study were slightly younger (PBMCs = 55.6±18.5 years, BAL = 60.6±19.0) than our older patients (77.7±9.8 years). Still, here older patients had elevated frequency of $CD4^+$IL-$17A^+$ T cells in pneumonia patients, and these patients also showed increased frequency of RORγt-expressing $CD4^+$IL-$17A^+$ T cells than healthy controls. Surprisingly, old patients had higher $CD4^+$IL-$17A^+$RORγt^+ frequency in unstimulated PBMCs pool than young patients. As mentioned earlier, reports of expression of RORγt^+ in $CD4^+$IL-$17A^+$ T cells in healthy old adults are contradictory [147,394], but in COPD patients, RORγt^+ expression was increased in lung tissues [410]. Considering the observed overall increase of $CD4^+$IL-$17A^+$ T-cell frequency during pneumonia, an elevated frequency of RORγt^+-expressing $CD4^+$IL-$17A^+$ T cells was probable. It remains to be confirmed however, if the frequency of $CD4^+$IL-$17A^+$RORγt^+ is different between young and old patients with pneumonia, as our young cohort consisted of only 3 patients.

There are reports of $CD4^+$ T cells producing both IFN-γ and IL-17A in mice during pneumococcal pneumonia [307], and in healthy old volunteers [147]. But in this thesis, these cytokines were stained for using different colour panels, and it was therefore not possible to

verify if double IFN-γ/IL-17A cells were present in our patients. In our older patients, frequency of $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IFN\text{-}\gamma^+T\text{-}bet^+$ T cells were also increased, reinforcing the hypothesis of a skew towards pro-inflammatory $CD4^+$-helper subsets during pneumonia disease. $CD4^+IFN\text{-}\gamma^+$ cells have been detected in older patients with pneumonia, in lower frequencies at day 1, then increasing by day 7 [411]. In addition, in children with IPD, an elevated frequency of $CD4^+IFN\text{-}\gamma^+$ frequency was detected in the convalescent phase of disease [412]. Thus, these elevated frequencies of $CD4^+IFN\text{-}\gamma^+$ in the periphery during pneumonia infection could be a consequence of cellular migration towards the infected lungs.

Our data also show that the frequency of $CD4^+$ T cell subsets following TIGR4 and 6B sometimes differed. This could be due to the concentration of the antigen (multiplicity of infection of 10 for TIGR4 and protein concentration for 6B, 5 μg/ml), or that some cell subsets could be responding to specific antigens whereas other subsets could be activated by conserved antigens in both strains. It is also possible that some outliers found in our dataset could be evidence of pneumococcal-specific responses. However, because no identification of the causative pathogen of pneumonia was performed, nor nasal swabs taken to test if patients were carriers of pneumococcus in the nasopharynx during disease, these hypotheses could not be confirmed.

Nevertheless, the polarisation of $CD4^+$T cells in response to *S. pneumoniae* is still subject of study. In an *in vitro* system, monocyte induction of Th1 or Th17 responses to *S. pneumoniae* were associated with bacteria viability. Live pneumococcus induced monocytes to secrete IL-12p40 following internalisation, and possibly TLR4 activation, thus triggering $CD4^+$T cell differentiation into Th1, while heat-killed bacteria induced TLR2 activation, largely mediated

by peptidoglycan alone, leading to differentiation into Th17 subset. Internalization of bacteria by monocytes was poorly related to induction of a Th17 response [295]. Triggering of a Th1 response negatively regulates Th17 differentiation, in the presence of IFN-γ and IL-12 [295,413,414]. However, older adults with pneumoniae showed elevated frequencies of both Th1 and Th17 subsets, and there was a positive correlation between increasing age and elevated frequencies of $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD4^{+}IL\text{-}17A^{+}$. Although high IL-1β levels have been associated with increased Th17 cell frequency, which have elevated expression of IL-1R in aged mice [415], in the cohort of this thesis, serum IL-1β was only detected in 1 patient. However, serum cytokine levels are likely to vary with the day of sample collection.

In contrast with the age-associated increase in T-helper-inflammatory subsets $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD4^{+}IL\text{-}17A^{+}$ during pneumonia in the old, the frequency of Treg or $GARP^{+}$Treg did not follow this trend. During pneumococcal infection in mice, expansion of the Treg population is required for control of bacterial dissemination from the lungs and resistance to infection, which are both impaired by inhibition of TGF-β1 [303]. Although the ratio of Th17/Treg frequencies has been reported to increase with age [304], in this thesis this ratio was unaltered in older adults during pneumonia. There has been a study reporting that Treg frequency was reduced in older patients, produced lower levels of IL-10 and TGF-β, which consequently led to impaired suppression of effector CD4 and CD8 T cells [416]. In contrast, here older patients had comparable frequencies of Tregs to healthy controls but showed a higher frequency of activated Tregs (GARP+) in patients of both age groups at baseline. These contrasting results could be associated with the mean age of the cohort studied (no information was given in the published paper), population origin, use of fresh instead of thawed PBMCs, stimulation length or other methodological differences.

In COPD patients, the frequency of GARP^{+}Tregs was comparable with that of healthy controls, although patients had elevated GARP mRNA transcripts compared to controls [417], and in patients with lung cancer, tumor-infiltrating Tregs had elevated GARP expression [418]. Here, while older adults with pneumonia had a higher frequency GARP^{+}Treg in the absence of stimulation than healthy controls, the frequencies of Tregs and GARP^{+}Treg were reduced following stimulation with *S. pneumoniae* 6B. This was not observed in young patients that showed higher GARP^{+}Treg frequency following TIGR4 or 6B stimulations or in the unstimulated condition. In contrast, CD3 and CD28 *in vitro* stimulation successfully activated Tregs of older patients, and production of IL-10 and TGF-β1 was higher than for healthy old individuals. This suggests that direct stimulation of co-receptors CD3 and CD28 is still functional in Tregs of older patients, but antigen-dependent activation, as mediated by an APC, may be impaired. This potential overactivation of Tregs from old patients could have detrimental effects, such as reduction of co-stimulatory molecules CD80 and CD86 in myeloid dendritic cells, as demonstrated in aged mice [419].

