AN AUGMENTED PASSIVE IMMUNOTHERAPY TO TREAT PNEUMOCOCCAL DISEASES

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BY

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Declaration

This thesis is the result of my own work and effort. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or other qualification.

Research in this thesis was carried out at the Liverpool School of Tropical Medicine (Liverpool, UK), Institute of Infection and Global Health (Liverpool, UK), Department of Infection, Inflammation and Infection (Leicester, UK), Centres for Disease Control and Prevention (Atlanta, USA) and the Malawi-Liverpool-Wellcome Trust Research Laboratories (Blantyre, Malawi).

Abstract

Background: Bacterial infections, particularly those of the respiratory tract, are a serious cause of mortality worldwide despite vaccinations and optimized treatment strategies. Prior to the introduction of antibiotics, passive immunotherapy was widely used to treat a range of bacterial infections. A successful opsonin based treatment, however, requires effective clearance by phagocytic cells. Augmented passive immunotherapy (API) is a novel treatment strategy that combines pathogen specific immunoglobulin (IVIG) and the immunomodulating peptide P4 to treat fulminant bacterial infections. Using *in vivo*, *in vitro* and *ex vivo* models of pneumococcal infection, this thesis aimed to describe host responses to API.

Methods: The *in vivo* recruitment and activation of phagocytes following peptide treatment in the absence of infection was characterised using flow cytometry and electron microscope imaging. These findings were translated to murine infection models by treating pneumococcal septicemia with intravenous peptide administration and pneumococcal pneumonia with intranasal peptide administration. *In vitro* phagocytosis killing assays were used to determine the ability of P4 to augment bacterial killing of a range of pneumococcal serotypes and describe the involvement of phagocytic Fcγ receptors. *Ex vivo* assays of phagocytic function of human neutrophil and alveolar macrophages were used to translate murine and *in vitro* findings.

Results: Peptide administration in the absence and presence of infection led to rapid recruitment of monocytes, neutrophils and macrophages and their activation as demonstrated by increased FcγR expression. Intravenous peptide administration during septicemia led to significantly increased survival rates in models of invasive and acute invasive pneumococcal diseases in young and aged mice of various genetic backgrounds. Intranasal peptide administration during pneumonia prevented the onset of septicemia and subsequent host mortality. Increased survival was associated with reduced bacterial burden in affected tissue. Peptide treatment of neutrophil cell lines, human neutrophils and alveolar macrophages modulates FcγR expression resulting in augmented phagocytic killing of opsonised pneumococci.

Conclusion: Augmented passive immunotherapy is a synergistic treatment that enhances natural host immune responses to infection by opsonizing pathogens and inducing their effective phagocytic clearance.

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Although scientific input by my parents was limited, this work would not have been possible without their *Durchhalteparolen* and *phrases de motivation*. Sylvie, Hans, merci pour tout – ohne euch wäre ich nicht hier. To my brother Jan - our discussions and face *à* face have always been therapeutical and motivating– thank you for everything.

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Contents

List of abbreviations

API	Augmented passive immunotherapy
aIPD	Acute invasive pneumococcal disease
AM	Alveolar macrophage
BHI	Brain heart infusion
BA	Blood agar
BAL	Bronchoalveolar lavage
CFU	Colony forming unit
DEPC	Diethylpyrocarbonate treated water
FBS	Fetal bovine serum
HAM	Human alveolar macrophage
IPD	Invasive pneumococcal disease
IP	Intraperitoneal
IN	Intranasal
IV	Intravenous
IVIG	Intravenous immunoglobulin
LPS	Lipopolysaccharide
MR	Mannose receptor
OPKA	Opsonophagocytosis killing assay
P4	Immunoactivating peptide
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
PsaA	Pneumococcal surface adhesin A
Pnc	Pneumococcus
PM	Peritoneal macrophage
PMNL	Polymorphonuclear leukocytes
ROS	Reactive oxygen species
SR	Scavenger receptor
ST	Serotype
TLR	Toll-like receptor

CHAPTER I.

Introduction

Preface

Opsono - *Preparing ...*
phago - *... to devour ...*
cytosis *... a cell.*

The term phagocytosis was first coined in 1882 by Russian scientist Elie Metchnikoff who at the time was working in the laboratory of Louis Pasteur in France. Metchnikoff noted that "wandering cells" in starfish larvae would engulf particles he had previously injected [1]. This was the first time phagocytosis had been described and recognized as being part of the immune response to infection:

> *"These wandering cells in the body of the larva of a starfish, these cells eat food ... but they must eat up microbes too! Of course the wandering cells are what protect the starfish from microbes! Our wandering cells, the white cells of our blood – they must be what protects us from invading germs." Elie Metchnikoff, 1882*

At the time these findings were controversial as competing researchers working in the laboratory of Robert Koch in Germany hypothesized that immunity against infection was delayed, and mediated through components present in serum. Previously, Koch's laboratory had described the first success in immunotherapy by generating therapeutic antibodies for transfer in animal models of diphtheria and tetanus. In addition, Koch's group noticed that serum generated following vaccination would lyse bacteria in the absence of immune cells, further supporting their theory of the importance of serum in immunity [2].

Debate as to the nature of immune responses to infection would continue until British scientists Wright and Douglas followed up on Metchnikoff's paradigm-shifting discovery. They performed experiments in which immune cells were allowed to phagocytose bacteria with normal or heat inactivated serum [3]. Their conclusions would support the notion of a cellular innate immune response hypothesized in the Pasteur laboratory:

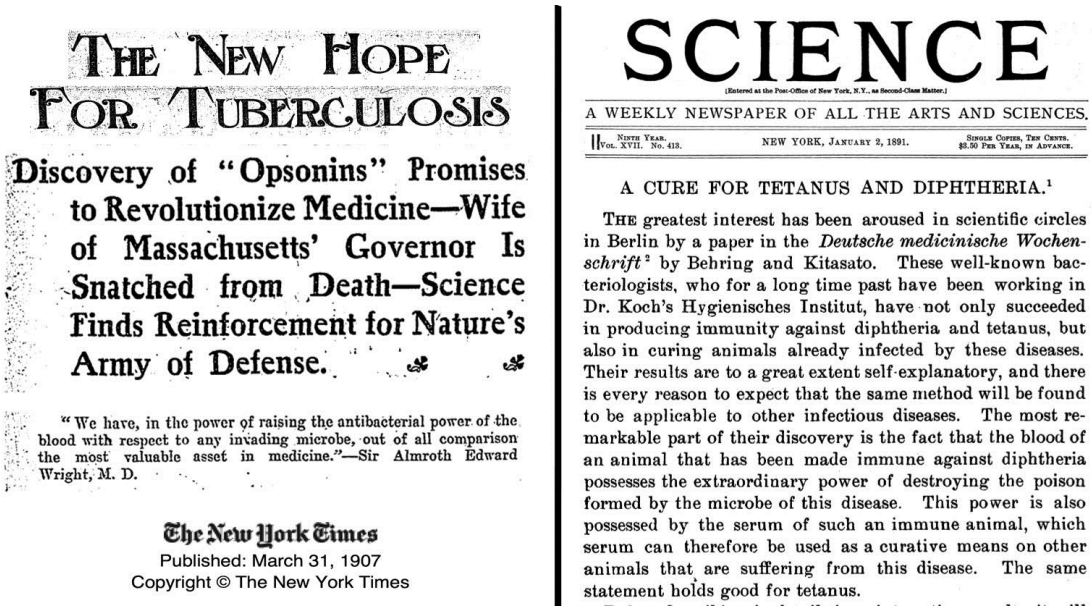
> *"We have here conclusive proof that the blood fluids modify the bacteria in a manner which renders them a ready prey to the phagocytes. We may speak of this as an "opsonic" effect and we may employ the term "opsonins" to designate the elements in the blood fluid which produce this effect." Wright et al, 1903*

Confirmatory evidence eventually led to the 1908 Nobel Prize in Medicine awarded to both Metchnikoff (Pasteur) and Ehrlich (Koch) for their work in describing phagocytes as part of an innate immune response utilizing components of a delayed immune response. During his acceptance speech, Ehrlich suggested a mechanism by which these two systems could interact:

> *"There are a series of … substances, probably mostly of protein nature, which … lure … these structures [into cells] by … the opsonins on the one hand, and the … toxins … on the other. I describe all the [substances] … as nutriceptors and would regard these … as the source of the antibodies which are theoretically and practically so important." Paul Ehrlich, Nobel Lecture, 1908*

While Ehrlich was wrong in stating that "nutriceptors" were the source of antibody, he correctly suggested that receptors would aid phagocytes to interact with opsonized particles. These were the first descriptions of an interaction between innate cellular immunity and adaptive serum based immunity that would open the door to the technologies of vaccination and serum therapy.

The media recognized the impact these discoveries had on medicine and health and hailed their potential as new treatments (Figure 1). Bacterial diseases, particularly tuberculosis and pneumonia, were causing a high burden of mortality at the time. Clinical treatments using crude plant extracts or other anti-microbial compounds such as arsenic and hexamine were largely ineffective.



THE NEW HOPE FOR TUBERCULOSIS

Discovery of "Opsonins" Promises to Revolutionize Medicine—Wife of Massachusetts' Governor Is Snatched from Death—Science Finds Reinforcement for Nature's Army of Defense.

"We have, in the power of raising the antibacterial power of the blood with respect to any invading microbe, out of all comparison the most valuable asset in medicine."—Sir Almroth Edward Wright, M. D.

The New York Times
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SCIENCE

[Entered at the Post-Office of New York, N.Y., as Second-Class Matter.]

A WEEKLY NEWSPAPER OF ALL THE ARTS AND SCIENCES.

Vol. XVII. No. 413. NEW YORK, JANUARY 2, 1891. Single Copies, Ten Cents. $5.00 Per Year, in Advance.

A CURE FOR TETANUS AND DIPHTHERIA.[1]

THE greatest interest has been aroused in scientific circles in Berlin by a paper in the *Deutsche medicinische Wochenschrift*[2] by Behring and Kitasato. These well-known bacteriologists, who for a long time past have been working in Dr. Koch's Hygienisches Institut, have not only succeeded in producing immunity against diphtheria and tetanus, but also in curing animals already infected by these diseases. Their results are to a great extent self-explanatory, and there is every reason to expect that the same method will be found to be applicable to other infectious diseases. The most remarkable part of their discovery is the fact that the blood of an animal that has been made immune against diphtheria possesses the extraordinary power of destroying the poison formed by the microbe of this disease. This power is also possessed by the serum of such an immune animal, which serum can therefore be used as a curative means on other animals that are suffering from this disease. The same statement holds good for tetanus.

Figure 1 – (A) Article published in the New York Times in 1907 highlighting the discovery of opsonins in serum by British scientists Wright & Douglas, and a successful clinical application of passive immunotherapy. (B) Article published in Science in 1891 describing the first use of passive immunotherapy against tetanus and diphtheria by German scientist Behring and his Japanese co-worker Shibasaburō.

Since then, our understanding of opsonins, opsonic receptors, phagocytes and passive immunotherapy has vastly improved but the fundamental principles suggested by Metchnikoff, Wright and Ehrlich remain the same. This thesis revisits these principles by describing the current knowledge of opsonophagocytosis, phagocytes and serum therapy and assesses the potential of a novel augmented passive immunotherapy to treat pneumococcal diseases.

A. Opsonophagocytosis

Phagocytosis is the process in which specialized immune cells called phagocytes engulf foreign particles, pathogens, dead or dying cells. In order to facilitate phagocytosis of pathogens, serum proteins (opsonins) bind to and mark pathogens for removal [4]. Immunoglobulin, complement and non-specific opsonins are proteins that can opsonize pathogens and together can recognize >10 billion potential targets [5]. To some extent, phagocytosis can occur independent of opsonins but this is limited to small particles, or recognition of molecules highly conserved throughout pathogen species. Figure 2 depicts five ways in which particles can be internalized into phagocytes.

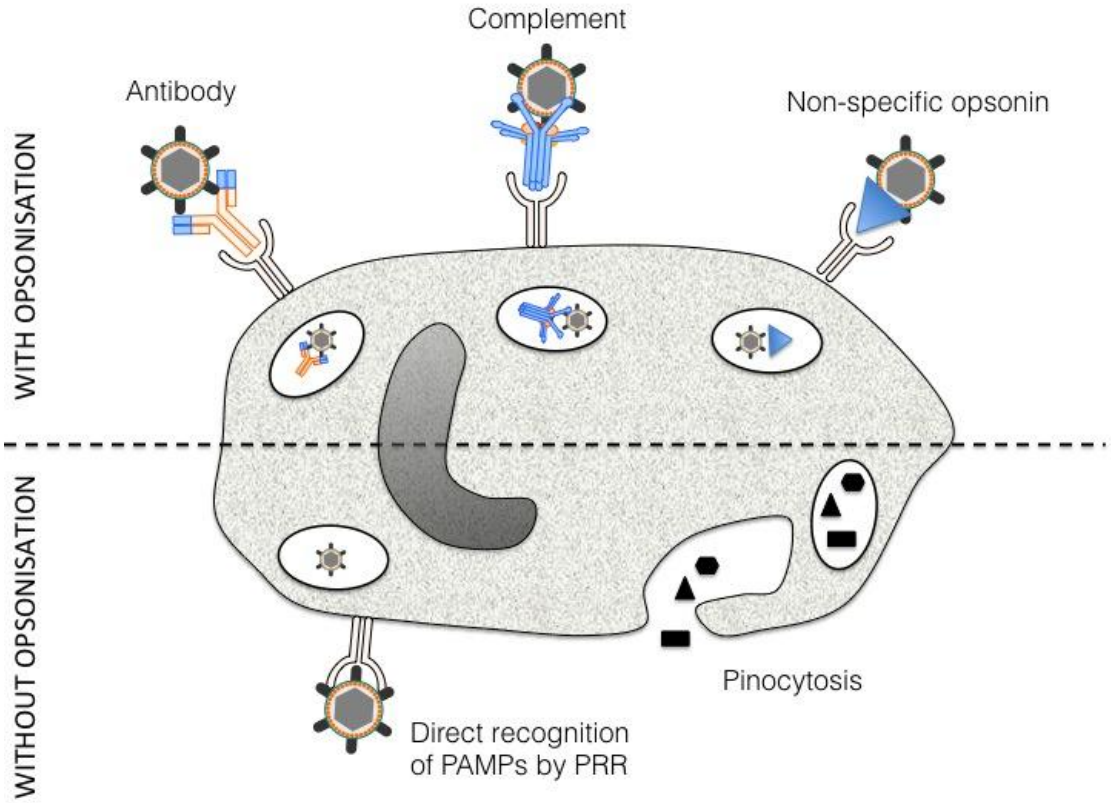


Figure 2 - There are several ways in which phagocytes can engulf particles. Either with the help of opsonins including antibody, complement and non-specific opsonins (top) or without opsonins through the direct recognition of Pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRR) or through pinocytosis (bottom).

The process of opsonophagocytosis can be broken down into several steps. First, an invading pathogen is exposed to serum proteins that are able to recognize and bind to it thereby forming an immune complex (1.1). This is then recognized by surface bound phagocytic receptors found on immune cells (1.2). Ligation of these receptors leads to the internalization of the immune-complex and subsequent destruction of the pathogen (1.3).

1.1 Opsonins

1.1.1 Immunoglobulin

Immunoglobulin (antibodies) are "Y" shaped globular plasma proteins produced by B-cells as part of the adaptive immune response to infection [6]. Immunoglobulin consists of two identical *heavy* and two identical *light* polypeptide chains, where both heavy and light chains have *constant* and *variable* domains (Figure 3) [7].

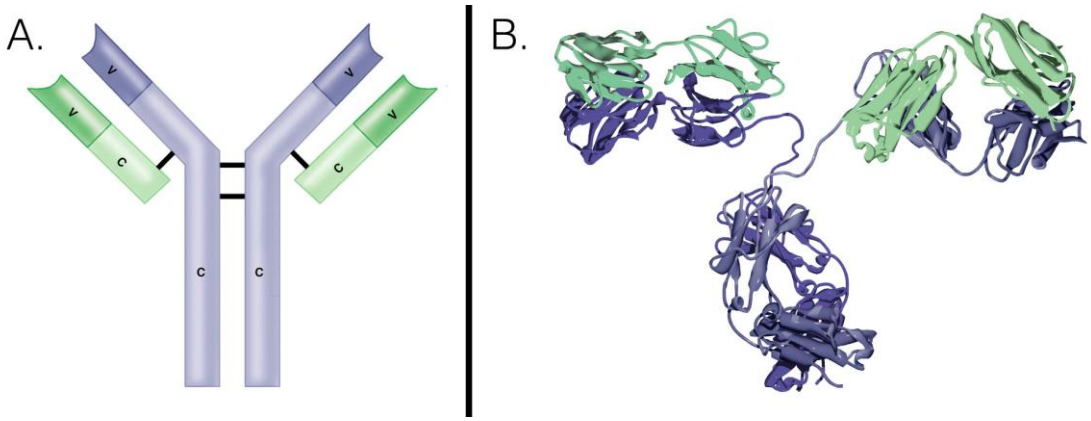


Figure 3 – Immunoglobulin structure. (A.) Schematic of Immunoglobulin showing the heavy (blue) and light (green) chains. Each chain is further divided into variable (marked as 'V') and constant (marked as 'C') domains. (B.) 3D model of Immunoglobulin showing the heavy (blue) and light (green) chains. Adapted from [8] and protein data bank (PDB) 1IGT.

The structure of immunoglobulin can be divided depending on its function. The top "v" shape is called the Fab region (*Fragment, antigen binding*) in which the variable domains are able to recognize and bind to specific molecules

(antigens) on a pathogen. Antigens are non-self molecules that usually make up important parts of a pathogen, such as capsule or membrane proteins used by bacteria to adhere to epithelial cells. The bottom "I" shape is responsible for binding to immune cells and is called the Fc region (*Fragment, crystallizable*), composed of the constant domains of two heavy chains that bind to Fc receptors on immune cells.

Immune complex

Antibody binding one or more antigens leads to the formation of an immune complex that can aggregate thereby facilitating more antigen binding by antibodies (Figure 4). Once formed, immune complexes are able to activate the complement system, initiate their own clearance through phagocytosis by effector cells [9] or be excreted into mucosal surfaces [10]. Failure to clear immune complex leads to their deposition into tissue such as kidney, skin, lungs joints, or blood vessels and the development of autoimmune diseases including systemic lupus erythematosus (SLE) and arthritis [11].

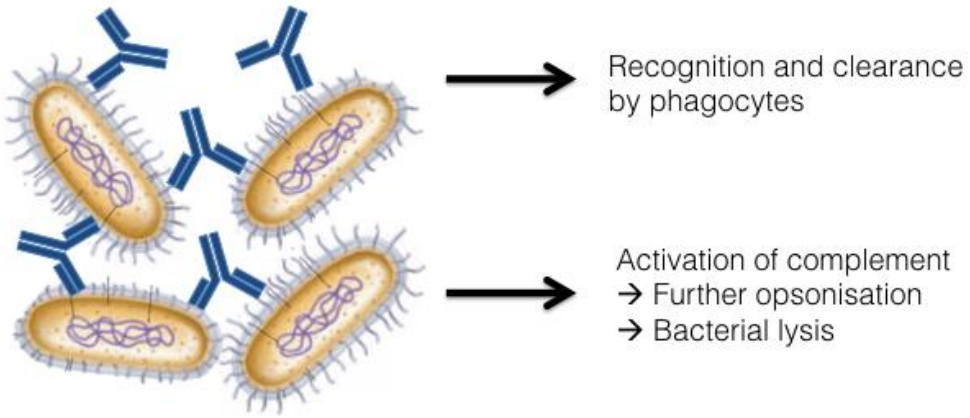


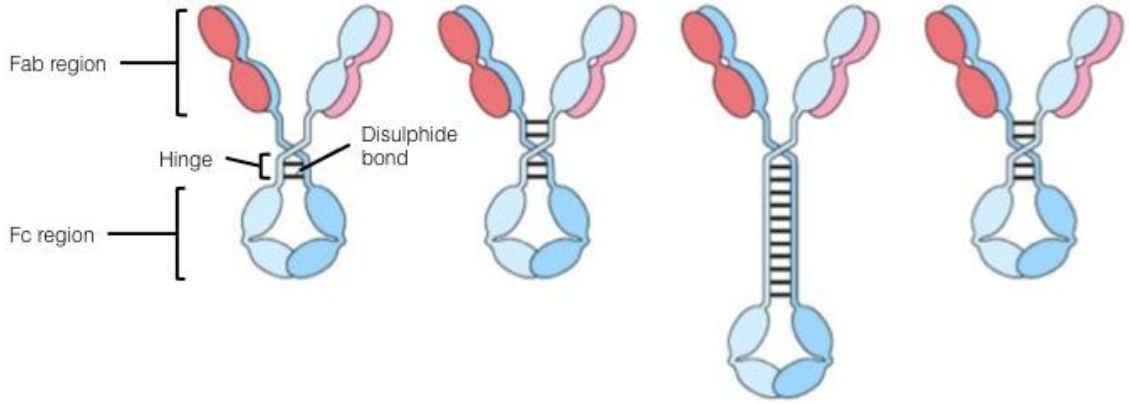
Figure 4 - Two possible outcomes following the formation of an immune complex. Antibody binds to antigen on pathogens and aggregates forming an immune complex. This is then either recognized by phagocytes leading to phagocytosis or activates the complement system leading to further opsonisation and bacterial lysis.