Although these are data from only 7 patients, the frequency of GARP^{+}Treg cells was positively associated with high levels of IL-33 in older patients. IL-33 is associated with protection of the mucosal-barrier [379] and is released by epithelial cells from the lung during COPD [380,381]. Using a mice model, Nascimento and colleagues (2017) demonstrated that IL-33 has been associated with sepsis-induced immunosuppression [420]. During sepsis, higher levels of IL-33 induced expansion of type 2 innate lymphoid cells that, by secretion of IL-4 and IL-13, drove macrophage differentiation into the alternative, IL-10-secreting phenotype. These macrophages then drive expansion of Foxp3^{+}Treg cells in sepsis-surviving mice and this increase in the Treg population lead to susceptibility of secondary

pneumonia caused by *Legionella pneumophila* [420]. The extension of the replicability of these findings in humans are yet to be confirmed. However, the finding of high activation of Tregs in older patients presented in this chapter as well as the reported impaired recovery of neutrophil migratory function in older patients following clinical recovery of CAP [124] are comparable with this mechanism. Thus, this hypothesis and whether IL-33 present in older patients contributes to drive Treg activation during disease remains to be elucidated in future work and will be further discussed in chapter 5.

Chapter 5

General Discussion

5.1 General Discussion

5.1.1 Innate immunity to *S. pneumoniae* in healthy older adults

Immunosenescence is associated with high infection rates to a range of bacterial and viral pathogens [41,421,422] and reduced vaccination responses [65,172] in older adults. The well-established decline in neutrophil function in old age [122,126,127,129,141], was attributed as one of the causes of increased susceptibility to *S. pneumoniae* infections [36,109,124,254]. However, this hypothesis had not been tested in older individuals, either in patients with pneumonia or following exposure to the bacteria experimentally. Also, which aspects of age-related neutrophil functional decline might compromise immunity to *S. pneumoniae* were not known, and reports of neutrophil phagocytosis in older adults showed contrasting results for *E. coli* [122,129], *S. aureus* [129] and Group B *Streptococcus* [62].

From the findings presented in Chapter 3, healthy older individuals maintained neutrophil ROS production in response to *S. pneumoniae* TIGR4, but ROS generation was reduced towards 19A and 23F. While pre-opsonisation of bacteria with pooled sera recovered this deficient oxidative burst to 19A, this remained low for serotype 23F. Despite reduced ROS production to these serotypes, NET generation was not affected, and in fact it was elevated in response to 23F in the older subjects. These data suggest that the reduced ROS was not physiologically relevant for NET generation. To better comprehend what mediates these different responses between serotypes in future studies, the use of mutant strains, *i.e.* TIGR4 strains expressing different capsules, will be a useful tool to determine the role of pneumococcal capsules on neutrophil activation in older adults. Furthermore, it has been proposed that NET generation in response to *S. pneumoniae* is mediated by induction of

autophagy via PI3kinase class III and activation of the autophagy gene Atg5 [277]. Interestingly, impaired signalling of PI3K class I in neutrophils from old donors led to reduced chemotaxis [126], so an investigation of PI3K class III signalling in old individuals could clarify if the age-related increase of NET generation to 23F is caused by altered PI3K class III signalling (Figure 5.1 A).

The data presented here also showed that neutrophil ROS production in response to PMA was not altered with age. This difference from the data for pneumococcal serotypes, could be associated with reduced activation of TLR2 and TLR4 in response to the pneumococcal PAMPs, whereas PMA activates PKC directly bypassing the TLR signalling step [423] (Figure 5.1 A). However, the serotypes tested did not modulate neutrophil TLR2 expression in neutrophils from old donors, but showed a trend towards reduced TLR4 expression. Interestingly, resting neutrophils of old donors generated higher levels of IL-8, suggesting that while surface TLR2 and TLR4 expression were not significantly modulated by age, altered age-related signalling of these receptors [94,97] may be associated with IL-8 production in the resting state and with age-associated altered ROS generation to *S. pneumoniae* serotypes. Elevated serum IL-8 was also found in the aged cohort, in agreement with the well documented increase in systemic inflammation with age, termed inflammaging [41].

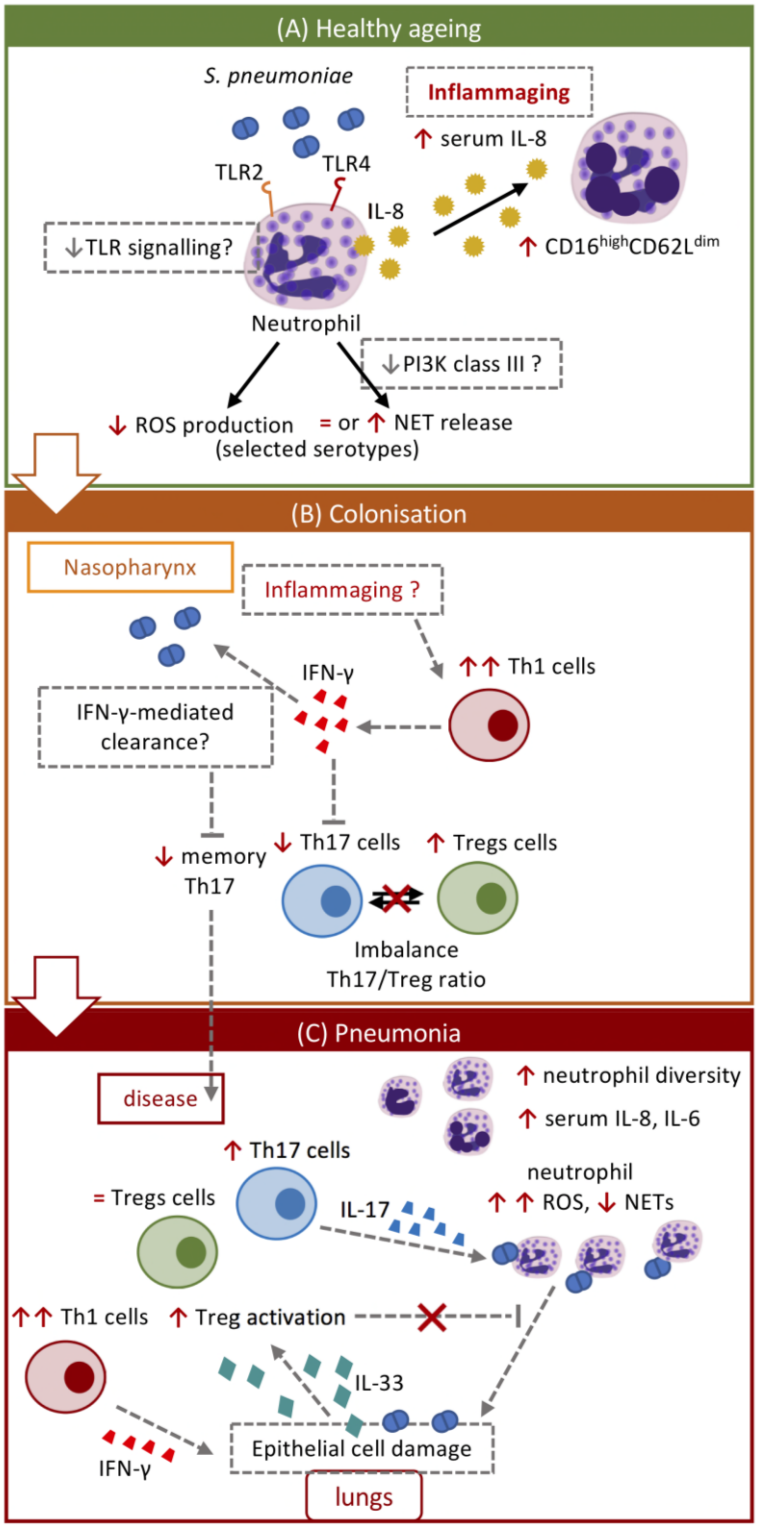

Figure 5.1. Proposed age-related changes in neutrophil and CD4+ T-helper cell responses to *S. pneumoniae* that lead to pneumonia infection. A) Impaired TLR2, TLR4 and Pi3K class III signalling in neutrophils promote reduced ROS generation and NET release to pneumococcal serotypes; inflammaging drives $CD16^{high}CD62L^{dim}$ neutrophils to the bloodstream. **B)** During colonisation, carriage is cleared by Th1-mediated response, due to age-related shift towards this subset, without induction of Th17 immune memory. **C)** Lack of pneumococcal-specific central memory Th17 cells allows *S. pneumoniae* to start infection in the lungs. Age-related skew towards pro-inflammatory Th1 and Th17 subsets promote high neutrophil activation and ROS production, which damages lung cells. IL-33 produced by epithelial lung cells activate Tregs to control inflammatory response, but Tregs are unable to control neutrophil activation, thus continuing inflammation. Red and black arrows indicate data described in this thesis. Grey arrows and boxes indicate hypotheses.

A positive correlation was found for serum IL-8 and the frequency of $CD16^{high}CD62L^{dim}$ neutrophils in the blood. It is not known yet what drives the differentiation of this neutrophil subtype or when this subset appears in the bloodstream of healthy older adults, but as this subset was first described in an acute model of inflammation [118,119] their appearance may be associated with inflammaging. This neutrophil phenotype has several features which suggest reduced function, such as poor adhesion to endothelium [119], possible suppressive properties towards T-cell proliferation [118] and reduced phagocytosis and ROS generation [121], all of which could increase susceptibility to infections.

Monocyte diversity and function were also examined. Non-classical monocytes showed increased surface CCR2 expression with age, which could lead to accelerated egress of these cells from the bone marrow [336]. However, the frequency of the different subsets was not altered with ageing, suggesting that elevated CCR2 expression on non-classical monocytes did not impact the overall proportion of peripheral monocytes subsets. Stimulation of monocytes with *S. pneumoniae* showed no age-related effects, as cells from old donors showed similar reductions in CD11b and CCR2 expression as well as production of TNF-α and IL-6 as young participants. Monocytes are most likely to encounter pneumococci in the tissue instead of blood, and loss of surface CCR2 and CD11b could be a strategy to retain these cells in infected tissue to mediate bacterial clearance or differentiate into macrophages. Unaltered monocyte TNF-α and IL-6 production in the older donors indicates that they can still mediate neutrophil recruitment to the infection site. This is supported by a report of elevated neutrophil influx to the lungs in older patients with pneumonia [109].