Immunoglobulin opsonisation

There are four classes of immunoglobulin able to opsonize pathogens (IgG, IgA, IgM and IgE) each with separate specificities. Immunoglobulin can be complexed into dimers to form IgA or pentamers to form IgM. IgA is the most abundant antibody in mucosal surfaces (S-IgA, secretory IgA) where it acts to prevent the colonization of bacteria. In serum, IgA is able to opsonize invading pathogens and initiate inflammation as well as phagocytosis [12]. IgM is primarily found in serum during the early stages of infection. On its own, IgM is an ineffective opsonin but contributes to opsonisation through the formation of immune complexes and activation of complement [13]. IgE is present in low quantities and mostly opsonizes parasitic infections [14]. The most important immunoglobulin in defence against infection is IgG as it is able to opsonize a wide range of pathogens and mediates effective phagocytosis. IgG is the most abundant immunoglobulin in serum and the only immunoglobulin capable of crossing the placenta thereby transferring passive immunity to a foetus. IgG also makes up >95% of total immunoglobulin in passive immunotherapy [15]. As such IgG as an opsonin will be reviewed in detail for the purpose of this thesis.

Immunoglobulin G

There are four different isotypes of IgG, IgG1, IgG2, IgG3 and IgG4, with structural differences shown in Figure 5 [16]. These isotypes vary in the size of their hinge region and also with respect to the antigen for which they are specific to. IgG1 and IgG3 target protein antigens and are potent activators of the complement system. IgG2 is most likely to bind to carbohydrate antigens, common throughout bacterial species, and has a weak ability to activate the complement system. IgG4, which is unable to activate the complement system, makes up ~5% of total IgG and is mostly associated with the response to chronic stimulation due its anti-inflammatory properties [17].



	IgG1	IgG2	IgG3	IgG4
% of all IgG	60%	25%	10%	5%
Role in host response	Protein antigens	Carbohydrate antigens	Protein antigens	Response to chronic stimulation, anti-inflammatory
Fc-receptor affinity	High	Low	High	Intermediate
Complement activator	2nd highest	3rd highest	Highest	No activation

Figure 5 - The four isotypes of human IgG and their characteristics in terms of total % of IgG, role in host response, Fc-receptor affinity and ability to activate the complement system. Adapted from [18].

The binding of immunoglobulin to an antigen is the first step in the opsonophagocytosis of a pathogen, but it can also be used to deactivate the function of a toxin. For example, immunoglobulin is produced against the *S. pneumoniae* virulence factor pneumolysin, a pore forming toxin that causes much of the damage associated with pneumococcal disease [19]. Binding of antibody to pneumolysin leads to the deactivation of the protein and therefore limits potential damage [20]. Similarly, snake venom induces the production of neutralizing anti-venom IgG in the host which is also used in therapeutic sera [21].

Importantly, however, antibodies are proteins that bridge the gap between the innate and adaptive immune system. Both systems rely on each other as without immunoglobulin there is minimal recognition of pathogens by

phagocytes (with few exceptions [22]) and without phagocytes there is minimal killing of opsonized pathogens.

1.1.2 The complement system

The complement system forms part of the innate immune system and is a general term used to describe a set of more than 30 soluble plasma proteins (>15% of total globular plasma protein) and their receptors which assist, or *complement*, the humoral immune response to infection [23]. Activation of the complement system occurs through classical, alternative, or lectin pathways in a cascade like fashion. In general terms, a complement cascade consists of inactive proteins activated through cleavage by a protease, which in turn converts the next protein into a protease able to cleave the next protein and so on. Each cascade is activated differently, but converge at cleavage of the C3 protein, the most abundant of complement proteins. Activation of C3 leads to further protein cleavage and ultimately (a) the formation of a membrane attack complex (MAC), (b) recruitment of phagocytes through chemotaxis or (c) opsonisation for phagocytosis (Figure 6) [24].

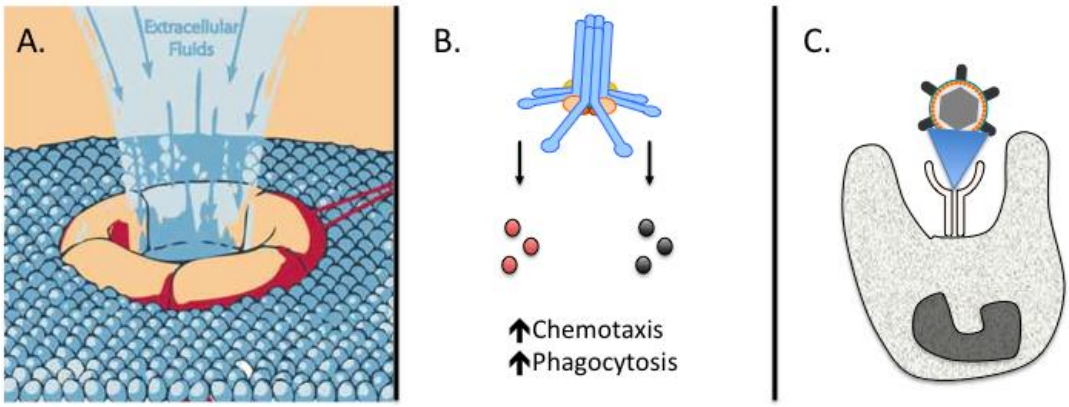


Figure 6 - Three possible outcomes of C3 cleavage following the activation of the complement system via the classical, alternative or lectin pathway. (A.) >13 complement proteins assemble on the membrane of a pathogen to form a pore causing leakage of fluids and cell death. (B.) Activated components of the complement system (C3a and C5a) have chemotactic properties that recruit phagocytes to the site of inflammation. (C.) Complement proteins deposit onto the surface of pathogens thereby marking (opsonizing) the pathogen for phagocytosis.

Activation of complement

Activation of the complement system occurs through classical, alternative, or lectin pathways. The classical pathway is activated through contact with antibody-bound antigens. The initial protein of the classical pathway is C1, a complex of proteins consisting of six molecules of C1q, two molecules of C1r and two molecules of C1s [25]. The constant domains of IgG and IgM immunoglobulin have binding sites for C1q that can activate the C1 complex, albeit at different concentrations. A single molecule of IgM, compared with several molecules of IgG, is needed to activate the C1 complex [17]. Activation of C1 complex leads to the cleaving of C4 and C2 proteins and the subsequent formation a C4b2a complex able to cleave and activate C3 protein (Figure 7).

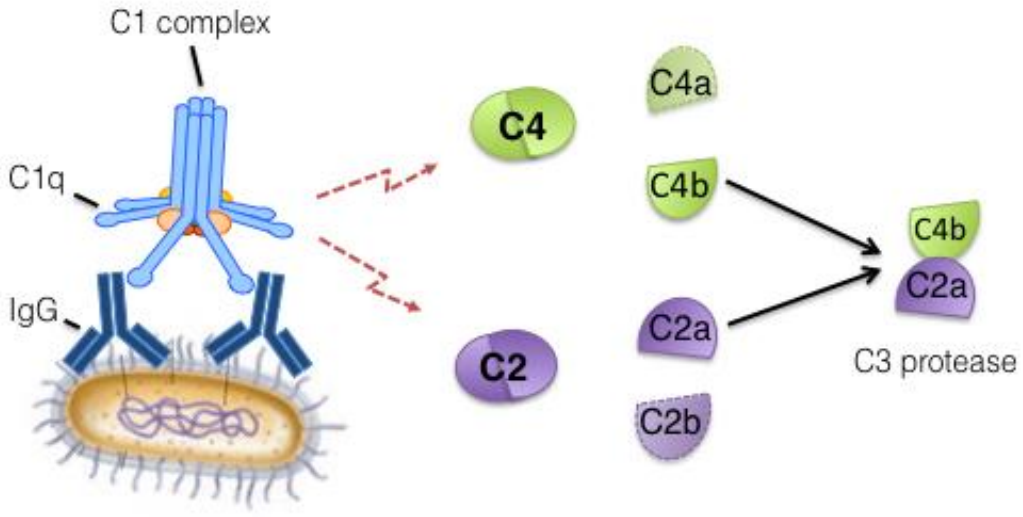


Figure 7 - Classical activation of the complement system. Immunoglobulin binds to antigen forming an immune complex, which binds to the C1 complex (via C1q) thereby activating it. Activated C1 is then able to cleave protein C2 and C4, creating subparts that form a C3 protease (C4b2a). This complex is then able to initiate the three functions of complement.

The lectin pathway, similarly to the classical pathway, is activated in the presence of opsonins [26]. Activation of the lectin pathway, however, occurs following non-specific opsonisation with either mannose-binding lectin or ficolin [23]. Activation of the lectin pathway has been shown to be critical in protecting against pneumococcal infections [27].

The alternative pathway is activated through low-level spontaneous conversion of C3 into C3b. This process, however, is tightly regulated to avoid damage to host cells. C3b is quickly deactivated either through contact with sialic acid (present on mammalian cells but not pathogens) or through binding to the regulatory protein Factor H, a soluble glycoprotein present in serum. Factor H binds glycosaminoglycans (GAGs) present on mammalian cells but not pathogens and prevents complement deposition through degradation of C3b and the alternative pathway C3 protease [28].

Complement opsonisation

Complement activation is followed by complement opsonisation. In particular, complement opsonisation occurs following cleavage of protein C3 into C3a, C3b and C3bi (inactive C3b). Internal thioester bonds of C3 exposed on C3b and C3bi following cleavage covalently bind to hydroxyl groups on carbohydrates and proteins commonly found on the surface of bacteria, viruses and protozoa [23, 24]. Complement mediated opsonisation via the classical pathway enhances antibody mediated phagocytosis and vice versa [29]. In the presence of anti-capsule antibodies on bacterial pathogens, complement deposition is greatly enhanced which in turn increases the efficacy of opsonophagocytosis [30]. In contrast, complement opsonisation via the lectin or alternative pathway leads to opsonophagocytosis at much lower rates than in the presence of antibody.

Complement inhibition

Pathogens have developed mechanisms to inhibit complement deposition. *Streptococcus pneumoniae,* for example, uses the virulence factors PspA and CbpA to deactivate complement [31]. PspA is anchored to phosphocholine moieties on the pneumococcal capsule thereby preventing C-reactive proteins (see 1.1.3) to attach and activate complement [32]. CbpA prevents complement deposition by binding human Factor H which degrades components of the complement C3b and the alternative pathway C3 protease [33].

1.1.3 Non-specific opsonins

C-reactive protein (CRP) is an evolutionary conserved plasma protein produced and secreted by the liver during early inflammation. CRP is primarily synthesized by hepatocytes [34], but under some circumstances also by epithelial cells from the respiratory tract [35], renal epithelial cells [36] and neuronal cells [37]. Levels of CRP in circulating blood are associated with the severity of infection and used as a clinical indicator of treatment success [38]. It was discovered in the 1930s that CRP binds phosphocholine expressed on the surface of dead or dying cells as well as some pathogens including *Streptococcus pneumoniae*, *Haemophilus influenziae* and *Leishmania donovani* [39]. Pathogen bound CRP can activate complement through the classical pathway [40] leading to complement opsonisation and to a much lesser extent the formation of a MAC [41]. In addition, pathogen bound CRP can associate with ficolins and activate the lectin-mediated complement pathway [42].

Mannose binding lectin (MBL, Mannose binding protein) is a soluble C-type lectin primarily secreted into the bloodstream by hepatocytes [43]. MBL can recognize repetitive carbohydrate ligands (mannose, glucose, L-fucose) commonly found on microbial surfaces such as *N. meningitides*, *S. aureus* and *S. pneumoniae* [44]. Once bound to a pathogen, MBL binds to MBL-associated

serine proteases (MASP1-2), thereby creating a protease complex able to activate the lectin pathway of complement leading to opsonisation and bacterial lysis [29]. The presence of MASP-2 is essential in activating the lectin pathway during pneumococcal infection [27]. Similarly, deficiencies in MBL production can lead to an increased risk of pneumococcal infections [45, 46].

Ficolins are lectin-like soluble serum proteins primarily produced by hepatocytes [47]. There are currently three known Ficolin proteins (L-ficolin, H-ficolin and M-ficolin) which can bind to pathogens and activate complement [48]. M—ficolin is the only ficolin isoform secreted by immune cells including monocytes [49] neutrophils [50], and alveolar epithelial cells [51]. Ficolins bind teichoic acid present in many cell walls of Gram(+) bacteria [52]. Similar to mannose binding lectin, ficolins associate with MBL/Ficolin-associated serine proteases (MASP1-2) and activate the lectin pathway of complement [53].

Surfactant proteins (SP) are lipoproteins produced to reduce surface tension and can recognize and bind to repetitive carbohydrate structures [54]. In the lungs, SPs are produced by type II alveolar cells and released to form pulmonary surfactants. The most abundant surfactants are SP-A and SP–D, which together can bind a wide range of pathogens [55, 56]. Surfactant proteins are unable to activate complement but rather help by binding to and aggregating pathogens [57], stimulating phagocytes [58], initiating phagocytosis through the binding of SP specific receptors [54] and through antimicrobial actions [59].

1.2 Phagocytic receptors

There are broadly speaking two types of receptors found on phagocytes that are involved in the process of phagocytosis. These are (a) those that recognize opsonic molecules bound to a pathogen (opsonic receptors) and (b) those that directly recognize components of pathogens (non-opsonic receptors). The opsonic receptors include Fcγ and complement receptors and the non-opsonic receptors include C-type lectins and scavenger receptors.

1.2.1 Fc receptors

Fc receptors (FcR) are membrane proteins present on monocytes, macrophages, neutrophils, natural killer cells, mast cells and B-cells. They bind the Fc region of immunoglobulin and are classified with respect to the class of immunoglobulin to which they bind; IgA bind Fcα receptors, IgE bind Fcε receptors, IgG bind Fcγ receptors and IgM bind Fcμ receptors. The critical FcR for inducing phagocytosis of opsonized pathogens is FcγR [60] and as such will be reviewed in detail.

To date, six Fcγ receptors have been identified in humans: FcγRI (CD64), FcγRIIA (CD32a), FcγRIIB (CD32b), FcγRIIC (CD32c), FcγRIIIA (CD16a) and FcγRIIIB (CD16b). An additional FcR able to bind IgG is FcRn (neonatal FcR), which transfers IgG into the placenta and breast milk and prevents IgG from degradation [61]. FcR-like proteins FcRL4, FcRL5 and TRIM21 also bind IgG (as well as IgA) but their role in immunity remains to be elucidated [62, 63].

Murine Fcγ receptors are similar to humans, consisting of FcγRI, FcγRIIB and FcγRIII but mice have an additional receptor (FcγRIV) not present in humans [64]. In both humans and mice, FcγRI binds with high affinity to the Fc portion of IgG, while the other receptors have low to medium affinity. Crosslinking of IgG to Fcγ receptors leads to the activation of an

immunoreceptor tyrosine based activation motif (ITAM) contained in the cytoplasmic domain of the FcγR molecule. An exception in both mice and humans is FcγRIIB, where cross-linking results in inhibition of an immune response through activation of an immunoreceptor tryrosine inhibitory motif (ITIM) [65]. The role inhibitory FcRs play in the immune system is still being elucidated but it has become clear that they form an essential role in regulating immune responses [66].

Although crosslinking between different Fcγ receptors varies with respect to both affinity and avidity of IgG to the relevant FcγR, downstream signaling through the ITAM domain is similar for each IgG/receptor interaction and is shown in Figure 8 [67]. First, tyrosine kinases assemble by the ITAM motif and begin the rapid phosphorylation of the tail subunit of ITAM [68]. This in turn recruits Src family tyrosine kinases, such as Spleen Tyrosine Kinase (SYK), which activate several downstream processes including phosphoinositide 3-kinase (PI3K) and son of sevenless homologues (SOS) leading to an influx of Ca^{2+} and other activation signaling pathways [69, 70]. SYK is believed to be essential for phagocytosis as it activates Arp2/3, a protein complex involved in mediating the branching of the actin skeleton used to engulf pathogens [71]. Inhibition of the SYK protein prevents actin dynamics and therefore blocks phagocytosis [72].

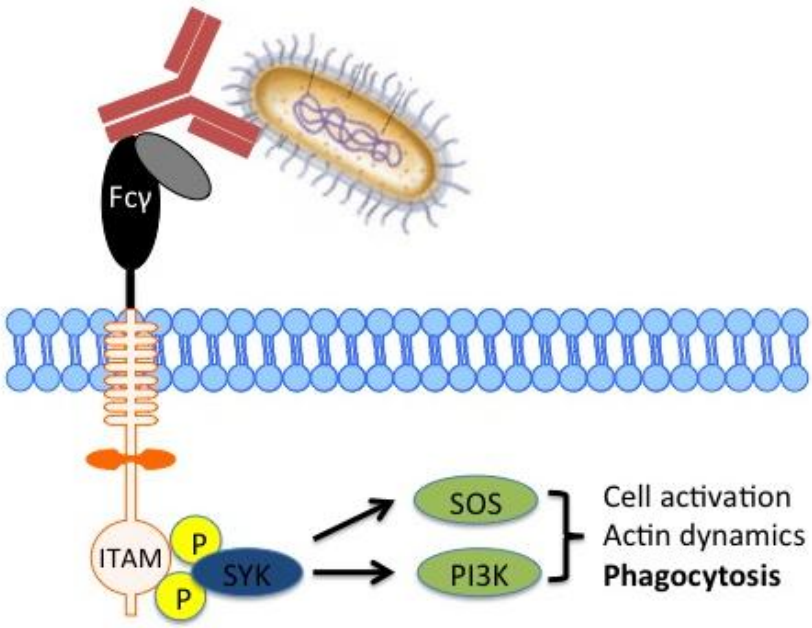


Figure 8 – Activating FcγR ligation. When an immune complex (consisting of an antibody bound to an antigen) bind to Fcγ receptors, the immunoreceptor tyrosine-based activation motif (ITAM) region of the receptor is rapidly phosphorylated by tyrosine kinases. Phosphorylated ITAM then becomes a docking site for Spleen Tyrosine Kinase (SYK) proteins, which are able to initiate a variety of intracellular signaling pathways via the activation of phosphoinositide 3-kinase (PI3K) and son of sevenless homologues (SOS). These signaling pathways ensure effective phagocytosis of the FcγR bound opsonized particle.

Importantly, Fcγ receptors are mobile proteins. Following activation, inactivated Fcγ receptors will migrate and cluster to the site of activation using lipid rafts [73]. This allows for further Fc-FcγR interactions and subsequent phosphorylation of ITAM motifs [74]. These findings led to the hypothesis that phagocytosis occurs in a zipper-like fashion where each Fcγ receptor ligation enhances the uptake [75]. Recently, however, it has been shown that increasing amounts of opsonizing IgG correlates with increased early signaling (receptor activation) but not with late signaling (actin dynamics) [76]. Instead, clustering and ligation of FcγR may serve to strengthen the attachment of opsonized particles while further downstream

signaling events dictate the activity and intensity of actin dynamics and subsequent uptake [77].

Current knowledge stipulates that regulation of FcγR expression is mediated through inflammation. That is, the presence of pro-inflammatory cytokines or mediators such as IFN-γ, TNF or lipopolysaccharide enhances the surface expression of Fcγ receptors [78]. As IgG competes with equal affinity for the activating FcγR as for the inhibitory FcγR, cells express activating FcγR at higher levels during inflammation in order to favour their binding [65]. While inflammation levels decrease and activating FcγR become occupied or internalized, inhibitory FcγR are ligated more frequently [79]. Ligation of inhibitory FcγR leads to phosphorylation of its ITIM motif and the recruitment of phosphatases including the tyrosine phosphatase SHP [66]. SHP inhibits downstream signaling of activating FcγR by dephosphorylating the ITAM motif, as well as inactivating SYK and PI3K leading to reduced expression of activating FcγRs and general dampening of immune responses [66].