The lack of severe age-related impairment of neutrophil ROS and NET generation to *S. pneumoniae* shown here suggests that older adults in good health may not be the group

most susceptible to pneumococcal pneumonia, but older adults in a frail condition may be. Frail elders have more pro-inflammatory cytokines in serum [424], altered neutrophil phenotype [425] and reduced macrophage killing of *S. pneumoniae* [95]. Therefore, more studies comparing immune function between healthy and frail elders in response to *S. pneumoniae* are needed. Interestingly, a recent study from this institute has shown that after clinical recovery from pneumonia infection, neutrophil function remains reduced in older patients but not young patients [124]. What could not be proven in this study was whether the older patients had reduced neutrophil function before the infection, or whether the infection reduced immune function.

5.1.2 Ageing and the immunising effect of pneumococcal colonisation in the nasopharynx

Having performed neutrophil and monocyte functional assays in a cohort of healthy old and finding no consistent and major differences *in vitro* to three different serotypes of *S. pneumoniae*, it was necessary to determine the impact of age in a more physiologically relevant model, the experimental pneumococcal colonisation. Immune cell behaviour in the nasopharynx can be different to the blood, as the complex microenvironment of the former includes interactions with epithelial cells and the local microbiome.

The lack of severe age-related impairments in neutrophil ROS production, NET generation and activation in response to *S. pneumoniae* indicated these cells could still potentially respond to *S. pneumoniae* in the nasopharynx. The very low carriage rates found in older adults [197] also suggests clearance is still being effective and potentially mediated by monocytes and neutrophils. However, it was not possible to examine neutrophils and

monocytes in the nasopharynx in this thesis, due to a conflict with planned studies at the LSTM. Neutrophil and monocyte function in the nasopharynx of old donors as well as the presence of $CD16^{high}CD62L^{dim}$ neutrophils should be addressed by future studies.

This thesis examined specific aspects of the innate and adaptive immune response to *S. pneumoniae* in this model using serotype 6B. Immunostaining of dendritic cells showed increased mDC1 frequency and reduced pDC in older adults. Considering the role of pDCs in anti-viral host response [63], the decline in the frequency of this population with ageing could contribute to susceptibility to viral infections, such as influenza, which often precedes pneumonia in older adults [390]. As shown in chapter 4, the expression of CD40 and HLA-DR remained unaltered in mDC1 cells of older subjects, suggesting that at the time volunteers were experimentally colonised, these cells could have normal antigen-presenting function, as both molecules are required for successful activation of naïve and memory T lymphocytes. mDC1s are also more efficient in activating naïve T cells than pDCs [426], thus it was expected the mDC1 population would have contributed to the activation of $CD4^+$ T cells involved in immunity against the pneumococcus. However, as described in chapter 4, older participants had lower frequencies of Th17 and of 6B-responding Th1, Th2 and Th17 cells, indicating that mDC1 from older individuals, although expressing the same levels of CD40 and HLA-DR as young subjects, may be functionally impaired, contributing to reduced $CD4^+$ T activation and differentiation in the elderly. Indeed, although previous studies have reported unaltered expression of co-stimulatory molecules CD80 and CD86 in mDC1 in older subjects [66,69,76,77], this subset induced low proliferation of T cells with ageing [65,73].

In experimentally colonised older participants, higher frequencies of IFN-γ^+T-bet^+ and IFN-γ^+ $CD4^+$ T cells were found in the circulation, as well as a reduced frequency of IL-$17A^+$RORγt^+

$CD4^+$ T cells. When data were divided into carriage-positive and negative volunteers, young carriage-negative volunteers exhibited a higher frequency of IL-17^+RORγ$^+$ cells than both young carriage-positive and -negative older volunteers. Interestingly, a statistically significant increase of 6B-responding cells was found in GARP$^+$Tregs. Pneumococcus-specific Th1 and Th17 responses have been found in tonsil tissue of children and young adults, and these responses were successfully supressed by Tregs via CTLA-4 and PDL-1 [227], inhibitory molecules required for these inhibitory functions [427]. These authors also showed the presence of PLY-specific Tregs in tonsil tissue [227]. This suggests that the population of pneumococcal-specific Tregs found in older volunteers of chapter 4, could potentially control IFN-γ$^+$T-bet$^+$ and IL-17A$^+$RORγt$^+$ responses to pneumococci, migrating alongside these inflammatory subsets towards the upper airway during colonisation.

Whereas the ratio of Th17/Treg cells increases with age, being low in children and higher in carriage-negative children, compared to carriage-positive [304], this association was not found here in older adults. In mice [303,401] and humans [262], carriage increases the frequency of Tregs, leading to stable colonisation, but stable colonisation is rarely observed in the old [197]. One hypothesis is that as ageing is associated with an increase in systemic IFN-γ$^+$CD4$^+$ T cell frequency [156–158], the microenvironment in which $CD4^+$T cells differentiate during experimental colonisation in older adults may favour differentiation into the IFN-γ-producing subset. As effector cells, IFN-γ$^+$CD4$^+$ T cells could then activate macrophages in the nasopharynx for clearance of colonising bacteria. Moreover, because induction of a Th1 response negatively regulates Th17 differentiation [295,413,414], the age-related skew towards the IFN-γ-producing subset could interfere with the dynamic balance

between Th17/Tregs, not favouring stable colonisation and establishment of Th17 cellular memory (Figure 5.1 B).

Indeed, as demonstrated in chapter 4, older volunteers had very low frequencies of central memory IL-17A^{+}RORγt^{+} cells following experimental colonisation, whereas this subset was present in carriage-negative and positive young participants. Furthermore, during pneumonia infection, the majority of IL-17A^{+}CD4^{+} T cells found in older patients were naïve, reinforcing the hypothesis that absence of memory IL-17A^{+}CD4^{+} T cells may be involved in susceptibility to pneumonia. Interestingly, a study demonstrated that following stimulation of PBMCs with pneumococcal proteins PcsB, StkP and PsaA, both Th1 and Th17 responses were detected in young and middle-aged adults, while most of the responses of old donor cells were Th1 or Th17 [301]. Thus, as suggested by Ferreira and colleagues [428], absence colonisation episodes in the elderly, potentially mediated by increased Th1 frequency as demonstrated in chapter 4, would contribute to poor functionality and lower levels of anti-pneumococcal antibody [62,301,318]. In the young, a balance between Th17 and Treg could promote clearance by Th17-mediated mechanisms or stable colonisation by Tregs. A second hypothesis is that the increased frequency of GARP^{+}Tregs is actively supressing Th17 cell activity in the nasopharynx of older adults [227,429].