The regulation of FcγR expression is also mediated by complement. Increased expression of activating Fcγ receptors is dependent on the presence of C5a, an anaphylactic protein activated in the final stages of the complement cascade [80]. Similarly, the production of C5a protein is dependent on the presence of Fcγ receptors [81] suggesting that complement acts in a way that regulates FcγR expression and vice versa.

1.2.2 Complement receptors

Complement receptors form part of the complement system. There is a range of complement receptors, mostly involved in the regulation of complement activation but also in complement's role of initiating inflammation, chemotaxis and adhesion [82]. Currently, five receptors are known to be actively involved in phagocytosis. These are CR1, CR3 (a.k.a. Mac-1), CR4, CRIg and C1qR (Figure 9) [25, 82, 83]. The localization of these receptors differs. CR1, is present on leukocytes and erythrocytes [84], CR3 on all phagocytes while CRIg is present only on macrophages [85].

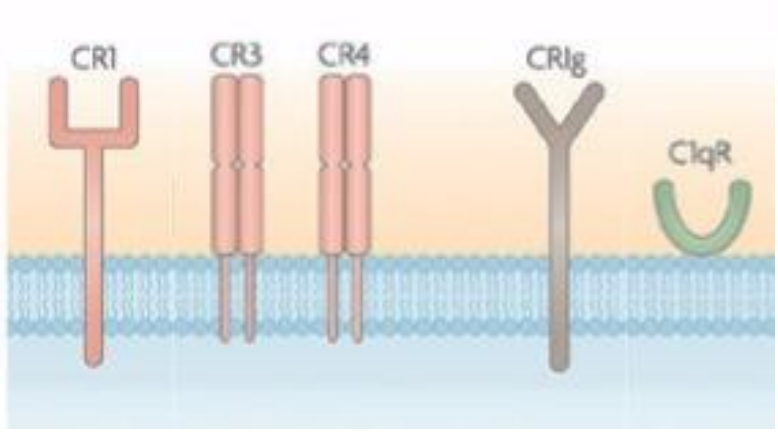


Figure 9 - Complement receptors CR1, CR3, CR4, CRiG and C1qR are involved in the phagocytosis of complement opsonised pathogens. Figure adapted from [82]

Current knowledge on the signaling pathways and effector function of complement receptors is limited. The best-studied complement receptor is CR3, which is able to bind to iC3b opsonized pathogens [24]. C3 has also been shown to initiate the phagocytosis of non-opsonized *Pseudomonas aeruginoas* [86]. In contrast to Fcγ receptors, ligation of complement receptors is not sufficient to initiate phagocytosis. Instead, phagocytic cells require prior activation in order to initiate phagocytosis via complement receptors [82]. Similarly to Fcγ receptors, however, CR3 signaling has recently been shown to be dependent on phosphorylation of SYK protein kinases [87] but

phagocytosis in SYK knockout macrophages still occurs suggesting a less important role for SYK phosphorylation in complement receptors than in Fcγ receptors [88]. As complement receptors have many more functions than phagocytosis, including the regulation of complement and activation of other immune cells [82], the exact nature of complement receptor mediated phagocytosis remains to be elucidated.

1.2.3 Non-specific receptors

Molecules found on microorganisms, but not on mammalian cells, which are generally essential to the structure or survival of the microorganism are called pathogen associated molecular patterns (PAMPs). These highly conserved, repeating structures are in turn recognized by Pattern Recognition Receptors (PRR) on immune cells. PRRs can be divided into endocytic PRR, where ligation results in phagocytosis, and signaling PRR, where ligation results in intracellular signaling. Common endocytic PRR found on phagocytes are C-type lectins and scavenger receptors while common signaling PRR include toll like receptors (TLRs) and Nod like receptors (NLRs).

Toll like receptors (TLR) are a family of proteins that play an essential role in the recognition of a wide variety of PAMPs [89]. There are currently 10 known human toll like receptors (TLR 1-10) expressed in various cells including monocytes, macrophages, neutrophils, dendritic cells, B-cells as well as epithelial cells [90, 91]. Surface expressed TLRs (TLR 1, -2, -4, -5, and -6) are responsible for the recognition of bacterial products while TLRs localized into intracellular compartments (TLR 3, -7, -8 and -9) recognize viral products [92]. Ligation of TLRs leads to a complex signaling cascade specific to each TLR [93] resulting in activation of many transcription factors including nuclear factor (NF)-κB, a classical, proinflammatory gene transcription regulator [92-94]. There is debate as to the role TLRs play in phagocytosis. TLR ligation up-regulates genes responsible for autophagy

(degradation of intracellular proteins using phagosomes) [95] and aids during phagosome maturation [96] as well as up-regulating receptors required for phagocytosis (FcR, complement, Scavenger receptors) [97, 98]. TLR-independent phagosome maturation, however, has also been reported [99]. While TLR ligation alone does not lead to uptake, it plays an important role in phagocytosis by activating immune cells through the recognition of pathogens and indirectly enhancing the process of phagocytosis [100-102].

C-type lectins (CL) are a large family of proteins that able to recognize and bind sugar moieties on bacteria [103, 104], fungi [105], and viruses [106]. Binding was originally thought to be dependent on the presence of Ca^{2+} but several CL have shown to bind independent of this [43]. The best-studied C-type lectin is the mannose receptor (MR). MRs are able to bind to repeating patterns of mannosyl- and fucosyl-containing glycoproteins, structures which are commonly found on yeast and certain bacteria including *M. tuberculosis* and *S. pneumoniae* [107]. MRs were originally believed to be restricted to macrophages but have since been detected on a variety of epithelial cells as well as monocyte derived dendritic cells [108, 109]. MR expression is modulated by cytokines including IL-4, IL-13, IL-10 (increased expression) and IFN-γ (decreased expression) [110]. Ligation of MRs in some studies led to phagocytosis [111] but it is generally accepted that receptor ligation assists, as opposed to being essential, in phagocytosis [112, 113]. Other transmembrane C-type lectins include DC-SIGN which binds fungi [105] and dectin which can bind yeast [114].

Scavenger receptors (SR) are transmembrane proteins able to detect and bind to lipopolysaccharide (LPS) and teichoic acid frequently found on bacterial pathogens. SRs also bind to apoptotic cells and some pathogens [115] as well as mop up, or "scavenge" small molecules. Scavenger receptor A (SR-A) and MARCO are the best studied. It has been shown that SR-A readily binds to lipoteichoic acid (LTA), a molecule frequently found on Gram(+) bacteria

[116]. Macrophages, which contain a range of SR, are able to bind to *Staphylococcus aureus* via SR-A leading to rapid phagocytic uptake of the pathogen [117, 118].

MARCO is a SR that is structurally similar to SR-A, but has the ability to bind gram-negative as well as gram-positive bacteria [119]. The molecular signaling pathways initiated by SRs still need to be elucidated but it has been shown that LPS activation and phagocytosis leads to rapid up-regulation of MARCO and SR-A expression [119, 120]. In line with other non-specific phagocytic receptors, the role of SR may serve to enhance the immune response to infection as opposed to having primarily antimicrobial functions.

1.3 Phagocytic uptake and killing

Following opsonisation by plasma proteins and the recognition of opsonized molecules by phagocytic receptors, phagocytes begin the task of taking up and degrading the bacteria. The uptake is mediated through actin while the killing of pathogens occurs in matured phagolysosomes.

1.3.1 Phagosome formation

FcγR and complement ligation leads to a clustering of receptors, *de novo* actin polymerization and the branching of new actin filaments. In the case of FcγR, this is mediated through Arp2/3 complexes engaging with Rho proteins, responsible for actin polymerization, while C3 receptors engage directly with Rho proteins [121]. Actin polymerization guides the cell membrane around the pathogen to be phagocytosed allowing additional opsonins to engage in a zipper-like fashion with clustered Fcγ and complement receptors. Once fully engulfed, the membrane-bound vacuole containing the pathogen, termed the phagosome, is internalized and detached from the cell main membrane [71].

The phagosome is not initially able to kill pathogens as it is primarily composed of the plasma membrane from which it was derived and its fluid contents reflect the extracellular milieu [122]. As such, the phagosome is rapidly modified to become a potent anti-microbial compartment, called the phagolysosome, able to kill and degrade internalized pathogens. This is a complicated multi-step process involving fusion, acidification and activation of enzymes [123]. In general terms, the degradation of bacteria following phagocytosis can be divided into oxygen-dependent and oxygen-independent processes.

1.3.2 Oxygen dependent degradation

The respiratory burst (oxidative burst) is the consumption of oxygen and glucose to produce reactive oxygen species (ROS) during phagocytosis. ROS can non-specifically oxidize proteins, lipids, DNA and carbohydrates as well as destroy iron–sulphur centres used in the respiratory chains of microorganisms [124]. In neutrophils and macrophages, ROS are generated through the activation of enzymes, including Phox and NADPH oxidase. Phox is an enzyme complex generated during phagosome maturation while NADPH oxidase is a membrane bound enzyme activated during phagocytosis [125]. Both enzymes are capable of transferring electrons from NADPH to oxygen. This reaction creates the ROS superoxide, hydrogen peroxide, hydroxyl radicals and a variety of their by products. In neutrophils, fusion of granules into phagosomes causes the release of the granule myeloperoxidase, which utilizes hydrogen peroxide and chlorite to produce the highly toxic compound hypochlorite [126]. Individuals unable to initiate an oxidative burst are affected by chronic granulomatous disease (CGD) and exemplify the importance of oxygen dependent degradation of bacteria in phagocytosis. These individuals frequently succumb to bacterial infections, as they are unable to properly clear pathogens [127].

1.3.3 Oxygen independent degradation

There are more than 100 proteins taking part in the degradation of internalized bacteria in phagosomes [123]. These range from cationic proteins which damage microbial membranes, lysozyme which splits the mucopeptide in the bacterial wall, lactoferrin which sequesters the iron essential for bacterial survival and a range of hydrolytic enzymes which break down bacterial proteins [128]. While enzymatic degradation helps in the breakdown of bacteria, it is generally believed that the respiratory burst accounts for most of the killing [127].

B. Phagocytes

2.1 Phagocytes

Phagocytes form the first line of defence against pathogens. They are either already present at the site of infection or migrate in large numbers to the site of infection to assist other resident phagocytes during inflammation. Through their ability to detect and kill pathogens, release inflammatory mediators and present antigens to antibody producing cells, phagocytes are important orchestrators of the immune response to infection.

2.1.1 Professional and non-professional phagocytes

Phagocytic cells are divided into professional and non-professional phagocytes. Professional phagocytes possess a range of receptors necessary for efficient and effective phagocytosis. Important phagocytic cells include monocytes, macrophages and neutrophils. The other professional phagocytes (dendritic cells, basophils and eosinophils) have primary roles other than the clearance of pathogens. Non-professional phagocytes, such as epithelial cells and fibroblasts, can also engulf particles but lack specialized receptors making them less effective. For the purpose of this thesis, monocytes, macrophages and neutrophils will be reviewed with respect to their functions of migration and phagocytosis to clear pathogens.

2.1.2 Phagocyte hematopoiesis

Hematopoiesis is the process by which stem cells in the bone marrow differentiate into blood cells including monocytes and neutrophils. Stem cells commit early on to become either monocytes via differentiation into monoblasts, or polymorphonuclear leukocytes (PMNL - neutrophils, eosinophils, basophils) via differentiation into myeloblasts.

The production of monocytes takes approximately 1.5-3 days and involves differentiation from monoblasts to pro-monocyte and then into early monocytes through stimulation with monocyte colony stimulate factor (M-CSF) and fms-like tyrosine kinase 3 ligand (Flt3-ligand) [129]. The production of PMNL takes approximately two weeks and involves differentiation into five cell types mediated by myeloid-specific growth factors (G-CSF and GM-CSF). In the first week, myeloblasts differentiate to pro-myelocyte and then into myelocytes. The second week of differentiation leads to metamyelocytes, then into band forms and finally into PMNL, which enter the bloodstream terminally differentiated. In comparison, monocytes have the ability to further differentiate into tissue specific macrophages and dendritic cells.

A simplified schematic of phagocyte hematopoiesis is shown in Figure 10.

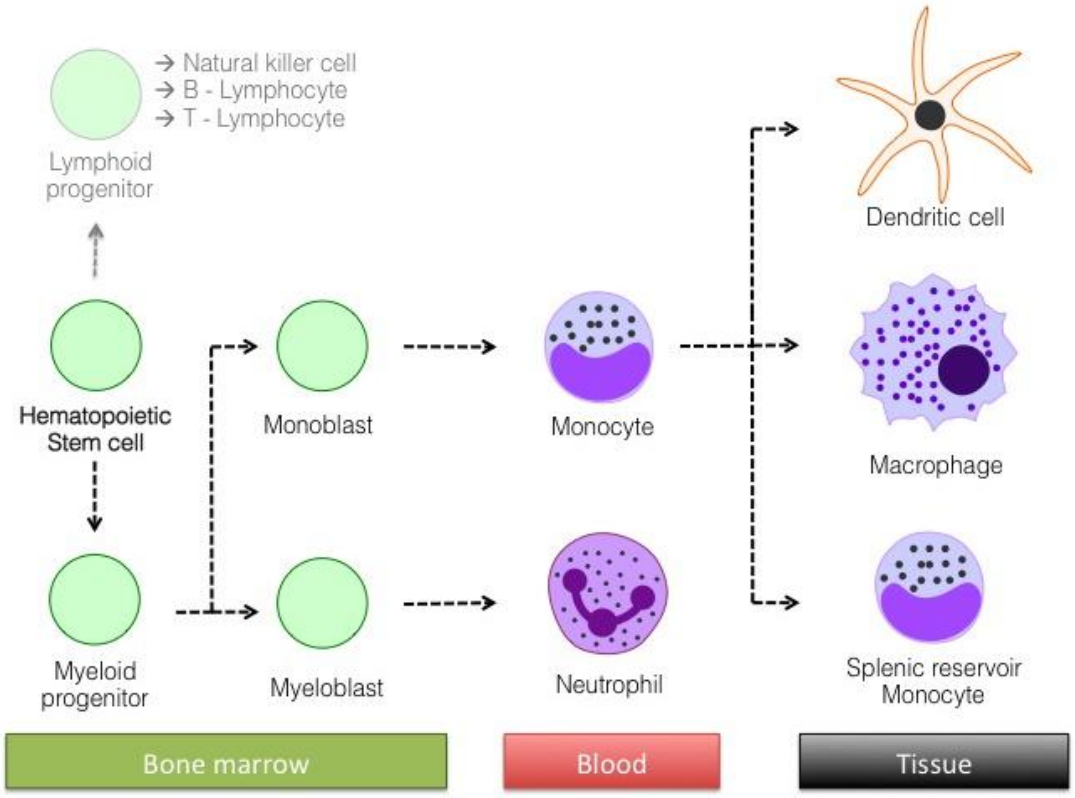


Figure 10 – Simplified schematic of the origin, differentiation and localization of neutrophils, monocytes, macrophages and dendritic cells. Self-renewing hematopoietic stem cells replenish phagocytes of the innate immune system. Following differentiation into myeloid progenitors, monocytes are formed via differentiation into monoblasts. Granulocytes (shown as neutrophils) are formed via differentiation into myeloblasts. Monocytes and neutrophils exit the bone marrow into blood as differentiated cells. In a steady state, monocytes further migrate to tissue where they patrol tissue or differentiate into dendritic cells or macrophages. A large proportion of monocytes also migrate to the spleen to form a splenic monocyte reservoir. Neutrophils remain in the blood circulation until they apoptose or are recruited to a site of infection.

2.2 Monocytes

Monocytes form part of the mononuclear phagocyte system (MPS) and can differentiate into dendritic cells and macrophages. Monocytes are usually identified by their large, invaginated nucleus following staining (Figure 11).

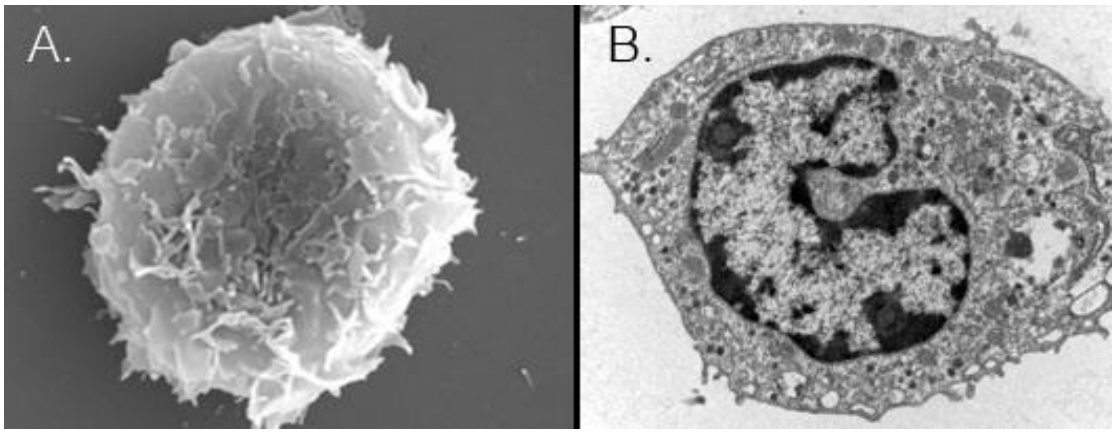


Figure 11 - Circulating blood monocytes. (A) Scanning electron microscope image of a human blood monocyte. Adapted from Microscope Service of Universidad Autònoma de Barcelona (UAB). (B) Transmission electron micrograph image of a transverse section of a murine blood monocyte showing the invaginated nucleus characteristic of monocytes. TEM Image taken as part of this thesis.

Monocytes are continuosly produced in the bone marrow and remain in the blood-stream for 1-3 days [130]. The function of monocytes is broad including replenishing tissue macrophages through differentiation, immune surveillance, promotion and resolution of inflammation and phagocytic clearance of pathogens [131, 132]. In the absence of infection, monocytes circulate in the blood, making up approximately 10% of circulating immune cells in humans (4% in mice), but are also stored in the bone marrow and spleen as a reservoir [133]. Monocytes give rise to macrophages and dendritic cells through differentiation during inflammation [134]. There are ongoing discussions in the description of monocyte subsets in murine and human hosts. A general distinction, however, can be made by classifying monocytes into resident and inflammatory monocytes.

2.2.1 Resident monocytes

Resident monocytes constitute a minority of monocytes in humans (10-20%) and approximately half of all monocytes in mice [134]. They are recruited to all noninflamed tissues where they patrol or differentiate into a range of macrophages as shown in Figure 12.

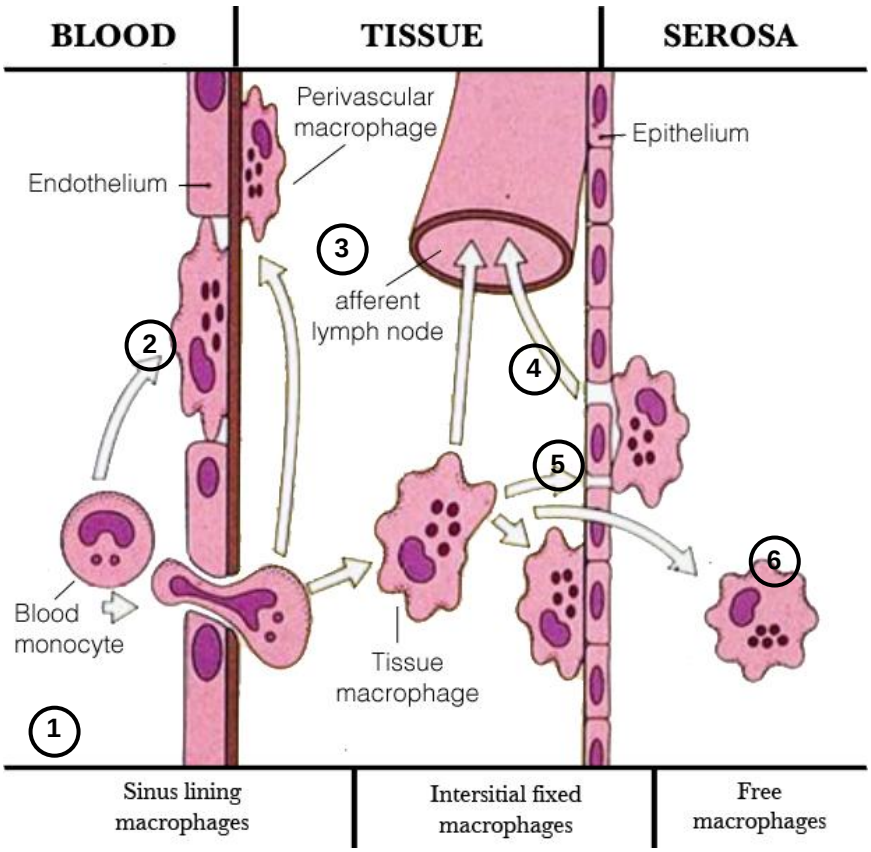


Figure 12 – Resident monocyte migration and differentiation into macrophages in the absence of infection. (1) Blood monocytes entering tissue differentiate into macrophages and either (2) adhere to the endothelium or enter tissue and (3) line/fix themselves to the perivascular endothelial layer. Differentiated macrophages in tissue can then (4) enter the lymphatic system via afferent lymph nodes or (5) adhere and fix themselves to epithelial layers. (6) Macrophages can further migrate within tissue to serosal spaces where they scavenge as free macrophages. Figure adapted from (DA Hums, DA Hughes).