These hypotheses can be initially tested by immunophenotyping of immune cells and cytokine dosage in nasal washes from experimentally colonised volunteers and by stimulation of isolated subsets. But perhaps, a more basic question may be to confirm if inflammaging, which is determined by the presence of pro-inflammatory cytokines in serum, is also detected in the nasopharynx of older adults, as observed in aged mice, where NALT gene expression of pro-inflammatory mediators such as CCL2 and TLR1 was increased [386].

5.1.3 Immunity to *S. pneumoniae* in older patients with pneumonia

The initial hypothesis of this thesis was that an impaired ability of neutrophils to successfully generate ROS and NETs in response to *S. pneumoniae* would be aggravated during pneumonia disease in the aged, culminating in failure to eliminate the bacteria, leading to accumulation of functionally impaired neutrophils in the lung [109]. However, the findings presented in Chapter 3 showed that neutrophils from healthy older donors did not have significant impairment in response to *S. pneumoniae* and that, during disease, in fact, neutrophils generated higher levels of ROS in response to the *S. pneumoniae* serotypes tested, while NET generation was reduced. Neutrophil IL-8 production had a trend towards reduction in older patients and was significantly lower following 23F incubation. Thus, neutrophil-IL-8 production in response to *S. pneumoniae* is unlikely to explain elevated neutrophil influx to the lungs found in older patients [109]. Conversely, monocytes from old pneumonia patients showed higher IL-6 production in response to 23F, compared to monocytes from healthy old subjects. Interestingly, the trend observed in neutrophil IL-8 production was opposite to monocyte IL-6 production, in which serotypes 4, 19A and 23F induced the highest to the lowest IL-8 production in neutrophils but showed the inverse trend in IL-6 production.

Another key finding was that patients had a heterogenous neutrophil population. The presence of $CD16^{high}CD62L^{dim}$ neutrophils was also found in the healthy aged cohort, but older patients additionally had immature granulocytes as a third neutrophil phenotype in peripheral blood. The release of immature granulocytes may be related to increased G-CSF, which mediates this transit [111] and is increased in patients with bacterial infections [430]. The release of immature granulocytes could be a strategy to compensate for a population of

functionally impaired $CD16^{high}CD62L^{dim}$ neutrophils, but immature neutrophils also have reduced function, as demonstrated in patients with sepsis [115]. Thus, if immature granulocytes are also functionally impaired during pneumonia, there could be accumulation of non-functional neutrophils in the lungs, as reported previously [109]. The change in neutrophil population during infection may therefore be a key factor compromising immunity in older patients.

Another relevant consideration is whether these neutrophil populations identified in peripheral blood in chapter 3 are also present in the lungs during pneumonia. A study of patients with acute lung inflammatory syndrome (ARDS) showed a high frequency of hypersegmented neutrophils in BAL [431], compared to blood, which were very similar to the phenotype described by Pillay *et al.* (2012) [118]. As the former study did not perform CD16 immunostaining, it is not possible to confirm whether these were $CD16^{high}CD62L^{dim}$ neutrophils, but cells did have the $CD11b^{high}CD62L^{dim}$ phenotype previously described [118]. Thus, there is evidence that neutrophil populations are distinct in the blood and lungs in ARDS, suggesting this diversity of neutrophil population may also occur in the lung of older patients. Moreover, blood neutrophils from ARDS patients which exhibited elevated ROS production, had this function controlled by PI3k inhibition [431]. Nevertheless, the extent to which $CD16^{high}CD62L^{dim}$ and immature neutrophils contribute to disease outcome and if they are present in the lung of patients during pneumonia needs further research.

Data from Chapter 3 showed that during pneumonia infection, neutrophils of older patients had elevated TLR2 expression. The literature suggests that TLR2 and CCR2 receptors may have a dynamic interaction. In mice, activation of neutrophil TLR2 by lipoteichoic acid (LTA), a polysaccharide present on the cell membrane and wall of the pneumococcus [232], was

shown to downregulate CCR2 expression in neutrophils, thus impairing their migration [432]. Conversely, it is reported that in older patients with pneumococcal pneumonia, neutrophil infiltration in the lungs is much higher than in young [109]. In this thesis neutrophil CCR2 surface expression was not assessed. In other cohorts, of healthy older adults [141] and COPD patients [433], neutrophil CXCR2 expression was comparable to that of healthy controls. In chapter 3, CCR2 expression was only found increased in non-classical monocytes of older patients, similar to a report of elevated CCR2 expression in intermediate monocytes of old donors [89]. Therefore, CCR2 expression could actually be increased in the old during pneumonia infection, thus driving neutrophils to the lungs. There, overactivation of neutrophils in response to the pneumococcus, such as elevated ROS production as reported in chapter 3, could lead to damage of lung epithelial cells and production IL-33 [380,381]. IL-33 can inhibit TLR2-mediated downregulation of CXCR2 in neutrophils [383], which could lead to continuous neutrophil migration to the lungs, leading to more tissue damage.