Replenishment of tissue macrophages is not the only role of resident monocytes. During early inflammation, they secrete high amounts of pro-inflammatory cytokines TNF-alpha and IL-1 in order to recruit other leukocytes and begin phagocytosing bacteria [135, 136]. Resident monocytes posess the phagocytic receptors FcγRI (CD16), FcγRII (CD32), FcγRIII (CD16), as well as complement and scavenger receptors resulting in high phagocytic activity [135]. In the later stages of inflammation, resident monocytes can switch to anti-inflammatory secreting cells and promote wound healing and angiogenesis [137].

2.2.2 Inflammatory monocytes

Inflammatory monocytes constitue the majority of monocytes in humans (80-90%) and make up half of all monocytes in mice [131]. During early inflammation, inflammatory monocytes are released from the bone marrow and splenic monocyte reservoir in response to CCL2 in blood [138]. Inflammatory moncytes express high levels of the C-C-receptor motif 2 (CCR2) allowing them to respond quickly to CCL2, which is released during inflammation by monocytes, macrophages and dendritic cells [139]. As inflammatory monocytes are released from the bone marrow and spleen, they are recruited to affected tissues and draining lymph nodes using adhesion molecules present on both the monocyte (LFA-1) and epithelial cells (ICAM-1) [140]. Murine inflammatory monocytes are efficient at phagocytosing opsonized pathogens and subsequently initiate an inflammatory response [141, 142]. Human inflammatory moncytes are also effective at phagocytosis but respond to LPS through the secretion of anti-inflammatory cytokines including IL-10 suggesting that a regulatory role is assumed following recruitment and later in inflammation [136].

2.2.3 Resident and inflammatory monocytes

The interplay between resident and inflammatory monocytes has been thoroughly studied using murine models of disease and is shown in Figure 13. Resident monocytes recognize invading pathogens through PRR and immediately begin phagocytosing bacteria and secreting pro-inflammatory cytokines to recruit leukocytes [130]. As inflammatory monocytes arrive to the site of infection, they express phagocytic receptors and begin clearing pathogens through phagocytosis and secretion of inflammatory cytokines [136]. Following the arrival of inflammatory monocytes, resident monocytes resume an anti-inflammatory role and begin the process of angiogenesis and clearance of apoptotic cells through the secretion of anti-inflammatory cytokines [130, 132, 135].

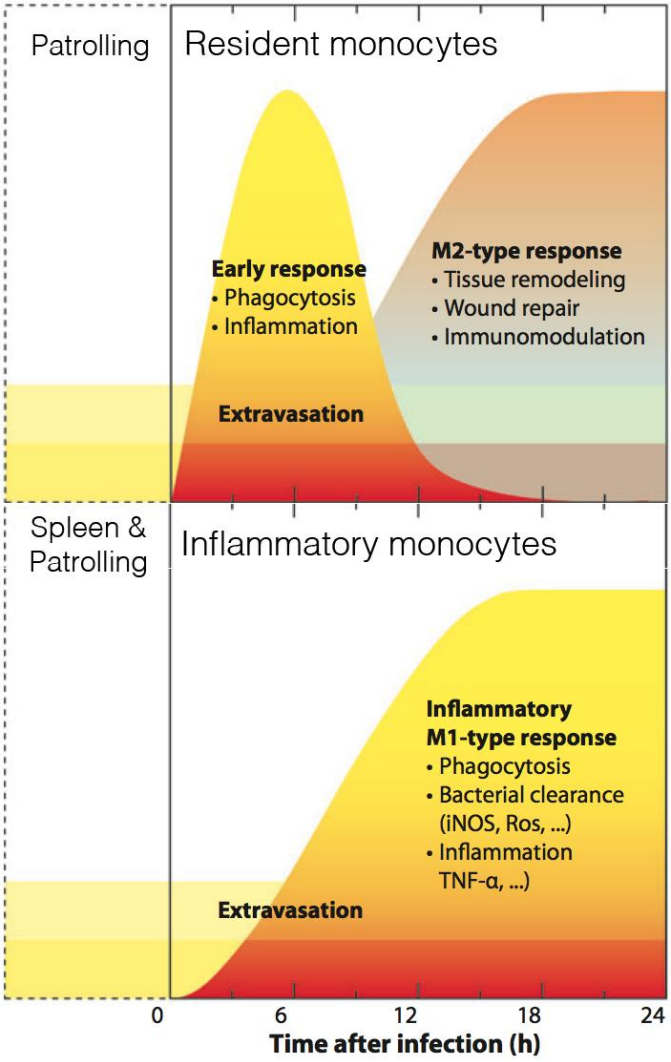


Figure 13 - Different roles and responses of resident and inflammatory monocytes to infection. Resident monocytes patrol the blood stream and tissue for potential pathogens. In case of an encounter, resident monocytes immediately begin phagocytosing and initiating an inflammatory response. This results in the influx of inflammatory monocytes that assist in bacterial clearance via phagocytosis and further inflammatory responses. Upon arrival of inflammatory monocytes, resident monocytes assume a wound repair, tissue remodeling and immunomodulatory role. Figure adapted from [131].

2.3 Macrophages

Macrophages are specialized phagocytic cells present in virtually all tissue that are derived from monocytes infiltrating into tissue during steady state and inflammation [134]. A common misconception is that macrophages are predominantly cells of the immune system. While they form an essential part in the defense against infection, macrophages are highly versatile assisting in tissue development, remodeling and repair in addition to immune surveillance. The wide range of tissue specific macrophages and their function in humans is shown in Figure 14.

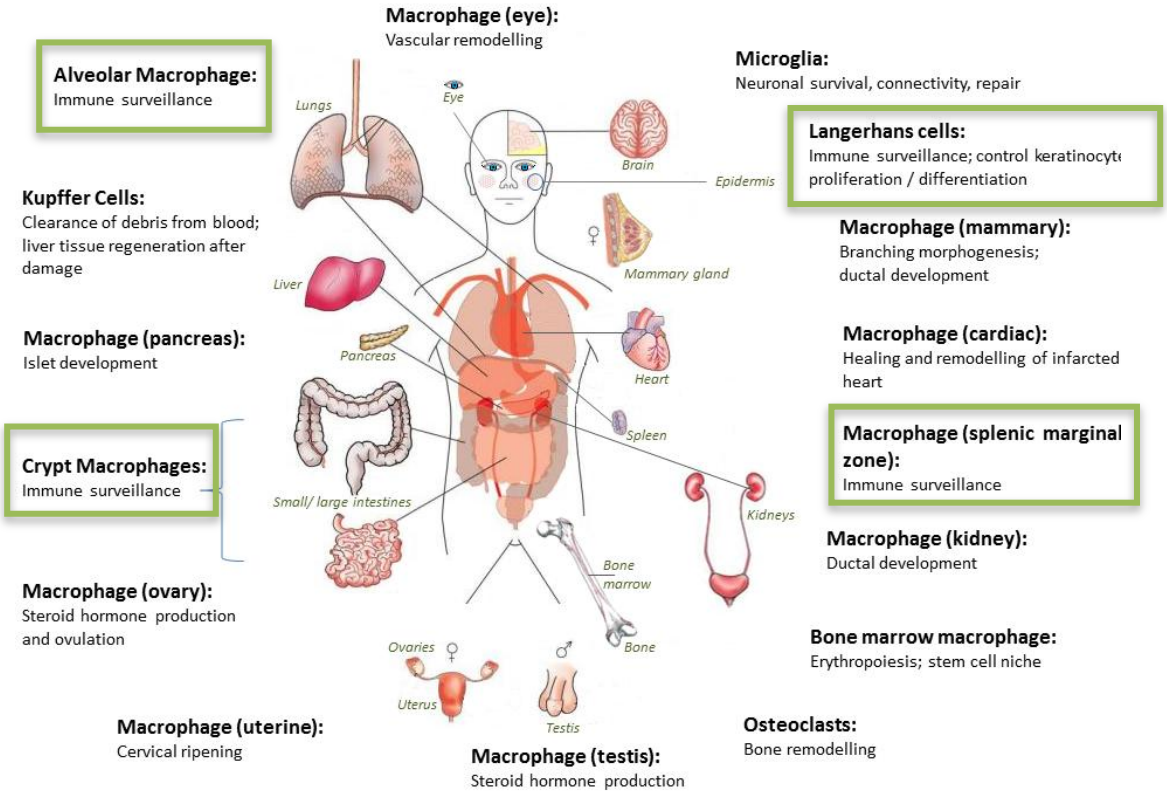


Figure 14 – Macrophages and their localization & function in the human body. Macrophages have many different functions depending on the tissue in which they reside. Common functions include tissue development and repair or immune surveillance. Areas of the body frequently exposed to the outer environment, such as the lungs and digestive tract, demand good immune surveillance and as such macrophages in these tissue have immune surveillance as their primary function (boxes). Other parts of the body, such as the spleen, strategically contain macrophages to monitor for pathogens in the circulatory system. The majority of macrophages however have functions that are essential for the homeostasis of the human body. Figure adapted from macrophages.com

The importance of macrophage function outside of the immune system is exemplified by mice deficient in colony-stimulating factors essential for the development and differentiation of macrophages [143]. In these mice, the development of the pancreas and nervous system as well as their fertility is detrimentally abnormal [143, 144].

2.3.1 Macrophage phagocytosis

Macrophages are prolific phagocytes. They possess a wide range of receptors, including PRR, MR, FcR and complement receptors that help decide on the outcome of phagocytosis [145, 146]. During steady state, macrophages scavenge cellular debris generated through tissue remodeling and remove apoptotic cells as well as red blood cells [147]. It is estimated that $2x10^{11}$ red blood cells are phagocytosed by macrophages each day, resulting in approximately 3 kg of iron and haemoglobin recycled per year [148]. Should a macrophage encounter a pathogen through phagocytic receptors, however, they change from being cells assisting in homeostasis to cells actively participating and orchestrating the immune response to infection [149]. In contrast, the phagocytosis of debris generated through tissue remodeling does not engage pathogen related phagocytic receptors and they remain cells participating in tissue homeostasis.

2.3.2 Macrophage populations

Following the engagement of PRR, macrophages can become either classically activated, wound healing or regulatory macrophages [150]. **Classically activated macrophages** have high antimicrobial capacity and perform the majority of phagocytic clearance of pathogens during infection as well as secreting pro-inflammatory cytokines [151]. Macrophages become classically activated in the presence of TNF and IFN-γ, cytokines produced by natural killer cells, T-helper cells and by nearby macrophages or in an autocrine

manner in the presence TLR stimulation [152]. **Wound healing macrophages** actively participate in reverting the damage caused by pathogens and the immune response. They are activated through exposure of IL-4 and IL-13; cytokines produced following tissue damage by PMNL and T-helper cells [153]. Wound healing macrophages have, for example, high arginase activity allowing them to mediate collagen formation for the production of extracellular matrices used to rebuild tissue [154]. **Regulatory macrophages** participate in the resolution phase of inflammation, having the ability to phagocytose apoptotic cells and restoring immune homeostasis thereby limiting the "collateral damage" [155]. They are activated through the exposure to IL-10 [156], a cytokine produced by, for example, regulatory T-cells as well as by macrophages following the phagocytosis of apoptotic cells [157] and immune complexes [158].

2.3.3 Alveolar macrophages

Alveolar macrophages (AM) are the specialized macrophages of the lung and play a major role in the innate response against infections that spread to the distal airways [159]. They are responsible for clearing pathogens and recruiting other phagocytes during respiratory disease. The infiltration of phagocytes and other effector immune cells into the lungs, however, can cause severe damage to tissue and hinder gas exchange. AMs therefore need to carefully regulate immune responses to avoid unnecessary activation. This is challenging as the human lung is frequently exposed to smoke, toxic gases, particulate matter and other non-pathogenic antigens that can inflame tissue and cause "false positives". In order to prevent this, AMs actively suppress their inflammatory activation through the down-regulation of phagocytic receptors [160]. In addition, AMs suppress the initiation of adaptive immune responses and prevent excessive inflammatory reactions to otherwise harmless antigens, as demonstrated in mouse models depleted of AMs which

show excess inflammation with both models of allergic and infection challenges [161].

The regulation of alveolar macrophages is mediated through the lung environment. AMs adhere to alveolar epithelial cells (AEC) in non-inflammatory conditions. This leads to the expression of $\alpha v\beta 6$, an integrin able to suppress macrophage function via the activation of TGF-β [162]. When AMs encounter a pathogen through PRR, they detach from AEC leading to the rapid down regulation of $\alpha v\beta 6$ and therefore TGF-β mediated immune suppression. This allows AMs to initiate immune responses including phagocytosing pathogens, secreting the pro-inflammatory cytokines IL-6 and TNF and recruiting phagocytes [163]. Immune suppression is re-instated when activated T-cells or inflammatory monocytes, who have assumed an anti-inflammatory role, encourage AMs to produce MMP-9, an activator of TGF-β, leading to their adhesion to AEC and subsequent expression of $\alpha v\beta 6$ [163].

2.4 Neutrophils

PMNL (Polymorphonuclear Leukocyte or granulocytes) include basophils, neutrophils and eosinophils and are cells in which small granules, as well as variably shaped nucleus, are visible in the cytoplasm (Figure 15). Neutrophils are the most abundant of all granulocytes making up approximately 50-70% of all circulating white blood cells and are the most important phagocytic granulocyte. In comparison, basophils and eosinophils make up 2-5% and 0.5-1% of total white blood cells, respectively.

Figure 15 - Granulocytes (also known as polymorphonuclear cells) showing their characteristic granules in the cytoplasm and varying shapes and sizes of nuclei.

Neutrophils exit the bone marrow as differentiated cells and, if left inactivated, circulate in the blood for an average of 5.4 days [164]. During inflammation, macrophages and epithelial cells, amongst other cells, secrete chemokines including formyl-methionylleucylphenylalanine (FMLP), leukotriene B_4 (LTB_4), and interleukin-8 (IL-8). These activate neutrophils and trigger their rapid migration in a gradient dependent fashion to the source of inflammation [165]. Protein expression is modulated during migration leading to cells becoming "sticky" and initiating a rolling motion through the forming and breaking of bonds between proteins expressed on neutrophils (integrins) and on epithelial cells (E-selectins, ICAM) close to the site of infection. Important integrins include the CD18 integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), as well as the CD29 integrin $\alpha 4\beta 1$ (CD49d/CD29), which bind to ICAM-1 [166]. Once at the high end of the chemokine gradient, tight adhesion occurs allowing the neutrophil to arrest movement and transmigrate into tissue through a process called diapedesis [166]. This process is also facilitated by increased vasodilation, increased permeability and blood flow as a result of inflammation. Once inside inflamed tissue, neutrophils phagocytose bacteria, release granules, form extracellular traps and modulate further immune responses. The lifespan of neutrophils in inflamed tissue averages between 1-2 days before they apoptose [167].

2.4.1 Neutrophil phagocytosis

Inside inflamed tissue, neutrophils perform the crucial role of removing pathogens though phagocytosis. Neutrophils express the major phagocytic receptors FcγRIIa and FcγRIIIB as well as complement receptor C3 while FcγRI is only expressed following TNF-alpha stimulation [168]. The importance of neutrophils in disease is exemplified in murine models of *S. pneumoniae, K. pneumoniae* and *L. pneumophila*, where depletion of neutrophils in otherwise resolving infection leads to host death [169-171].

2.4.2 Neutrophil granules

Neutrophils contain approximately 300 proteins (granules) classified into secretory, primary (azurophilic), secondary (specific) and tertiary granules [126]. These can either be shuttled into the membrane to act as ligands during migration, secreted to target extracellular pathogens at the site of infection (degranulation), or be shuttled into phagosomes to aid in the breakdown of phagocytosed pathogens [172]. Upon entering inflamed tissue, a short period of restraint ensues (15-45 minutes *in vitro*), prior to degranulation [173]. The period of restraint ensures that degranulation does not occur unnecessarily as it can be severely damaging to the host. Elastase, a primary granule for example, can deactivate virulence factors present on enterobacteria [174, 175] but also affects tissue integrity through the digestion of E-cadherin [176] which has been associated with the development of emphysema [177]. This, however, also aids in removing barriers of neutrophil-bacterial contact and to block escape routes for pathogens as well as leading to the leakage of serum proteins, such as complement and antibodies, into inflamed tissue [178].

2.4.3 Neutrophil extracellular traps

Neutrophils undergo cell death specifically to form extracellular matrices (called neutrophil extracellular traps; NET) that can entrap and kill pathogens [179]. Figure 16 shows the release of NETs by neutrophils to entrap bacterial pathogens.

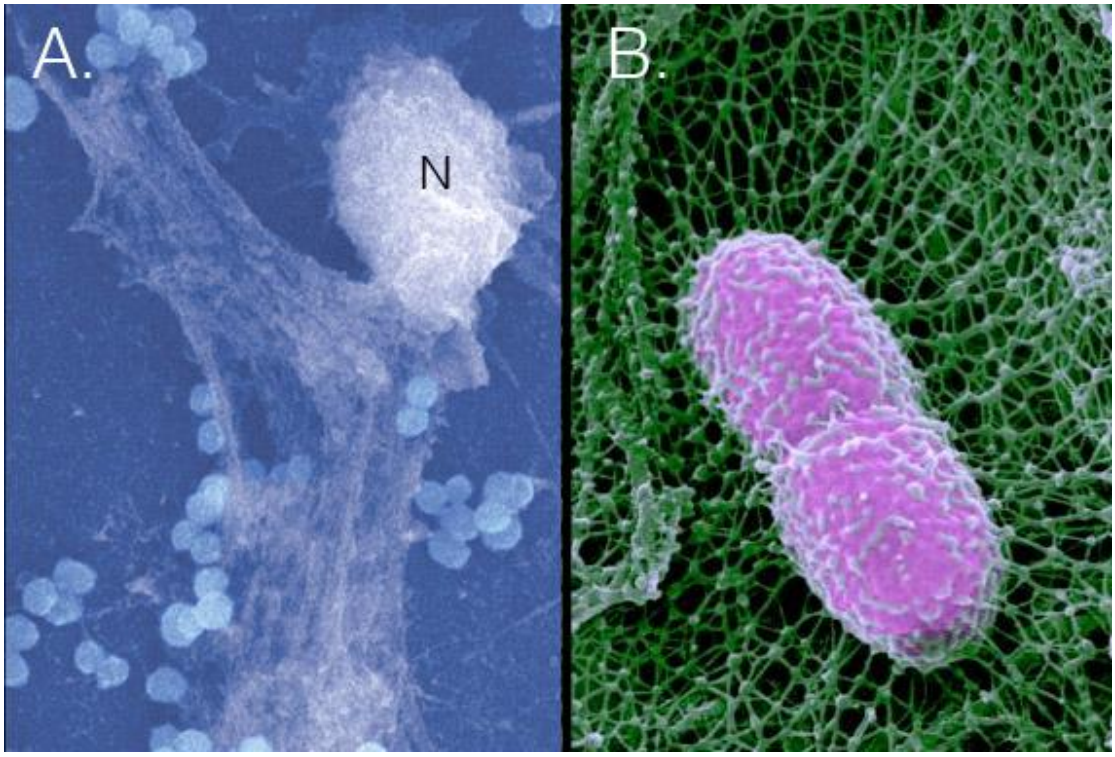


Figure 16 - Neutrophil extracellular traps (NET) are formed through programmed cell death in order to entrap and kill pathogens. (A) *In vitro* stimulation of human neutrophils leads to NET formation entrapping *Staphylococcus aureus* (N=neutrophil). Image reproduced from [180] (B) Scanning electron micrograph of *in vivo* NET formation in a murine lung infected with *Klebsiella pneumoniae*. Image reproduced from [181].

Activation via phorbol myristate acetate (PMA), IL-8, lipopolysaccharide or whole pathogens leads to the formation of NET through a novel pathway of programmed cell death, termed ETosis [182]. DNA and histones released by neutrophils undergoing ETosis form the backbone of NETs while secreted neutrophilic granules such as elastase, cathepsin G and myeloperoxidase bind to NETs and act as antimicrobial agents against any trapped pathogen [183].