High levels of IL-33 are of interest as these may be associated with activation of Tregs, as data from Chapter 4 suggests. $CD4^+$ T cell polarisation during pneumonia in the aged was marked by elevated frequency of subsets IFN-γ^+ and IL-$17A^+$, as well as an elevated frequency of activated Tregs at baseline. It is yet to be confirmed if IL-33 is a factor driving GARP expression in Tregs, but in regard to this association, Tregs expressing the IL-33 receptor ST2 are highly activated [434], suggesting that GARP and ST2 expression increase together and both correlate with increased function. Upon stimulation with anti-CD3 and anti-CD28, the frequency of $GARP^+$Tregs of older patients increased as well as IL-10 and TGF-β1 production, demonstrating that during pneumonia infection, these cells remain functional in older patients. However, while the frequency of pro-inflammatory $CD4^+$ T cell

subsets IFN-γ^+ and IL-17A$^+$ increased with advancing age of patients, Treg frequency did not. IFN-γ can induce apoptosis in epithelial lung cells [435,436], and could be one factor causing IL-33 release from damaged lungs cells (Figure 5.1 C).

In addition to IL-33-mediated neutrophil recruitment via TLR2 and CCR2 [383], recent findings have demonstrated this cytokine can mediate detrimental effects in mice, such as amplification of lung macrophage IL-12 and NK cell IFN-γ production, leading to an increased Th1-cell-like inflammatory response in a COPD model [380], to sepsis-induced immunosuppression by contributing to expansion of Treg population, leaving mice susceptible to secondary pneumonia [420] and promoting loss of immunologic tolerance in the lungs of mice, mediated by Tregs expressing GATA3 after IL-33 exposure [437]. Therefore, investigation of the role of IL-33 during pneumococcal pneumonia should add to the comprehension of disease mechanisms in the aged.

5.1.2 Limitations of the thesis studies

One limitation of the pneumonia disease study was that the causative pathogen of pneumonia infection in patients recruited was not established. Although it was expected that *S. pneumoniae* would be the main pathogen among patients, this could not be confirmed, as this was a pilot study and did not include detailed microbiological investigations by the hospital. Another issue was the difficulty of recruiting young pneumonia patients, as very few were admitted to the hospital during the recruitment periods. Most young people with pneumonia are not ill enough to be admitted to hospital. Presence of comorbidities is also an issue in older patients and thus it is hard to say with certainty which deficits seen were due purely to age rather than multimorbidity. It would

also have been interesting to verify if alteration in immune function and cell population frequencies at later time points such as 7 and 30 days later and 6 months, to determine if alterations found were transitory, as well as if pneumococcal carriage can be found post disease.

The use of an experimental human carriage model in older volunteers is a novel and valuable approach to study pneumococcal colonisation. This was the first study to use samples from an older cohort for the study of $CD4^+$ T cells. For safety reasons, recruitment of older volunteers started at age 50, and progressed towards older participants once volunteers did not become ill. As a result, not all participants were over 65 years. Also, as this work was done in collaboration, the samples from young participants available post-colonisation were from a later time point. These factors could explain the lack of significant differences between pre and post data as well as among age groups.

Finally, this thesis proposed to assess age-related changes in neutrophil microbicidal functions by measuring ROS production and NET generation in response to *S. pneumoniae*. Although this objective was achieved, confirmation of bacterial killing still needs to be measured by a killing assay. Moreover, these functions and activation were assessed in blood neutrophils, and it is not possible to confirm that these may be fully replicated in the lungs, particularly during pneumonia infection. This requires further investigation, once previous studies showed that neutrophil with different phenotypes are found in BAL of ARDS patients, compared to blood [431], and that even in healthy individuals, transit of neutrophils to the lungs modify their phenotype towards a more activated one, marked by reduced CD62L expression and increased CD11b, CD54, CD32, CD88 and CD66 [438].

5.1.3 Future directions

This thesis provided information on neutrophil function in older adults during health and pneumonia, together with evidence of $CD4^+$ T cell polarisation during experimental pneumococcal colonisation in the nasopharynx and during pneumonia in the aged. However, many studies are still required for a detailed understanding of the mechanisms driving increased susceptibility to *S. pneumoniae* infection in older adults, so that therapeutic targets can be identified. Some suggested research is:

1. Perform neutrophil killing assays to determine if *S. pneumoniae* killing correlates with ROS generation;
2. Test neutrophil assays using isogenic TIGR4 strains expressing different capsules, to determine the extent of the role of pneumococcal capsule in neutrophil activation in older adults;
3. Examine monocyte and neutrophil viability after incubation with *S. pneumoniae* and PLY in a larger sample size to verify if viability of old donor cells is compromised upon contact with the pneumococcus or its toxin;
4. Pre-incubation of isolated neutrophils from healthy older adults with serum (or plasma) from old patients, to determine if ROS production and NET generation are modulated by humoral inflammatory mediators;

5. Investigation of PI3K class III signalling in NET generation in response to *S. pneumoniae* in older adults, to verify if activity of this class of PI3K is impaired with ageing;
6. Test if treatment with IL-10 and TGF-β1 could normalise neutrophil ROS and NET generation in old patients with pneumonia, to examine if neutrophils are still sensitive to regulatory cytokines released by Tregs during disease;
7. Measure expression of CCR2 and ST2 (IL-33) receptor in neutrophils of healthy old and pneumonia patients to verify if expression of these receptors is changed with ageing and disease;
8. Analyse the immune cell populations in the nasopharynx of older adults, before and after experimental colonisation. (Currently ongoing study at Dr. Daniela Ferreira's lab at LSTM).
9. Analyse neutrophil populations (immature, $CD16^{high}CD62L^{dim}$) and isolated neutrophil microbicidal functions in response to *S. pneumoniae* in frail older adults.
10. Investigate the effect of age on mTOR signalling on NET release [439] and neutrophil hypersegmentation [440], in light of the beneficial effects of mTOR inhibitor therapy in older adults, which successfully enhanced their response to influenza vaccination [441].