NETs are abundantly produced at sites of infection, including in the lining of alveoli during pneumonia [184] and in the middle ear during otitis media [185]. Not surprisingly, however, pathogens such as *Streptococcus pneumoniae* have developed methods to free themselves from NETs via the expression of endonucleases [184] and the production deoxyribonuclease (DNase) [186].

2.4.4 Neutrophil Immunomodulation

Cytokines and granules released by neutrophils at the site of infection can act in an immunomodulatory manner as shown in Figure 17.

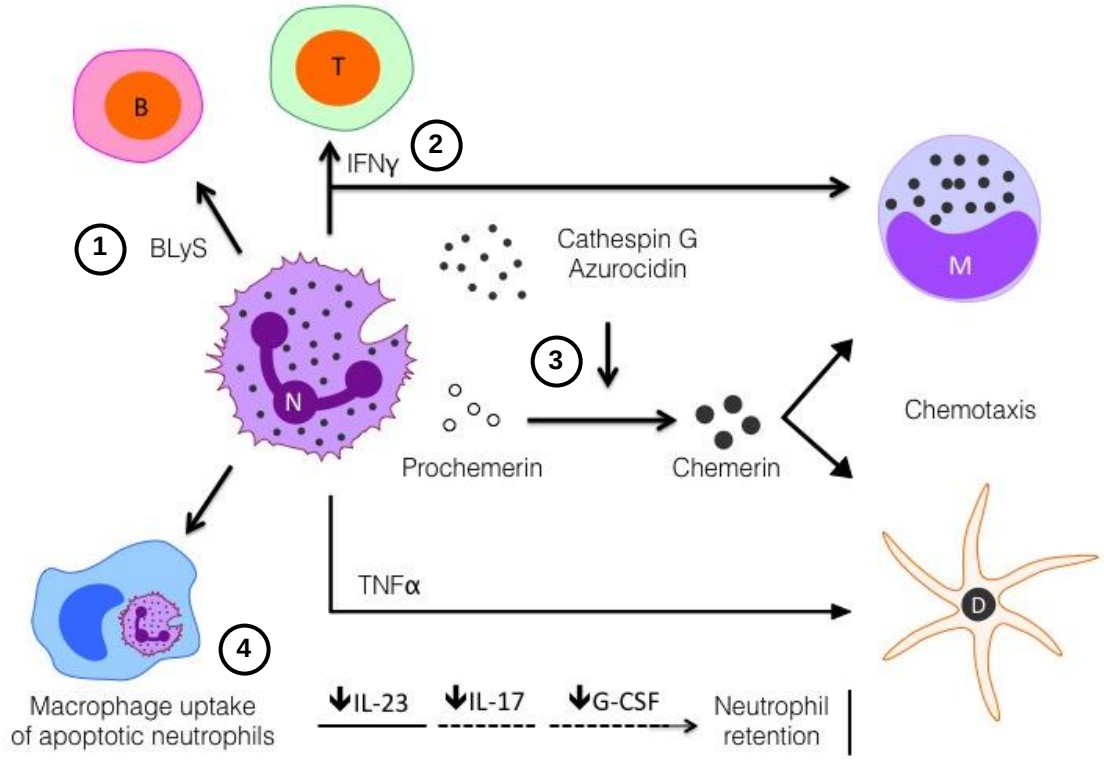


Figure 17 – Schematic diagram depicting examples of neutrophil immunomodulation in inflamed tissue (N=neutrophil, M=monocytes, B=B-lymphocyte, T=T-lymphocyte, D=pre-dendritic cells). (1) BlyS and (2) IFNγ secreted by neutrophils leads to the activation of B-cells and T-cells, respectively. (3) Cathepsin G, a granules secreted by neutrophils leads to the activation of Chemerin, a chemoattractant for monocytes and dendritic cells. (4) Apoptotic neutrophils are cleared by macrophages leading to reduced IL-23 secretion by macrophages. This in turn leads to reduced IL-17 secretion by stromal cells in the bone marrow and therefore reduced secretion of G-CSF by neutrophil progenitors. G-CSF is important for the release of neutrophils from the bone marrow.

Neutrophils secrete low amounts of cytokines on a per-cell basis, and are often overlooked as important contributors to cytokine signaling. During an infection, however, neutrophils are the predominant cell type in inflamed tissue and, as a group of cells, contribute significant levels of cytokines.

B Lymphocyte stimulator (BLyS), a cytokine belonging to the TNF ligand family, is secreted by neutrophils and acts as strong activator of B-cells [187]. Similarly, TNF-α is secreted in order to stimulate T-cell and monocyte activity. These combine to augment the adaptive immune response to infection. Granules also play an important role in the recruitment of immune cells to the site of infection. Azurophilic granules, such as azurorcidin, act as direct chemoattractants for mononuclear phagocytes and granulocytes [188, 189]. Cathepsin G is able to convert prochemerin, a weak chemoattractant, into chemerin, a potent chemoattractant for granulocytes [190]. In addition to recruiting more cells to the site of infection, neutrophils can also indirectly limit their recruitment through programmed cell death. Uptake of apoptotic neutrophils by macrophages leads to the secretion of IL-23 and, through a signaling cascade, the retention of neutrophils in the bone marrow [191].

C. Respiratory infections

> *When pneumonia is at its height, the case is beyond remedy if he is not purged, and it is bad if he has dyspnoea, and urine that is thin and acrid, and if sweats come out about the neck and head, for such sweats are bad, as proceeding from the suffocation, rales, and the violence of the disease which is obtaining the upper hand.*
> Hippocrates, ~ 460 BC – 370 BC

3.1 Lungs: entry point for pathogens

The above quotation shows that respiratory tract infections have long been a clinical problem. In order to understand why the human lung is frequently affected by pathogens, one must appreciate the level of exposure the respiratory tract is subjected to on a daily basis. Given a rate of 12-16 breaths per minute, it can be estimated that an adult breathes on average 17,000 – 23,000 times a day. In the same time, approximately 7,200 liters of blood pass through the lungs for gas exchange on a surface area estimated to be about $70m^2$ [192]. Gas exchange requires close contact between the inhaled oxygen and circulating blood and as such the body is physically only protected by a thin membrane in the lung compared to the multilayered protection afforded by human skin. This, alongside the temperature and humidity at which air enters the lung (37°C, 100%) creates an ideal setting for pathogens to enter the host and flourish.

3.2 Pneumonia

Pneumonia is defined as infection and inflammation leading to alveolar consolidation of the lung that can be caused by bacteria, viruses, fungi and parasites. Very young, old and immunocompromised populations are most frequently affected by pneumonia. The most common bacterial cause of

pneumonia is *Streptococcus pneumoniae*, alongside *Haemophilus influenzae* and *Staphylococcus aureus*. Pathogens enter the lungs either via direct inhalation or by seeding from biofilms of the nasopharynx. The presence of a pathogen in the lung is detected by alveolar macrophages or by pulmonary dendritic cells and causes the recruitment of leukocytes and inflammation. This leads to consolidation (fluid) in the alveoli, which hinders proper gas-exchange and interrupts the vital oxygen supply to tissues. Bacteria are the most common cause of pneumonia and these patients frequently have preceding or concurrent viral infections, particularly in immunocompromised individuals [193].

3.2.1 Childhood pneumonia

Pneumonia is listed by the WHO as the primary cause of child (<5yrs) deaths worldwide with >1 million deaths each year. It is estimated that 7-13% of childhood pneumonias progress to severe life-threatening diseases [194]. There are up to 155 million new cases of childhood pneumonia each year where the majority is in developing countries (Table 1) [195].

Table 1 - Estimated yearly new cases of childhood pneumonia by world regions. Adapted from [194]

World Region	Estimated no. new cases per year (millions)
African	35.12
Americas	7.84
Eastern Mediterranean	19.67
European	3.03
South-East Asia	60.95
Western Pacific	29.07
Total *for developing countries*	151.76
Total *for developed countries*	4.08

3.2.2 Adult pneumonia

Although pneumonias are most frequent in children, adults are also susceptible where the risk of infection increases with age and is highest in populations >65 years [196]. 10-20% of cases of pneumonia in adults require hospitalization [197]. The 30-day mortality rate in adult patients admitted to ICU in the UK was 23-35% in 2008 and all-cause mortality as high as 28% [198].

3.2.3 Sepsis secondary to pneumonia

Sepsis secondary to pneumonia is caused when respiratory infections cannot be contained in the lung by the immune system. When bacteria enter the normally sterile blood circulation, an immense inflammatory reaction is initiated leading to and exaggerated immune response and potential organ failure. In developed countries, approximately 10% of hospital admissions of pneumonia are bacteraemic where 60% of these cases are caused by *S. pneumoniae* [199-202]. In the US sepsis is a common and frequently fatal condition, with as many deaths annually as those from acute myocardial infarction [203]. It is especially common in the elderly and is likely to increase substantially as the U.S. population age [203]. Since the 1980s, Gram(+) bacteria have been the most commonly isolated bacteria in sepsis in the US [204].

3.2.4 Costs of pneumonia and sepsis

The direct healthcare costs associated with pneumonia in the UK are high, previously estimated at £441 million annually [205]. The annual cost of treating pneumonia in the US is estimated to be $4.8 billion for patients aged >65 years and $3.6 billion for patients aged <65 years [206]. The estimated annual total cost of treating sepsis in the US were $16 billion nationally [203] and £2.5 billion in the UK. Pneumonia and sepsis secondary to pneumonia is

a significant health problem in children and adults and is associated with the use of extensive healthcare resources, occupying 30% of ICU beds in the UK [207].

3.5 Streptococcus pneumoniae

Streptococcus pneumoniae (pneumococcus), is the main cause of bacterial pneumonia, one of the leading causes of infectious disease deaths in sub-Saharan Africa, and the biggest killer of children < 5 years worldwide [208]. *S. pneumoniae* was first discovered in 1881 by Louis Pasteur in France [209] and George Sternberg in the US [210] after injecting human saliva into rabbits and demonstrating pneumococci as the pathogen causing bacteraemia. This indirectly led to the development of the Gram stain in 1884 [211] which allowed for improved identification of pathogens in tissue preparations. Using the Gram stain it was established that in the late 19th and early 20th century, *S. pneumoniae* was the causative pathogen of most human lobar pneumonias [212, 213] as well as meningitis [214] and otitis media [215].

3.5.1 Microbiology

Originally named *Diplococcus pneumoniae* due to its morphological traits [216], *S. pneumoniae* is a capsulated, Gram(+) diplococcus that grows in chains [217]. It has a repertoire of 95 known different thick polysaccharide capsules (serotypes) that cover the peptidoglycan cell wall [218]. The presence of capsule is critical to the pneumococcus as it is used to evade opsonisation, and subsequent phagocytosis [219]. In addition to capsule, *S. pneumoniae* possesses a range of protein virulence factors used for colonization and invasion of the host [220]. Table 2 gives an overview of the function and mechanism of a selection of these. Pneumococcal virulence factors are embedded in the polysaccharide capsule as shown in Figure 18.

Table 2 - Selected virulence factors of *S. pneumoniae* and their mechanism of virulence & bacterial function.

Virulence Factor	Mechanism of Virulence	Bacterial Function	Ref
PspA	Inhibition of Complement	Stabilization of capsular charge	[221]
PspC	Inhibition of complement	Adhesion, binding to IgA & C3	[221]
PsaA	Inhibition of complement	ABC transporter (Mn^{2+})	[222]
CbpA	Inhibition of complement	Adhesion	[223]
PavA	Host barrier translocation	Modulates virulence factors	[224]
Hyaluronate lyase	Spreading	Degrades connective tissue	[225]
Neuraminidase	Biofilm formation	Degrades important glycans	[226]
Pneumolysin	Inflammatory	Pore forming excreted toxin	[227]
Autolysin	Inflammatory	Cytolysin	[228]

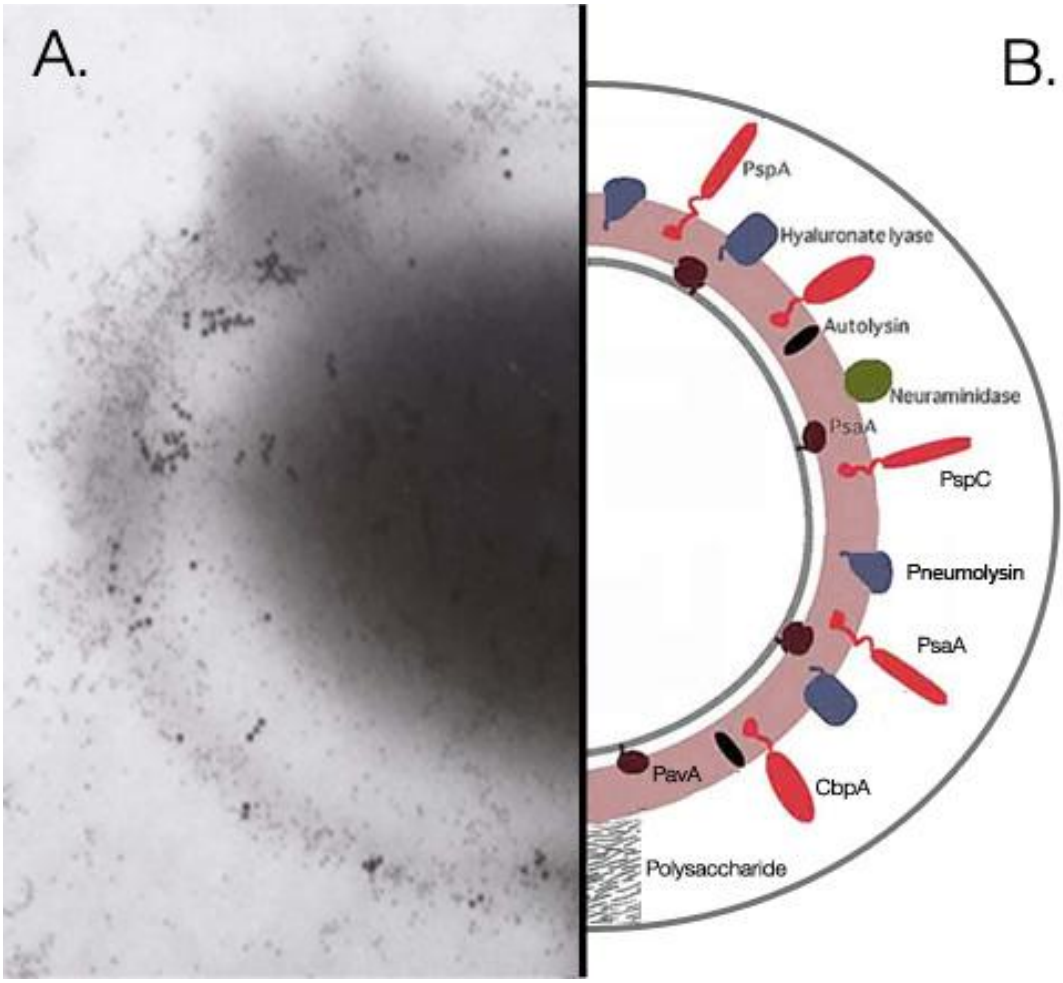


Figure 18 – Electron microscope and schematic image of *Streptococcus pneumoniae*. (A) Ferritin-labeled pneumococci highlighting the polysaccharide capsule as a shadow. Black dots around and within the capsule show the presence of pneumococcal virulence factor PspA by use of anti-PspA immunogold antibodies. Adapted from © Barry Gray. (B) Schematic diagram of *S. pneumoniae* showing a selection of pneumococcal virulence factors embedded into the polysaccharide capsule. Virulence factors are used to adhere to epithelial cells or evade phagocytosis amongst other functions (see Table 2 for further detail). Adapted from [229]

3.5.2 Pneumococcal surface adhesin A (PsaA)

As P4 peptide (see 4.2) is derived from Pneumococcal surface adhesin A (PsaA), this virulence factor will be reviewed in detail. PsaA is a highly conserved 37 kDa protein comprising of 309 amino acid residues. It is a virulence factor of *S. pneumoniae* and can be found embedded in the membrane of all pneumococcal serotypes. PsaA is an essential ABC transporter and is involved in the adhesion to epithelial cells. Pneumococci in which the PsaA gene has been knocked out have >80% less capacity to adhere to type II pneumocytes (A549 and Detroit 562 cell lines) than the parent strain [222]. PsaA− insertion deletion mutants of *S. pneumoniae* D39 were also significantly less virulent than *S. pneumoniae* D39 parent strain, as demonstrated in a low-dose intra-peritoneal (IP) challenge model in mice. This may be due to several known characteristics of PsaA− mutants, including their impaired growth in an Mn2+ deficient environment, their reduced capacity to adhere to lung cells, and their hypersensitivity to oxidative stress [230-232]. Amongst other proteins such as PspA, PspC, PLY and NanA, antibodies against PsaA are found in patients recovering from pneumonia, otitis media and invasive pneumococcal disease (IPD) and mice immunized with combinations of Pnc virulence proteins, including PsaA, survived longer than the control group after pneumococcal challenge [233]. PsaA is capable of provoking both the humoral and cellular lineage of the immune system. Zhang et al. (149) showed that B-cells capable of producing IgA, IgG, and IgM in response to PsaA were present in the adenoidal mononuclear cells (MNC) in children, thereby demonstrating the participation of humoral immunity against pneumococci. PsaA also augments the immune response through the priming of T-helper 1 (Th-1) immunity [234]. A 28 amino acid fragment of PsaA forms the peptide P4 (see chapter I, section 4.1).

3.5.3 Transmission

S. pneumoniae is spread via intimate contact, aerosol droplets or large-droplet secretions from symptomatic and asymptomatic carriers [235]. The pneumococcus can also colonize hands and fomites [236], although this only contributes to a small percentage of infections due to short survival. Transmission occurs frequently in close contact settings such as day-care centres [237], nursing homes [238], schools [239] and prisons [240]. Highly infectious strains have been shown to be able to move from city to city [241] and country to country [242].

3.5.4 Colonization

Transmission of *S. pneumoniae* is followed by the colonization of the nasopharyngeal tract by the bacterium. In order to effectively colonize the nasopharynx, pneumococci down-regulate expression of the polysaccharide capsule to allow membrane proteins to interact and adhere to epithelial cells [243]. Pneumococci remain in the nasopharynx asymptomatically for a limited duration or invade the host causing pneumococcal diseases. Nasopharyngeal carriage is more common in children than in adults [244, 245] and is known to be higher in developing countries than those found in developed countries [246]. In the Gambia, carriage rates are as high as 80% among babies aged <1 month, compared to 20% in the U.K [247]. Other developing countries such as Zambia, Pakistan and The Philippines also exhibit high carriage rates [246].

3.5.5 Disease

Following aspiration from the nasopharynx to the lower respiratory tract, pneumococci undergo phase variation to increase or reduces expression of capsule depending on its environment [248]. This process is assisted by pneumolysin [249], a virulence factor able to inhibit cilliary beating of epithelial cells [250]. Pneumococci then repeat the adherence and invasion

steps to bronchoepithelial cells using a variety of virulence factors. Invasion into the peribronchiolar space is facilitated by the activation and damage of epithelial cells through inflammatory mediators [251]. *In vitro*, *S. pneumoniae* have been shown to bind to type-2 alveolar cells leading to their transfer from the lungs into blood by modulating microfilament assembly [252]. *In vivo*, adherence and invasion of bronchoepithelial cells by 4 h post-infection was observed as well as varying levels of pneumococcal penetration and internalisation into host cells and translocation through epithelial layers [253]. Once in blood, pneumococci can infect all organs and cause sepsis or cause meningitis through the infection of the meninges via blood, or directly from the upper respiratory tract [244].

3.5.6 Treatment

Antibiotics have been the treatment of choice for various diseases caused by *S. pneumoniae* and Penicillin has been the mainstay of treatment. Resistance rates to penicillin are rising with over one third of isolates in the US being affected, while Central Europe is seeing 10% resistance rates [254]. In Senegal, resistance rates are as high as 62% although significantly lower in Ivory Coast (22%) and Morocco (9%) [255]. Furthermore, treatment with antibiotics is not the optimal solution, however, as it can cause complications in some patients. Especially in children, pneumococci reach high levels in cerebrospinal fluid before symptoms begin to appear. Treating such patients with antibiotics causes a sudden release of cell wall fragments that are responsible for the damage seen in cases of untreated meningitis, leading to further inflammation and dangerously high levels of inter-cranial pressure [256].

D. Augmented passive immunotherapy

4.1 Passive immunotherapy

Passive immunotherapy (serum therapy) is the transfer of pre-formed antibodies from an immune individual to a naïve or vulnerable individual thereby transferring immunity. Behring & Kitasato first described passive immunotherapy in 1890 despite their limited knowledge of the structure and function of antibodies [2]. This marked the beginning of passive immunotherapy against a variety of infections including pneumonia, meningitis, mumps, measles, polio, Spanish flu and many more [257-259]. Passive immunotherapy was the standard treatment for lobar pneumonia in the early 20th century and was reported to reduce hospital mortality from 90% to 45% [257, 260]. The discovery of antimicrobial compounds in the 1930s, however, led to the disuse of passive immunotherapy (Figure 19). Antibiotics had several advantages: they were cheaper, less toxic and more effective.

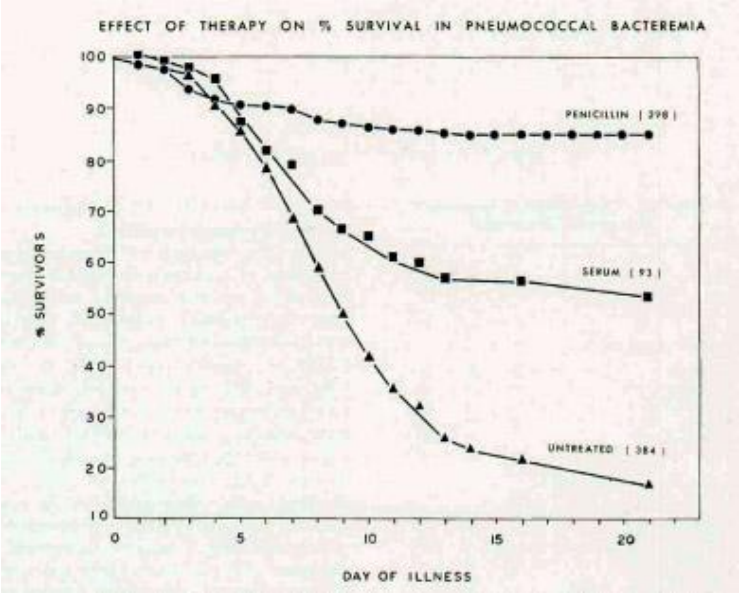


Figure 19 – Study by Austrian & Gold (1964) from a hospital in Brooklyn (NY, USA) showing survival from pneumococcal bacteraemia following treatment with penicillin (90%, n=298), serum (55%, n=93) or untreated (15%, n=384). Successes in the use of antibiotics to treat pneumococcal diseases contributed to the disuse of serum therapy [261].

4.1.1 Historical serum production

Use of animals for the generation of therapeutic serum was implicated in the development of "serum sickness", probably caused by hypersensitivity reactions to non-human antibody proteins and failure to clear immune complex leading to deposition in small vessels. Serum sickness was reported in 10-50% of recipients and manifested itself with rashes, proteinuria, and arthralgia [258]. In addition, the logistical challenges and costs of animal husbandry, purifying and refrigerating antibodies further added to the disuse of serum therapy. Initially, studies showing improved efficacy using a combined treatment of antibiotics and serum in animal models of pneumococcal [262], meningococcal [263] and scarlet fever [264] suggested using serum as an adjunct therapy. This, however, was not adapted as other studies debated the efficacy of adjunct treatments and serum still remained difficult to obtain [257].

4.1.2 Modern serum production

Improvements in antibody processing did not materialize until 1975 with the development of hybridoma technology to produce unlimited amounts of monoclonal antibody (mAb) and more recently the development of humanized antibody technology (hAb) [265] which abolished the issue of "serum sickness" [266]. Furthermore, improvements in the isolation of antibodies from human sera have led to the development of intravenous immunoglobulin (IVIG), a purified and processed serum able to be administered intravenously without toxicity (Figure 20). To produce IVIG, blood is collected from volunteers or paid individuals in blood donor collection centres or source collection plasma centres. Serum is isolated from blood through either automated apheresis or fractionation [267]. Automated apheresis separates blood and plasma through density, centrifugation or affinity methods and results in Fresh Frozen Plasma (FFP) that is suitable for direct patient infusion or further processing for IVIG production.

Fractionation is a multistep process that renders soluble plasma proteins insoluble which can then be separated through centrifugation [267]. Serum obtained through this method needs further processing before it can be administered safely into humans. IVIG usually contains serum pooled from >1000 human donors. A recent study looked at the efficacy of collecting large amounts of serum during an H1N1 epidemic. They found that plasma could be collected at a rate of more than 2000 L/week and the final IVIG product contained H1N1 antibody titers substantially higher than those collected before the emergence of the pandemic H1N1 virus [268].

Figure 20 - Immune serum then and now. (A.) Vial of anti-diphtheria immune serum dated 1895. Immune serum was produced by inoculating horses or goats with increasing doses of diphtheria toxins. Serum was then collected and bottled without further processing. (B.) Today, serum is collected from >1000 human blood donors and antibody isolated through fractionation or apheresis. Isolated antibody is then processed to produce an immune serum able to be administered intravenously (IVIG).

4.1.3 Passive immunotherapy today

Today, passive immunotherapy to treat infectious disease is regaining interest due to the advent of multi-drug resistant infections and the improved methods of obtaining serum. Modern IVIG has a broad repertoire of anti-carbohydrate antibodies [269] and also contains specific opsonizing antibodies against a range of pathogens including methicillin and vancomycin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci

and penicillin-resistant *Streptococcus pneumoniae* [270]. Gram(-) pathogens are also readily opsonised using modern IVIG [270]. The formulation of IVIG is such that, for example, anti-CMV, anti-*Haemophilus influenzae* and anti-*Streptococcus pneumoniae* antibodies have a median half-life of 26.4 days following administration into humans [271].

IVIG is increasingly being used to treat infectious diseases in clinical settings. A recent meta-analysis of randomized controlled studies looking at the use of IVIG in treating sepsis of children and neonates concluded that exogenous immunoglobulin exerts a significant effect on mortality in sepsis and septic shock [272]. Similarly, a meta-analysis of studies looking at the use of IVIG to treat adult sepsis demonstrated an overall reduction in mortality with the use of IVIG for the adjunctive treatment of severe sepsis and septic shock [273]. Large-scale clinical trials are necessary, however, to determine the efficacy of current IVIG formulations in treating sepsis and stimulate further research in passive immunotherapy against infectious diseases.

One problem of using passive immunotherapy against infectious diseases is the necessity for frequent and/or continual administration of antibodies. This limitation is partly due to the inherent kinetics of an immune response during passive immunotherapy [274]. It involves a wide range of cellular events including effector cells such as neutrophils, monocytes, macrophages, dendritic cells and T and B cells. The extent of immunological stimulation of the effector cells is greatly influenced by the quality and quantity of antigen specific antibodies. The kinetics of an immune response in passive immunization is therefore greatly influenced by the time taken by the effector cells to respond to the antibodies, which in turn determines the frequency and amount of antibody required [275]. Expediting the response time could lead to a rapid and efficient elimination of target pathogens [274].

4.2 P4 peptide

P4 is a 28-amino acid peptide fragment of the pneumococcal virulence factor PsaA. [276]. P4 contains a functional epitope for the adherence of PsaA to nasopharyngeal cells [276]. A 3D model of the P4 epitope within PsaA is shown in Figure 21. This epitope adheres to epithelial cells via a binding moiety on human E-Cadherin receptors (an important glycoprotein in calcium-dependent binding at tight junctions between epithelial cells) [277].

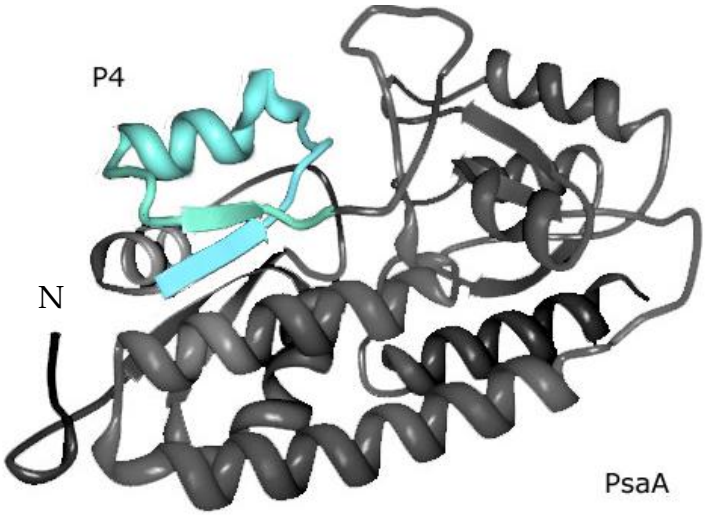


Figure 21 - Three-dimensional structure of Pneumococcal surface adhesin A (PsaA, Protein Database ID: 1PSZ) with the immunomodulating peptide fragment P4, highlighted in blue (Residues 251-278). N = N-terminal.

4.2.1 Structure

P4 peptide sequence is highly conserved within PsaA proteins from all pneumococcal serotypes and also amongst several other bacterial species. The LpeA protein from *Listeria spp.* in particular exhibits remarkable similarities both in structure and function to PsaA. LpeA has recently been shown to be used by *Listeria spp.* to invade macrophages through mechanisms still to be elucidated [278]. Table 3 (adapted from [279]) shows the sequence homologies of P4 peptide to non-streptococcal proteins. With the exception for possible

roles of binding and adhesion, other functional similarities remain to be determined for proteins other than LpeA.

Table 3 - P4 sequence homology with non-streptococcal proteins. Adapted from [279]

Source organism	Putative function	Sequences producing significant alignments*
S. pneumoniae	P4 peptide sequence	[251]LFVESSVKRRPMKTVSQDTNIPIYAQIF[278]
S. pneumoniae	PsaA	[245]LFVETSVDDRSMETVSKETNVPIAGTIF[272]
S. pneumoniae	ABC transporter substrate-binding protein	[251]LFVETSVDDRSMETVSKETNVPIAGTIF[278]
Bacillus anthracis	Manganese binding protein and adhesin	[257]LFVETSVDRRSMETVSKETNVPIAGTIF[284]
B. cereus	Adhesion lipoprotein	[253]LFVETSVDRRSMETVSKETNVPIAGTIF[280]
B. cereus E33L	Adhesion lipoprotein	[253]LFVETSVDRRSMETVSKETNVPIAGTIF[280]
Listeria innocua	Adhesion binding protein and ABC transporter	[252]LFVETSVDPRSMETVSKETNVPIFAKIF[279]
L. monocytogenes	Adhesion binding protein and ABC transporter	[252]LFVETSVDPRSMETVSKETNVPIFAKIF[279]
Leuconostoc mesenteroides	ABC transport protein and adhesin	[255]LFVESSVSPKAMEKVSKETGLPIYSKIY[282]

4.2.2 Functions *in vitro*

P4 peptide has been shown to activate human nasopharyngeal cells, modulating their cytology and cytokine secretions thereby enhancing adherence and internalization of pneumococcal bacteria *in vitro* [279]. P4-mediated activation was also demonstrated with other eukaryotic cells, including mouse macrophages (RAW 261.4) and freshly isolated polymorphonuclear neutrophils from healthy human donors [279] [280]. When exposed to the peptide, HL-60 cells undergo various phenotypical changes such as increased pseudopods, foamy, granulated cytoplasm with condensed nuclei and large vacuoles with internalized materials (Figure 22)

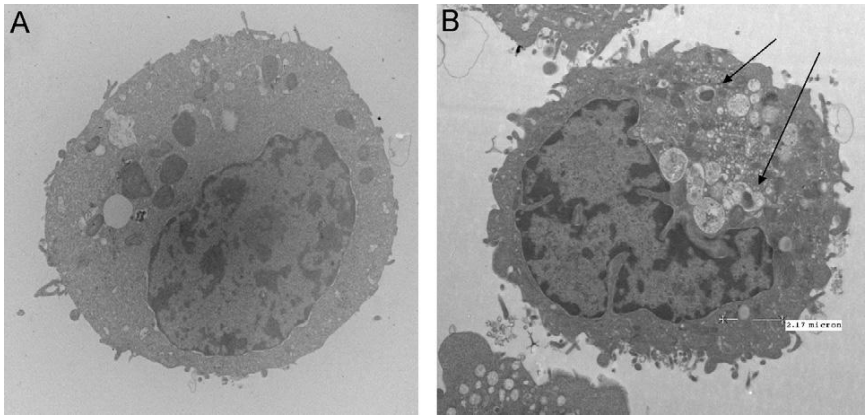


Figure 22 - HL-60 cells in a normal state (A) exposed to the P4 peptide (B). The arrows highlight increased intra-cellular granules present. Adapted from [279].

The increased activation seen in phagocytes following P4 exposure also leads to enhanced phagocytosis. In opsono-phagocytosis assays, in the presence of species specific antibody and complement, P4 is able to increase the uptake and subsequent killing of bacteria by >70%. This has been established in the HL60 cell line as well as mouse & human derived peripheral blood neutrophils.

4.2.3 Functions *in vivo*

Additionally, Rajam *et al* (280) were able to show the potential of using P4 mediated passive immunotherapy *in vivo*. In a fatal pneumococcal mouse model, P4 therapy was able to rescue 80% of moribund mice by giving them a combined treatment of P4 and pathogen specific antibodies. In contrast, only 10% of the control strain and 30% of the mice treated purely with antibodies and complement survived [280]. Both *in vivo* and *in vitro*, P4 shows no signs of toxicity.

Further research at the CDC has shown that enhanced phagocytosis *in vitro* confers therapeutic benefit *in vivo* [281]. Rajam *et al*. used reference *in vitro*

opsonophagocytic killing and uptake assays to show that P4 enhanced opsonophagocytosis of *S. pneumoniae* by human promyelocytic leukaemia cells (HL-60) differentiated into granulocytes [280]. In an *in vivo* extension of this study, mice were infected with *S. pneumoniae*, then injected with IVIG and/or P4 at 72 and 96 hours post-infection. Survival was 10% in untreated mice, ~30% in mice given IVIG or P4 alone, and 80% in those treated with both IVIG and P4.

Adding a low dose of ceftriaxone to P4 and immunoglobulin increased mouse survival to 100% [280]. P4-activation of *in vitro* opsonophagocytosis has also been demonstrated with *Neisseria meningitidis* and *Staphylococcus aureus*, in the presence of pathogen-specific antibodies [282]. In a murine model of overwhelming *S. aureus* infection, intravenous P4 with immunoglobulin rescued 70% of mice, compared with 0% survival of untreated controls [282].

Augmented passive immunotherapy combines commercially available pooled immunoglobulin G (IVIG) and the phagocyte stimulating peptide P4 to rapidly and effectively augment an immune response before the effect of antibiotic treatment would be apparent.

4.2.4 Summary of P4 publications

There are currently 7 publications related to P4 peptide. Their main findings are summarized in Table 4.

Table 4 – Main findings from previous P4 peptide publications.

Year	Main findings	Ref
2007	P4 treatment of epithelial cells leads to **cytological changes** allowing for increased binding and internalization of *S. pneumoniae.* NP cells reduce their secretion of FGF-β, but increase their secretion of IL-6, IL-8 and VEGF **cytokines** following peptide treatment.	[279]
2008	P4 treatment of HL-60 neutrophil cell line leads to **increased phagocytosis** in a dose-dependent manner. P4 and antibody treatment of *S. pneumoniae* infected mice **increased survival** from 10% to 80%.	[280]
2009	P4-antibody therapy as an **adjunct to antibiotics** reduced the need for dual administration and led to 100% survival (compared to 90% P4-antibody alone and 20% control). **Repeat therapy** was effective following infection of previously rescued mice. **Septic blood neutrophils** from mice showed enhanced phagocytosis following peptide treatment.	[281]
2010	Peptide-antibody treatment of two strains of **aged mice** led to significantly increased survival.	[283]
2011	Survival was improved from 20% to 80% in mice with *S.pneumoniae*-influenzea co-infection and treated with P4. Survival correlated with increased clearance of bacteria and virus and **decreased lung consolidation**.	[284]
2011	API **increased survival** of *S. aureus* infected mice infected mice from 20% to 70% P4 stimulation led to enhanced **phagocytosis of *S. aureus*** by HL-60 neutrophil cells *in vitro*.	[282]
2012	Increased survival following API treatment of sepsis due to increased **phagocytic activation and recruitment**. Intranasal administration of P4 to **treat pneumococcal pneumonia**. *Ex vivo* and *in vitro* **macrophages increase phagocytosis** following peptide treatment.	[285]

E. Aims and objectives

There were four main aims in this project, which are addressed in their relevant chapters. The first aim was to characterise the phagocytic host response to peptide administration in the absence of infection (chapter III). The second aim was to translate these findings into infection studies by assessing phagocytic host responses to infection and treatment (chapter IV). The third aim was to determine potential mechanisms by which P4 augments phagocytic killing (chapter V). The final aim was to translate the murine and *in vitro* findings to primary human phagocyte studies (chapter VI).

CHAPTER II.

Materials & Methods

2.1 Microbiology

2.1.1 Bacterial strains

Table 5 details the strain and type of pneumococci used in this thesis. Laboratory serotype 2 strain D39 pneumococci were passaged once in a murine host to standardize their virulence prior to *in vivo* studies (see chapter III). For each serotype, a single culture broth was batch processed and pneumococci stored in single-use aliquots for < 6 months. A total of eight pneumococcal serotypes were used for the *in vitro* and *ex vivo* studies. Both laboratory and clinically derived strains of pneumococci were used.

Table 5 – The type, source and use of *S. pneumoniae* strains for *in vivo* studies.

Serotype (Strain)	Type/Source	Use
2 (D39) *In vivo passaged*	Laboratory (NCTC 7466)	Acute IPD studies Carriage studies
3 (WU2)	Laboratory (ATCC WU2)	IPD studies Aged mice studies
2 (D39)	Laboratory (NCTC 7466)	*Ex vivo, in vitro*
4 (TIGR4)	Laboratory (ATCC BAA344)	*In vitro*
2, no capsule (D39)	Laboratory (7466Δcps)	*In vitro*
4, no capsule (TIGR4)	Laboratory (BAA344Δcps)	*In vitro*
19F (ST180)	Clinical (Invasive)	*Ex vivo, In vitro*
6B (ST138)	Clinical (Invasive)	*Ex vivo, In vitro*
14 (ST124)	Clinical (Invasive)	*In vitro*
23F (11902)	Clinical (Invasive)	*Ex vivo*

2.1.2 Standard media

Blood agar base (BAB) culture plates

16 gram of BAB medium (SIGMA) was mixed with 400 ml of distilled water and autoclaved at 15 psi (103 kPA) for 20 minutes. Following autoclaving and once the medium reached ~56°C, 20 ml of Sterile Defibrinated Horse Blood (SIGMA) was added and gently mixed. The resulting medium was then poured into sterile petri dishes (90 mm), left to dry >3 hours then stored inverted either at RT or at 4°C. 400 ml of medium resulted in ~25 BAB culture plates and were stored for <7 days.

BAB culture plates +5% v/v horse blood with gentamicin

BAB culture plates were prepared as above with a minor modification: Immediately after the addition of Horse Blood, 2 μg/ml of gentamicin (SIGMA) was added to the broth and gently mixed.

Brain heart infusion (BHI) broth

37 gram of BHI medium (SIGMA) was mixed with 1 litre of distilled water and autoclaved at 15 psi (103 kPA) for 20 minutes. Following autoclaving, the medium was checked to ensure it's pH value was 7.4 ± 0.2 and stored at RT for <6 weeks.

BHI serum broth

BHI broth was prepared as above with a minor modification: After autoclaving, 20 ml of Fetal Bovine Serum (FBS) was added to 80 ml of medium to reach 80% v/v BHI medium with 20% v/v FBS. BHI Serum Broth was prepared fresh for each use.

Cryopreservation solution

In order to optimally cryopreserve isolated leukocytes, a freezing solution was prepared using the ratios of 75% RPMI medium (Sigma), 15% Foetal bovine serum (FBS, Gibco) and 10% dimethyl-sulphoxide (Sigma).

2.1.3 Viable count of bacteria (Miles & Misra Method)

The Miles & Misra is an accurate dilution method for determining the number of colony forming units (CFU) present in a given sample [286] [287]. It relies on serial dilutions, culturing and visual counting of CFU. The number of CFU detected in a given dilution is used to extrapolate the CFU / ml of sample.

20 µl of sample (e.g. aliquot, infection dose, blood or tissue homogenate) for viable count testing was added to 180 µl sterile PBS and serially diluted (10-fold) until dilutions of 10^6 were reached. BAB culture plates were divided into six sections, and 60 µl (3 x 20 µl) of each dilution was plated into each corresponding sector and incubated at 37°C 5%CO_2 (Figure 23).