5.1.4 Conclusions

The work described in this thesis has shown for the first time age-related changes in neutrophil ROS production and NET in response to different serotypes of *S. pneumoniae*, and

that these functions are changed during pneumonia in old individuals. Additionally, heterogeneity of the neutrophil population in blood was demonstrated in older adults during pneumonia. Using the experimental human pneumococcal colonisation model, it was demonstrated for the first time that older adults fail to generate memory IL-17^{+}RORγ^{+}CD4+T cells, have lower frequencies of this subset, together with age-related increase in the Th1 subset, and 6B-responding Tregs. These populations were also detected during pneumonia infection, in which frequencies of pro-inflammatory CD4^{+} T cell subsets increased in the very old, whereas Treg population did not, although they retained the production of their regulatory cytokines. Overall, our data brings new information to the original hypothesis that impairment of neutrophil function was one of the underlying causes of susceptibility of older adults to pneumococcal pneumonia, showing that reduced function is not found in response to all serotypes and that not all functions are impaired. Moreover, despite the ongoing infection, older patients can produce a high pro-inflammatory CD4-mediated immunity, which can be detrimental to lung tissue.

Chapter 6 - References

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Appendices

Appendix I – Review article, posters, awards and presentations during the completion of this PhD

Published article

Gonçalves MT, Mitchell TJ, Lord JM. Immune ageing and susceptibility to *Streptococcus pneumoniae*. Biogerontology. 2016;17(3):449–65.

Abstracts

Gonçalves MT, Mitchell TJ, Lord JM. The effect of age neutrophil on responses to *S. pneumoniae*. 10th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD 2016). Glasgow, Scotland, 26-30 June 2016.

Gonçalves MT, Mitchell TJ, Lord JM. Effect of age on neutrophil bactericidal functions. Festival of Graduate Research, University of Birmingham, March 2017.

Presentation

Gonçalves MT. The effect of age neutrophil on responses to *S. pneumoniae*. E-poster presentation: 10th International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD 2016). Glasgow, Scotland, 26-30 June 2016.

Prize won

Gonçalves MT, Mitchell TJ, Lord JM. Defining the mechanisms underlying reduced immunity to *Streptococcus pneumoniae* with age. Best Poster (College of Medical and Dental Sciences). Research Poster Conference, University of Birmingham (Birmingham, UK), 2016.

Appendix II – Neutrophil and monocyte median fluorescence intensity (MFI) data and statistical analysis

Table A.2. Age-related changes in neutrophil receptor expression and intracellular IL-8 production

			Healthy young			Healthy old			Older patients		
CD11b	**Control**	**stimulations**	**Control**	**stimulations**	**p value**	**Control**	**stimulations**	**p value**	**Control**	**stimulations**	**p value**
		LPS		18460 ± 3267	**0.0026**		18355 ± 1294	**0.0026**		16023 ± 3139	**0.0026**
		TIGR4		15489 ± 3503	**0.0026**		13319 ± 4011	**0.0026**		13269 ± 3260	**0.0026**
	Unstimulated	**19A**	4233 ±	12909 ± 3058	**0.0026**	4195 ±	11072 ± 4187	**0.0026**	7540 ±	11951 ± 3292	**0.0026**
	vs	**23F**	1066	11888 ± 2629	**0.0026**	1294	12306 ± 2328	**0.0026**	2259	10457 ± 2750	**0.0026**
		HK TIGR4		8586 ± 2874	0.0416		5000 ± 1316	0.0416		8833 ± 2015	0.0208
		Δcps		4696 ± 1706	0.0364		4131 ± 1422	0.1562		7186 ± 2027	0.0364
CD62L	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		305.9 ± 103.6	**0.0026**		325.1 ± 106	**0.0026**		721.6 ± 325.6	**0.0026**
		TIGR4		2913 ± 3853	**0.0026**		4920 ± 4104	**0.0026**		8737 ± 3742	**0.0026**
	Unstimulated	**19A**	19214 ±	8585 ± 4049	**0.0026**	22377 ±	12411 ± 7410	**0.0052**	15807 ±	11166 ± 4064	**0.0026**
	vs	**23F**	6096	10222 ± 5237	**0.0026**	4023	9208 ± 6609	**0.0026**	3010	13505 ± 3547	**0.0078**
		HK TIGR4		14287 ± 780.5	0.1666		21078 ± 2767	0.0416		21078 ± 2767	0.0625
		Δcps		19527 ± 5641	0.1562		22477 ± 4298	0.1666		16903 ± 3018	**0.0026**
TLR2	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		1222 ± 437.6	**0.0052**		1021 ± 400.5	**0.0026**		1843 ± 611.1	**0.0052**
		TIGR4		1705 ± 315.9	0.1145		1667 ± 348.5	0.1145		2714 ± 881.2	**0.0026**
	Unstimulated	**19A**	1651 ±	1585 ± 250.5	0.0963	1633 ±	1642 ± 235.9	0.1354	2317 ±	2590 ± 849.2	**0.0026**
	vs	**23F**	121.1	1793 ± 309.6	0.0364	295.7	1657 ± 299.4	0.08853	729.1	2443 ± 778.3	0.0182
		HK TIGR4		1909 ± 148.1	0.0416		1401 ± 85.24	0.125		2233 ± 934.3	0.0416
		Δcps		1725 ± 229.7	0.0625		1676 ± 176.9	0.0833		2259 ± 836.5	0.0677
TLR4	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		233.1 ± 31.09	**0.0026**		240.3 ± 24.7	0.0468		326.5 ± 88.84	0.0156
		TIGR4		382.3 ± 54.28	0.0625		492.4 ± 200.4	0.026		503.8 ± 121.5	0.1145
	Unstimulated	**19A**	415.6 ±	483.7 ± 66.02	0.026	308	364.7 ± 87.95	0.0625	503.8	487.5 ± 79.26	0.1145
	vs	**23F**	120	490.6 ± 89.76	**0.0052**	118.7	344.9 ± 68.27	0.0625	121.8	546.7 ± 66.21	0.0937
		HK TIGR4		431.7 ± 109.8	0.0833		291.3 ± 33.5	0.1666		385.7 ± 92.46	0.0416
		Δcps		410.7 ± 211.4	0.1354		279.3 ± 122.6	0.0625		338.8	0.0156
IL-8	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		619.9 ± 244.1	**0.0026**		830.4 ± 388.4	**0.0026**		526.3 ± 269.4	**0.0026**
		TIGR4		1772 ± 1218	**0.0026**		1376 ± 790.6	**0.0026**		826.7 ± 503.7	**0.0026**
	Unstimulated	**19A**	272.3	1298 ± 774.5	**0.0026**	401.9 ±	1193 ± 634.5	**0.0078**	239.9 ±	661.4 ± 427.4	**0.0026**
	vs	**23F**	23.45	822.4 ± 507.9	**0.0026**	112.6	897.9 ± 327.1	**0.0078**	98.16	403.4 ± 249.3	0.013
		HK TIGR4		358 ± 173.4	0.0416		378 ± 33.45	0.125		173.8 ± 87.94	0.0416
		Δcps		268 ± 38.03	0.1145		343.4 ± 52.21	0.0468		192.7 ± 81.48	**0.0078**