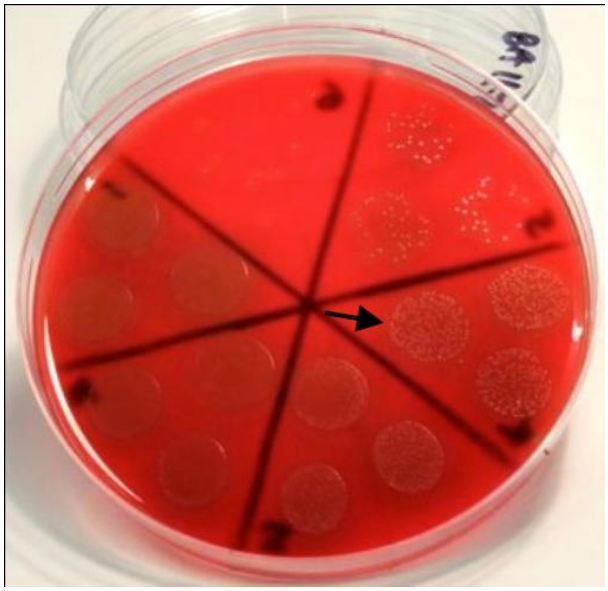


Figure 23 - Miles and Misra. Arrow indicates sector in which 30-300 CFUs were counted.

The following day, sectors in which 30-300 CFU were visible were counted and the CFU/ml or CFU/mg calculated as follows:

To evaluate numbers of CFU in blood or infection dose:

$$\frac{CFU}{ml} = average\ number\ of\ colonies\ in\ sector\ \times\ dilution\ factor\ \times \frac{1000}{60}$$

To evaluate numbers of CFU in lung or nasopharyngeal tissue:

$$\frac{CFU}{mg} = \frac{CFU}{ml} \times\ 10\ or\ 5\ (lung\ in\ 10ml, nasopharnyx\ in\ 5ml) \div tissue\ weight$$

2.1.4 Preparing stocks of pneumococci

Pneumococci from laboratory bead collections were streaked for isolation on BAB culture plates and grown overnight at 37°C (5% CO_2). In addition, an optochin disk was placed on the initial site of inoculation (Figure 24). A zone of inhibition around the optochin disk indicates susceptibility of the inoculum to optochin; a hallmark of the pneumococcus and the most common method of distinguishing between pneumococcus and *Streptococcus viridans*.

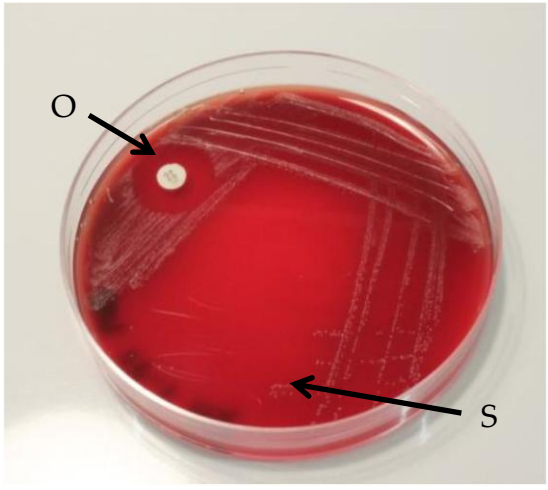


Figure 24 - Streaking pneumococci for isolation. *S. pneumoniae* grown on BAB culture plates and tested for optochin susceptibility. (O) shows the zone of inhibition surrounding the optochin disk while (S) shows single colonies.

Following overnight culture, a sweep-full of colonies was used to inoculate 10 ml BHI (pre-warmed to 37°C) in a sterile universal tube. The inoculum was incubated for 16-18 hours at 37°C (5% CO_2) until OD_{500} 1.4-1.6.

The BHI pneumococcal culture broth at correct OD was centrifuged at 1500 *x* g for 15 minutes, the supernatant carefully removed and the remaining pellet re-suspended in 1ml BHI serum broth. 700 µl of the re-suspended pellet was added to 10ml of BHI serum broth to reach an OD_{500} 0.7 (adjusted if necessary by addition of BHI serum or pellet) and incubated statically for 5 hours at 37°C (5% CO_2) until $OD_{500} \geq 1.6$. Following incubation, the culture broth was centrifuged at 1500 *x* g for 15 minutes, the supernatant discarded and the pellet re-suspended in 10 ml fresh BHI Serum Broth.

The resulting serum broth containing pneumococci was then divided into 500 µl single use aliquots in sterile cryotubes and slow-frozen at -80°C. After 48 hours in the freezer, aliquots were thawed and numbers of pneumococci / ml determined using the Miles and Misra Method.

2.2 Murine strains

Table 6 shows the age, sex, genetic background and supplier that were used in this thesis. Mice were allowed to acclimatize for one week upon arrival and kept in groups of 5 in individually ventilated micro-isolator cage (IVC) racks. Food and water was provided *ad libitum*. All *in vivo* experiments were conducted following guidelines from ethical review and animal welfare committee and under authority of UK Home Office licence (project licence PPL:80/2111, personal licence PIL:40/9700) or the Institutional Animal Care and Use Committees (IACUC) at the Centres for Disease Control and Prevention.

Table 6 - Strain, age, sex, supplier of the mice and their application used for *in vivo* studies.

Strain	Age	Sex	Supplier	Application
MF1	8-12 weeks	Female	Charles River UK	Acute IPD studies Carriage studies *Ex vivo* studies Animal Passage Virulence test
Swiss Webster	8-12 weeks	Female	Charles River USA	IPD studies
Swiss Webster	15 months	Female	Charles River USA	Aged mice IPD studies
BALB/c	11 months	Female	Charles River USA	Aged mice IPD studies

2.2.1 Monitoring murine behavior

Following infection, the physical appearance of mice was assessed frequently to judge the course of infection and the wellbeing of mice. The scoring system [288] used to assess the ability of mice to groom themselves, their posture and their activity is detailed in Table 7.

To ensure objective scoring, cage-identification cards were concealed prior to scoring. Once mice reached a point score of "lethargic ++" they were culled in accordance to the severity limits of the UK Home Office Licence under which these experiments were performed.

Table 7 - Description and score used to assess the development of infection in murine models.

Score	Description
Normal	Mouse is moving around the cage normally and has a normal coat.
Hunched +	Mouse is slightly arched over in the middle and walking on tip toes.
Hunched ++	Mouse is very arched over and walking on tip toes.
Starry +	Coat of the mouse is not groomed well around the neck area and fur is upright.
Starry ++	Coat of the mouse is not groomed all over and fur is upright.
Lethargic +	Mouse is slower at moving around the cage.
Lethargic ++	Mouse is not moving around the cage unless encouraged.
Moribund	Coat of the mouse is not at all groomed, mouse has stopped moving around that cage, and mouse has labored breathing.

2.3 Viable count of leukocytes (haemocytometer)

A haemocytometer is a thick glass microscope slide with laser-etched grids creating chambers with known volume. As such, the volume of any cell solution added to these chambers is known and the number of cells / ml can be extrapolated. Trypan blue is used to determine cell viability (Figure 25).

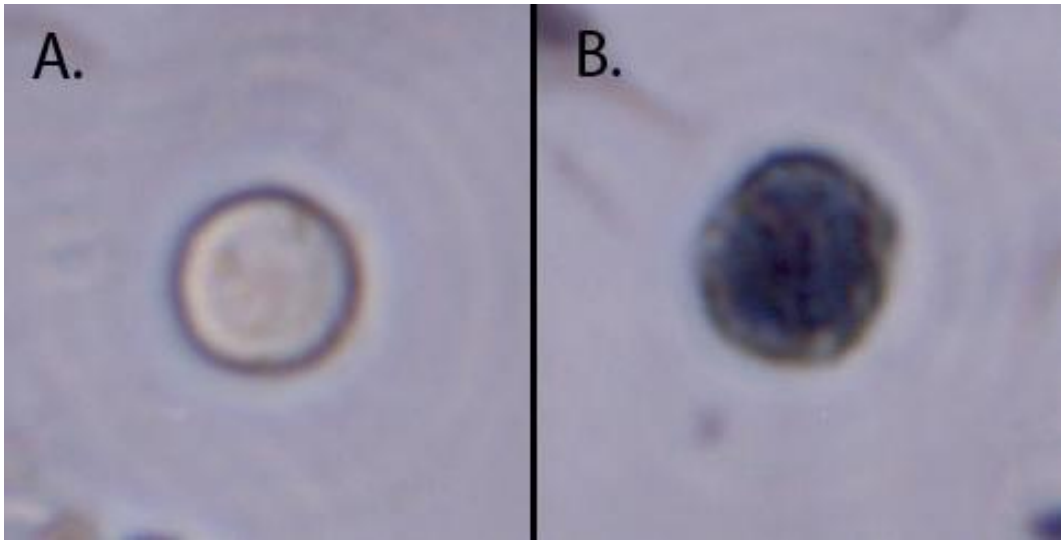


Figure 25 - Assessing viability using Trypan Blue. Dead cells do not maintain membrane integrity and take up Trypan blue dye thereby indicating non-viability. (A) Shows a viable HL-60 cell not taking up the dye while (B) has clearly taken up the dye indicating the cell is not viable.

Cell suspensions were thoroughly mixed and two 50 µl samples added to 50 µl Trypan blue solution (diluted 1:4 with PBS). The resulting suspension was added to the haemocytometer chamber and cells counted in specific grids using a light microscope (Figure 26).

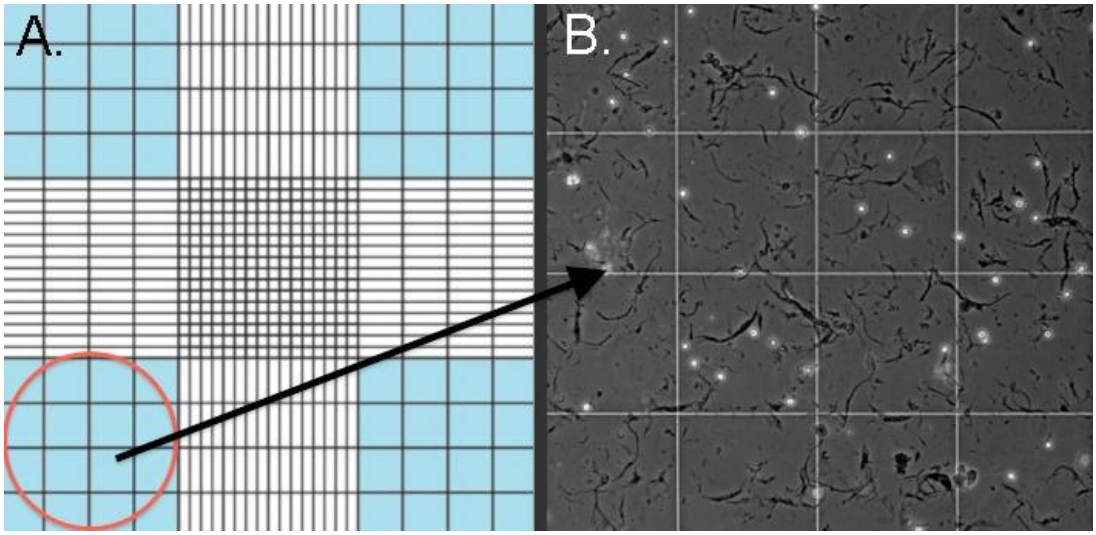


Figure 26 –Schematic diagram of a haemocytometer and its gridded chambers. (A) The blue areas (and red circle) represent area in which cells are counted. (B) High contrast image focused on one section (highlighted in red on left panel) of a haemocytometer. White dots indicate cells. Image taken at 40x magnification.

Using the number of alive & dead cells counted in each grid, and by incorporating the dilution factors, the number of cells / ml and their viability was calculated as follows:

$$\frac{Cells}{ml} = \left(\frac{total\ number\ cells\ in\ four\ grids}{4} \times 10^4\right) \times 2$$

$$\%\ non-viable = \frac{total\ number\ Trypan\ Blue\ positive\ cells\ counted}{total\ number\ cells\ counted} \times 100$$

2.4 Infection studies

2.4.1 Lung and nasopharyngeal tissue

Lung or nasopharyngeal tissue were harvested and placed into universal tubes containing 10 ml or 5 ml sterile PBS, respectively. Tissue weight was determined by comparing weight of tube before and after the addition of lung or nasopharyngeal tissue. Once the weight was recorded, tissue was mechanically disrupted by homogenization with an Ultra-Turrax T8 homogeniser (IKA) for ~1 minute. The resulting homogenate was briefly vortexed and two 20 µl samples were taken and assessed for the numbers of CFU using the Miles and Misra method.

2.4.2 Blood tissue

Blood was collected by cardiac puncture following terminal anesthesia or, on live mice, by removal of a small volume from the tail vein and immediately placed into eppendorf tubes containing 2 µl heparin (10 units / ml, Sigma) to avoid blood clotting. Two 20 µl samples were taken from the blood samples and assessed for CFU using the Miles and Misra Method.

2.5 Cell population studies

2.5.1 Tissue preparation (lungs)

Lung tissue was first weighed then placed in a sterile petri dish and cut into small pieces using a scalpel. To further break down lung tissue, thereby releasing immune cells, the disrupted lung tissue was enzymatically digested by placing it in a 1.5ml eppendorf tube containing 1ml of PBS and 10 mg/ml of Collagenase D (Roche) for 30 minutes at 37°C. Following digestion, cell suspensions were yielded by passing the lung tissue through a 40 μm pore diameter cell sieve (BD Biosciences) with the aid of a cell plunger and by flushing the sieve twice with 10 ml sterile PBS. The cell suspensions were then centrifuged at 300 *x* g for 10 minutes and the supernatant discarded. The resulting cell pellet was re-suspended in cryopreservation solution and immediately placed in a slow-freezing jar at -80°C. Aliquots of frozen cells were quickly thawed in a room temperature water bath when required.

2.5.2 Tissue preparation (blood)

Samples were first subjected to red blood cell (RBC) lysis using the BD Pharm Lyse kit (BD Biosciences). All reactions were done at RT. RBC-lysis solution was diluted 1:10 with distilled H_20 and added to blood samples at ratio of 1:3 (1ml blood: 2ml RBC-lysis). 10 minutes later, lysis was stopped by dilution with PBS. The tube was then centrifuged at 300 *x* g for 10 minutes, the supernatant discarded and the resulting cell pellet re-suspended In PBS for another washing step. The resulting cell pellet was re-suspended in cryopreservation solution and immediately placed in a slow-freezing jar at -80°C. Aliquots of frozen cells were quickly thawed in a room temperature water bath when required.

2.5.3 Leukocyte preparation

Leukocytes derived from blood or lungs were stored in DMSO-containing cryopreservation solution, which can be toxic to cells and interfere with antibody binding. Cells were therefore thawed and then washed twice by centrifuging aliquots at 300 *x* g for 10 minutes, discarding the supernatant and re-suspending the cell pellet in 5ml RPMI. Following the washing steps, the cell suspension was counted using a haemocytometer and the volume adjusted to obtain ~5 x 10^6 cells/ml of suspension (If necessary, cells were centrifuged at 300 *x* g for 10 minutes, the supernatant discarded and the pellet re-suspended in a volume to obtain the desired cell concentration).

2.5.4 Antibody staining

Labeled anti-CD antibodies purchased from Biolegend, USA, were used. 200µl of the cell suspensions were stained for 30 minutes at 4°C using 50µl optimized antibody concentrations (see Table 8) of CD45 FITC (hematopoietic cells marker, 30-F11), F4/80 APC (macrophage/monocyte marker, BM8), GR1 PE/CY7 (neutrophil marker, RB6-865), and CD16/32-PE (Fc-γRII/III marker, 93). Further staining involved using CD45 and isotype-matched control antibodies for each primary antibody. Following staining, excess/unbound antibody was removed by centrifuging cells twice at 300 *x* g for 10 minutes, discarding the supernatant and re-suspending the resulting pellet in 1ml PBS (first wash) and 200µl PBS (second wash). 300 µl of PBS was added to the final cell solution.

Table 8 - Antibodies and their relative isotype control used for Flow Cytometry staining of immune cells populations.

Antibody	Conjugate	Dilution
CD45 (haematopoietic cells)	FITC	1/300
F4/80 (monocyte/macrophage)	APC	1/400
Gr-1 (granulocyte/neutrophil)	PE/Cy7	1/300
CD16/32 (Fcy RII/III)	PE	1/250
Rat IgG2b - κ Isotype Ctrl	FITC	1/300
Rat IgG2b - κ Isotype Ctrl	PE/Cy7	1/300
Rat IgG2b - κ Isotype Ctrl	PE	1/250
Rat IgG2b - κ Isotype Ctrl	APC	1/400

2.5.5 Acquisition and analysis

Following staining, leukocytes were acquired using a FACSCalibur Flow Cytometer (BD). Prior to acquisition, a dot plot displaying the forward scatter parameter and FITC CD45 was created. A gate was placed around the $CD45^+$ population and 50,000 events within this gate collected.

Flow Cytometry data was analysed using the CellQuest Pro software supplied by the manufacturer (BD). $CD45^+$ events were gated to isolate hematopoietic cells from red blood cells and tissue debris. The $CD45^+$ population was plotted so that macrophages (F4/80) or neutrophils (Gr1) could be isolated based on their fluorescence (APC and PE-Cy7, respectively). Within the macrophage/neutrophil populations, PE fluorescence was used to measure relative Fcγ receptor expression.

For blood and BAL cell populations, the percentage of each cell type was determined by dividing the number of $F4/80^+$ or $Gr1^+$ events collected by the number of $CD45^+$ events acquired. For lung cell populations, cells/mg tissue was calculated by extrapolating from the 50,000 collected events the total

number of each population in the complete tissue sample and then dividing by the tissue weight.

For all samples, the fluorescence from isotype control stains was used as baseline during analysis. Figure 27 shows an example of using CD45-FITC fluorescence to isolate hematopoietic cells and Gr1-PE/CY7 fluorescence to isolate neutrophils from a blood sample and how baseline fluorescence was determined using isotype control fluorescence.

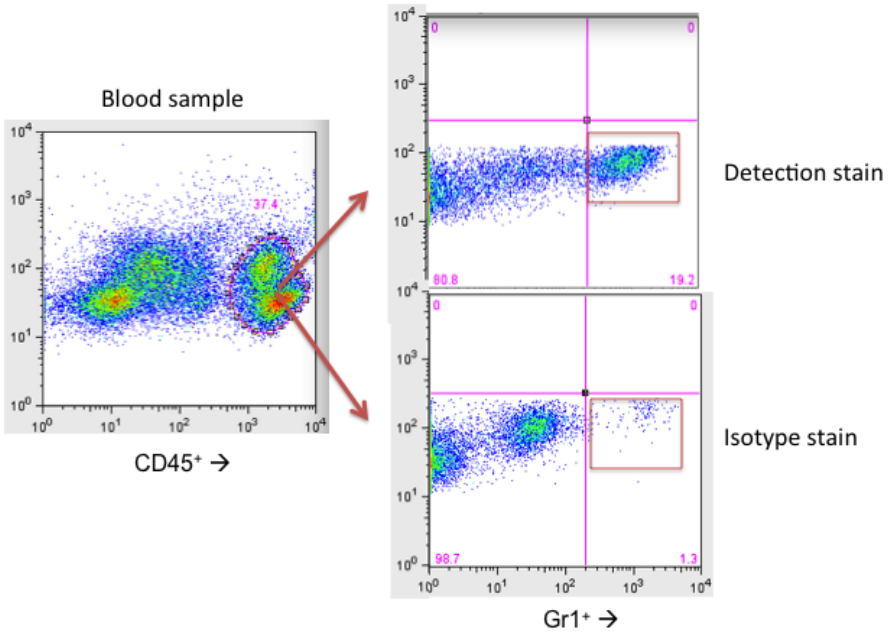


Figure 27 - Example of gating strategy to detect neutrophil populations and account for unspecific binding (isotype control) during analysis. Blood samples are first plotted against side scatter and CD45 FITC fluorescence. A gate is drawn around the CD45⁺ population and plotted on a new graph using side scatter and either Gr1 or PE-Cy7 isotype fluorescence parameters. Fluorescence measured in the isotype stain was used as background and subtracted from Gr1 fluorescence to determine actual expression.

2.6 P4 peptide

2.6.1 Peptide synthesis

The 28 amino acid peptide P4 (LFVESSVKRRPMKTVSQDTNIPIYAQIF) was synthesized and purified at the Centers for Disease Control and Prevention, Atlanta, GA and the Emory University Microchemical Facility. Peptide synthesis was performed in an Advanced ChemTech 396 multiple peptide synthesizer by means of standard and modified 9-fluorenyl-methoxycarbonyl (Fmoc) protocols [289-291] resulting in a free N- and C-terminus.