[Caption on the next page]

Expression of CD11b, CD62L, TLR2, TLR4 and intracellular IL-8 following incubation of neutrophils from healthy young (n=7), old (n=7) donors and older patients with pneumonia (n=7) following 4-hour stimulation with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40). Data are presented as median fluorescence intensity (MFI) and shown as mean ± SD. Differences between unstimulated control and stimulations were performed using Wilcoxon test. Significant differences ($p < 0.0084$) are indicated in bold font.

Table A. 3. Age-related changes in monocyte receptor expression and intracellular TNF-α and IL-6 production

			Healthy young			Healthy old			Older patients		
CD11b	**Control**	**stimulations**	**Control**	**stimulations**	**p value**	**Control**	**stimulations**	**p value**	**Control**	**stimulations**	**p value**
		LPS		44478 ± 21097	0.0208		24769 ± 7405	**0.0026**		22038 ± 3573	0.0911
	Unstimulated	**TIGR4**	32270 ±	10109 ± 7249	0.0104	20763 ±	11283 ± 2872	**0.0026**	22132 ±	12321 ± 3997	**0.0013**
	vs	**19A**	19302	17499 ± 11673	0.0104	7380	14396 ± 5022	**0.0052**	5761	13532 ± 3617	**0.0026**
		23F		25933 ± 21778	0.0208		14687 ± 4618	**0.0052**		18074 ± 4097	**0.0013**
CCR2	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		28248 ± 1391	0.1041		31301 ± 6105	0.0260		35069 ± 8094	0.1406
	Unstimulated	**TIGR4**	27549 ±	11206 ± 6759	0.0104	33130 ±	17729 ± 10924	**0.0026**	34691 ±	26973 ± 7606	**0.0013**
	vs	**19A**	5128	13437 ± 5986	0.0104	7888	19836 ± 7917	**0.0052**	6886	27230 ± 9324	**0.0013**
		23F		17010 ± 7599	0.0104		21647 ± 5847	**0.0026**		33366 ± 7122	0.0182
TNF-α	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		21658 ± 6798	0.0104		20244 ± 6240	**0.0026**		15500 ± 9881	**0.0013**
	Unstimulated	**TIGR4**	4430 ±	8379 ± 6029	0.0104	3116 ±	5014 ± 1973	**0.0026**	3539 ±	5377 ± 3226	0.0247
	vs	**19A**	3066	4601 ± 1330	0.1041	517	5213 ± 3239	0.0260	1814	6546 ± 3452	0.0091
		23F		6372 ± 1606	0.0729		6140 ± 1597	**0.0026**		9570 ± 7352	0.0130
IL-6	**Control**	**stimulations**	Control	stimulations	p value	Control	stimulations	p value	Control	stimulations	p value
		LPS		1190 ± 414.6	0.0104		1361 ± 240.4	**0.0026**		1404 ± 1112	**0.0013**
	Unstimulated	**TIGR4**	132.3 ±	177.6 ± 186.2	0.1354	94.74 ±	57.61 ± 13.51	**0.0026**	179.8 ±	44.94 ± 19.09	0.0208
	vs	**19A**	92.26	85.85 ± 48.89	0.0208	27.76	141.4 ± 123	0.1666	135.6	92.4 ± 58.4	**0.0013**
		23F		153.4 ± 68.27	0.1666		157.1 ± 109.2	0.0494		257.7 ± 200.7	0.0325

Expression of CD11b, CCR2 and intracellular TNF-α and IL-6 following incubation of monocytes from healthy young (n=7), old (n=7) donors and older patients with pneumonia (n=7) following 4-hour stimulation with LPS (50ng/ml) or *S. pneumoniae* serotypes (MOI 40). Data are presented as median fluorescence intensity (MFI) and shown as mean ± SD. Differences between unstimulated control and stimulations were performed using Wilcoxon test. Significant differences ($p < 0.0084$) are indicated in bold font.