2.6.2 Peptide control and storage

Each fresh batch of peptide was quality controlled for purity by the Emory University Microchemical facility; a minimum of 97% purity only was used for all studies. P4 peptide was best solubilized in DEPC-treated water (280) (Ambion, USA). Peptide activity was quality controlled using the standardized HL-60 OPKA for each received batch of peptide. Peptide solutions were prepared fresh on the day of experiment and stored at 4°C (melting ice) until use. For *in vivo* work, peptide solution was hand-warmed (37°C) for 1 minute prior to administration.

2.7 Opsonophagocytosis killing assay

Opsonophagocytosis killing assays (OPKA) were performed using a variety of phagocytes. Minor modifications were applied to the method in order to account for the difference in cell types. The opsonophagocytosis assay can be divided into three stages: opsonisation, phagocytosis and analysis.

2.7.1 Opsonisation

An aliquot of non-passaged pneumococci was thawed and diluted in sterile PBS to reach 5 x 10^4 CFU / ml. Intravenous immunoglobulin (IVIG, Gamunex, Lot 26NG042, Talecris) was used as a source of pathogen-specific antibody. 1 ml of the diluted bacterial suspension was added to a universal tube containing 1 ml of IVIG diluted 1:4 in sterile PBS. Additionally, 1 ml of the bacterial suspension was added to a universal tube containing 1 ml of sterile PBS as a non-opsonized control. Control and IVIG tubes were then added to a shaking (300 RPM) incubator at 37°C for 15 minutes to allow for opsonisation to occur.

2.7.2 Phagocytosis

For murine BAL, peritoneal, J774 and human BAL macrophages. Medium (see chapter methods for specifics) was carefully removed from the wells containing 5x10^4 adhered macrophages and replenished with 30µl fresh medium. 10 µl of baby rabbit complement (PelFreeze, USA) and 20µl of opsonized bacterial suspension (containing 5 x 10^2 pneumococci) was then added to the wells. Finally, 20µl of DEPC treated water or 20µl of P4 solution (10 µg peptide in 20 µl DEPC treated water) was added to the wells to make a final volume of 80 µl / well.

For HL-60 neutrophils, blood derived neutrophils and THP-1 monocytes. 20µl of opsonized bacterial suspension (containing 5 x 10^2 pneumococci) and 10 µl of baby rabbit complement were added into wells of a 96-well plate. 20 µl of cell suspension (containing 1 x 10^7 cells) was then added to each well giving a multiplicity of infection (m.o.i.) of 1:400 (1 pneumococcus to 400 phagocyte). Finally, 20 µl of DEPC treated water or 20 µl of P4 solution (10 µg peptide in 20 µl DEPC treated water) was added to the wells to make a final volume of 80 µl / well.

Control reactions. Control wells included the use of non-opsonized bacteria or the use of heat-inactivated complement (inactivated by incubating for 30 minutes at 56°C) with and without P4 solution. Wells containing opsonized bacteria and complement only were used to establish the bacterial dose.

Incubation time. For neutrophils (freshly derived & HL-60), monocytes (THP-1) and murine macrophages, cells were incubated for 45 minutes at 37°C (5% CO_2). For human alveolar macrophages, cells were incubated for two hours at 37°C (5% CO_2).

Culturing. Following incubation, 10 µl from each well was plated in duplicates onto blood agar plates and incubated for 18-24 hours at 37°C (5% CO_2). The remaining volume from the wells were collected, centrifuged at 300 *x* g and the resulting supernatant stored at -80°C.

2.7.3 Analysis

The following day, CFU on blood agar plates were counted. In order to determine the efficacy of killing of opsonized pneumococci, the number of bacterial CFU in the bacterial dose (opsonized pneumococci and complement) was compared to the number of bacterial CFU recovered from reaction wells (opsonized pneumococci, complement, macrophages and ± P4 solution). A successfully diluted bacterial dose (see opsonisation 2.9.1) resulted in 60 ±10 CFU. The killing index was then calculated as follows:

$$\%\ killing\ index = 100 - \left(\frac{average\ CFU\ recovered\ from\ reaction}{average\ CFU\ from\ bacterial\ dose} \times 100\right)$$

An example of counting CFU cultured overnight from the wells of an opsonophagocytosis killing assays is shown in Figure 28.

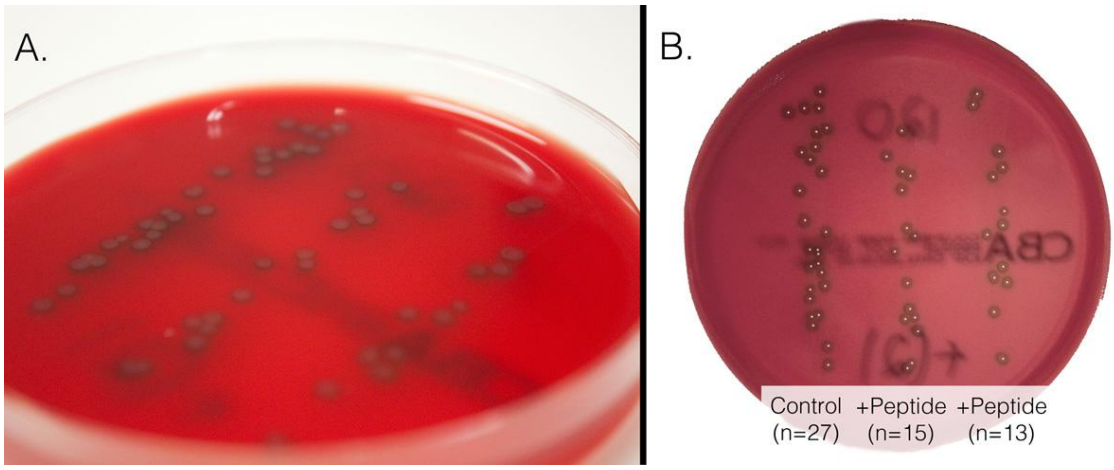


Figure 28 - (A) Pneumococcal CFU on blood agar cultured from the supernatant following an opsonophagocytosis killing assay. (B) Example of CFU counting to determine bacterial killing. 27 CFU were recovered from control wells compared to 15 and 13 from wells containing a stimulant.

CHAPTER III.

P4 peptide studies in healthy mice

A. Introduction

3.1 Introduction

Inflammatory mediators during infection significantly modulate cellular immune responses. The aim of this chapter was to assess *in vivo* responses to peptide administration in the absence of infection in order to establish baseline responses to peptide treatment in a healthy host prior to the infection studies in chapter IV.

3.1.1 Behavioral studies

It is well established that the administration of inflammatory mediators such as LPS or Interleukin-1 affects neuronal responses and induces "sickness-like" behavior in mammals [292-295]. Symptoms of sickness-like behavior include a loss of appetite, sleepiness, withdrawal from normal social activities, fever and fatigue and are thought to be part of a motivational system that prioritize the host's recovery from infection [296]. To determine whether peptide administration would induce such responses, P4 at the treatment dose and a dose 10 x higher was administered intravenously into mice and their behavior was monitored with respect to activity, breathing, ability to groom themselves and posture as previously established [288].

3.1.2 Cell response studies

Previous studies on augmented passive immunotherapy (API) *in vivo* described host survival and pathogen clearance but did not define host cellular immune responses to infection and treatment [274, 281, 283]. As infection and associated cytokines strongly modulate cellular responses, naïve healthy mice were chosen to determine baseline levels in cellular responses

following peptide administration. Both intravenous and intranasal routes of peptide administration were used and cellular responses defined in peripheral blood and BAL, respectively. Due to the nature of API (augmenting phagocytosis in the presence of pathogen specific immunoglobulin), monocytes, macrophages and neutrophils were chosen as the main phagocytic cells likely to respond to API.

3.1.3 FcγR expression

API relies on the presence of pathogen specific immunoglobulin G to be effective [274, 281, 283]. It is therefore likely that the phagocytic Fcγ receptor is involved during augmented phagocytic clearance of pathogens. To determine the phagocytic potential of neutrophils, monocytes and macrophages following *in vivo* peptide stimulation, Fcγ receptor expression of phagocytes in BAL and peripheral blood was determined using flow cytometry. In addition, to confirm flow cytometry results and determine the relative localization of Fcγ receptors, immunogold staining and electron microscopy was employed to visualize and enumerate Fcγ receptor expression.

3.1.4 Phagocytosis assays

Previously, murine peripheral blood neutrophils were shown to augment their phagocytic killing of opsonised pneumococci following peptide administration [281]. The phagocytic response of murine macrophages was unknown. Murine alveolar and peritoneal macrophages were therefore extracted from healthy mice and challenged with IVIG opsonised pneumococci in the presence or absence of P4. In addition to *ex vivo* macrophages, the murine macrophage cell line J774 was used to determine phagocytic killing of a range of IVIG opsonised pneumococcal serotype in the presence of P4.

B. Materials & Methods

3.2 Behavioral response to P4 dosage in the MF1 mouse host

P4 peptide was pre-tested in mice to determine behavioural responses to peptide administration. Two doses were chosen: (A) the treatment dose of 100 µg P4 peptide / 100 µl DEPC treated water and (B) a 10 x higher dose of 1 mg P4 peptide / 100 µl DEPC treated water. Three mice per group were injected twice, 6 hours apart and monitored based on their ability to groom themselves, their posture and their activity as previously established [288]. Mice that received peptide solution were observed thrice daily (8AM, 2PM, 8PM) for two days and their behavioral responses compared to healthy naïve littermates.

3.3 Cellular responses to peptide administration

The effect of *in vivo* peptide administration on phagocyte populations was observed in the absence of infection. P4 solution was administered either intravenously or intranasally (see Table 9 for peptide concentration) and peripheral blood phagocytes or BAL phagocytes isolated for Flow Cytometry staining.

Table 9 - Volume and concentration of intravenous and intranasal peptide administration in the absence of infection.

Administration	DEPC volume	P4 Peptide
Intravenous	100 µl	100 µg
Intranasal	40 µl	100 µg

3.3.1 Intravenous administration

Solubilized peptide (see Table 9 for peptide concentration) was injected into dorsal tail veins (n=5 per time point). Blood tissue was collected by cardiac puncture following terminal anesthesia at 1, 4 and 24 hours following injection. Blood samples were then purified and prepared for staining as described in chapter II.

3.3.2 Intranasal administration

Solubilized peptide (see Table 9 for peptide concentration) was administered intranasally to mice (n=5 per time point). Mice were first lightly anaesthetized using 2.5% v/v inhaled Isofluorane (1.6-1.8 L O_2/min) in an anaesthetic box. The absence of reflex reactions and twitching of whiskers confirmed anesthesia. Once anaesthetized, the mice were scruffed and P4 solution administered equally into both nostrils in a drop-wise fashion, allowing each drop to be inhaled before the next was administered. At 1, 4 and 24 hours following administration, BAL fluid was collected following terminal anesthesia using the methods described in this chapter. BAL samples were centrifuged and re-suspended in PBS for staining as described in chapter II.

3.4 Immunogold receptor staining

FcγR membrane protein was stained using colloidal gold and viewed using TEM to determine receptor localization on membranes and in phagosomes as well as relative numbers per cell. Primary antibody solution was prepared by diluting 1 μl of rat anti-mouse CD16/32 (Fc-γRII/III marker, 93) antibody in 250 μl PBS. Secondary antibody solution was prepared by diluting 1 μl of 10 nm colloidal gold-labeled anti-rat IgG antibody (Sigma, G7035) in 200 μl PBS.

3.4.1 Leukocyte preparation

P4 solution (100 μg/100 μl DEPC per mouse) was administered into tail-veins and blood was collected by cardiac puncture 24 hours later before cervical dislocation. Blood samples were subjected to red blood cell lysis and the resulting cell pellet washed, adjusted to obtain ~5 x 10^6 cells/ml of suspension, centrifuged at 300 *x* g for 10 minutes and the supernatant discarded

3.4.2 Immunogold staining

The resulting cell pellet was then stained for 30 minutes at 4°C using 50 μl of primary antibody solution. Following incubation, the cells were washed twice by centrifuging at 300 *x* g for 10 minutes and the resulting cell pellet stained using 50 μl of secondary antibody solution for 30 minutes at 4°C. Following incubation, the cells were washed twice by centrifuging at 300 *x* g for 10 minutes then fixed and submitted for transmission electron microscopy (TEM). Control samples were stained using secondary antibody solution alone were used to address non-specific binding.

3.4.3 Analysis

Images of peripheral leukocytes generated from TEM were randomly selected and blindly assessed by two independent observers for the presence of gold particles internalized and on the surface of membranes. An example of the distinct black spots indicating the presence of gold can be seen in Figure 29.

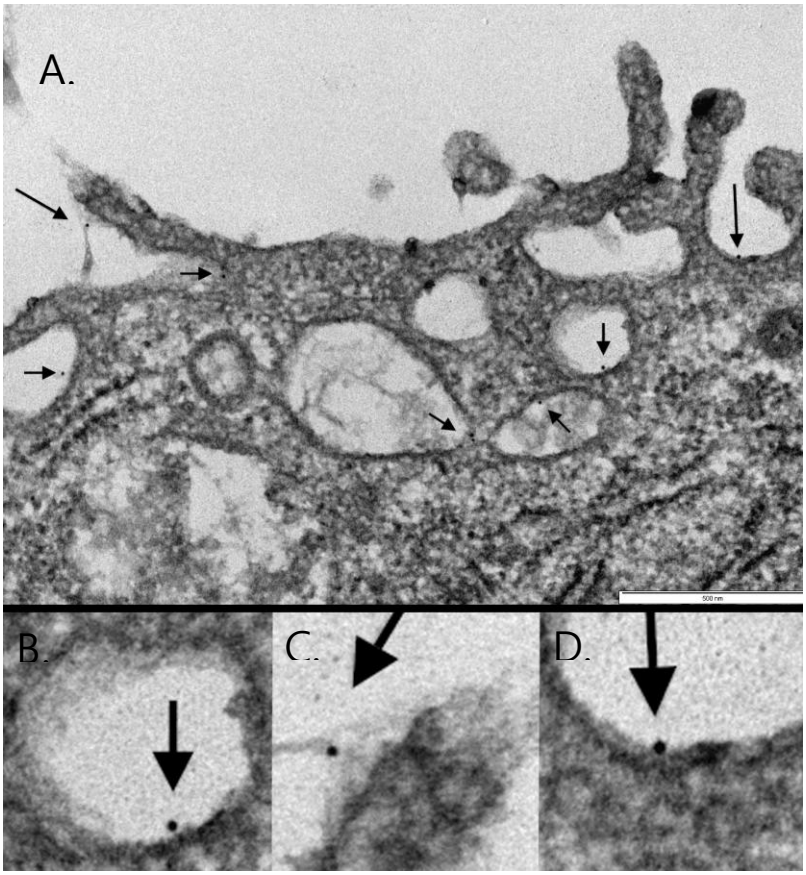


Figure 29 – Example of immunogold detection using TEM. Mice were intravenously injected with P4 solution and blood collected at 24 hours for immunogold staining. (A) shows part of the membrane of a murine monocyte cell. The arrows are indicating the presence of gold particles. The three panels below are close-up images from (A) and show the presence of gold particles (B) internalized into phagosomes, (C) attached to pseudopods and (D) about to be engulfed.

3.5 Murine macrophages: Alveolar, peritoneal, J774.2

Alveolar and peritoneal macrophages are the resident phagocytes that first respond to pneumonia or peritonitis respectively. P4 was tested for the ability to augment the killing of opsonized pneumococci by peritoneal and alveolar *ex vivo* derived macrophages. Additionally, the hypothesis that macrophages from the J744.2 murine cell line would be able to phagocytose higher amounts of opsonized pneumococci irrespective of bacterial capsule following peptide treatment was tested.

3.5.1 Standard media used for macrophage cell culture

Dulbecco's Modified Eagle Medium (DMEM, Sigma, UK]) was used for all macrophage cell culture. Medium was supplemented with 10% FBS and 5% antibiotic/mycotic (10,000 units penicillin, 10 μg streptomycin + 25 μg amphotericin B/ml [Sigma, UK]) solutions to maintain growth and prevent contamination. Additionally, DMEM supplemented with 10% FBS without the addition of antibiotic/mycotic solution was prepared for use in phagocytosis assays.

3.5.2 Obtaining peritoneal macrophages

Peritoneal macrophages (PM) were obtained by exposing the peritoneum of mice euthanized by cervical dislocation and washing the peritoneal cavity twice using 2.5ml medium and a 14-gauge syringe (Figure 30A) The resulting cell suspension was centrifuged at 300 *x* g for 10 minutes, the supernatant discarded and the pellet resuspended in 2 ml medium containing antibiotics. The number of PM/ml was determined using a haemocytometer. Cells were then plated onto tissue culture treated 96-well reaction plates at a concentration of 5 x 10^4/well and incubated at 37°C (5% CO_2) for 3 hours. Following incubation, wells were gently washed twice with sterile, pre-warmed (37°C) PBS to remove non-adherent cells and antibiotic-free medium was added to the wells.

3.5.3 Obtaining BAL and alveolar macrophages

BAL. Alveolar macrophages (AM) were obtained by lavaging murine lungs and retrieving the fluid. Following euthanasia by pentobarbital overdose, murine lungs were exposed and the trachea cannulated with a 24 gauge plastic catheter (BD Instye, Becton Dickinson). A 2ml syringe was adapted to the catheter and lungs lavaged twice with 1.5 ml medium as shown in Figure 30B.

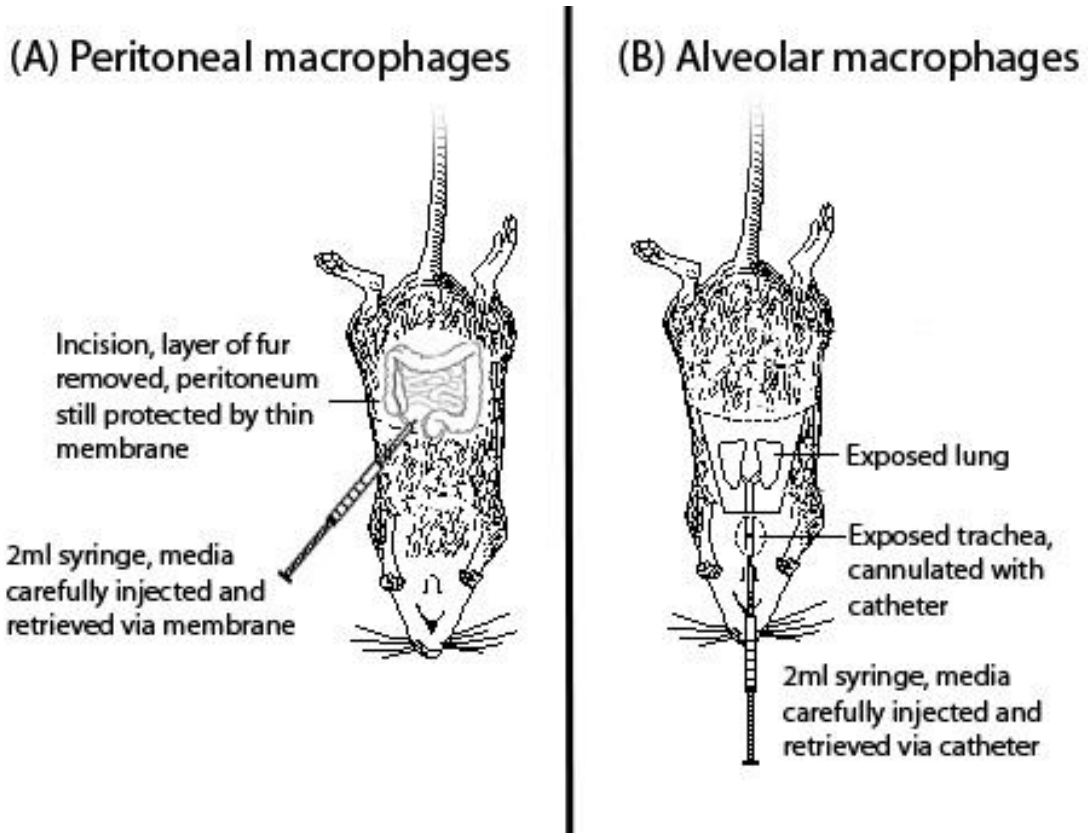


Figure 30 - Diagram of the method used to obtain *ex vivo* derived macrophages. (A) peritoneal macrophages were obtained by exposing the peritoneal membrane and carefully injecting and retrieving medium into the peritoneal cavity taking care not to damage organs. (B) Alveolar macrophages were extracted from mice by exposing and cannulating the trachea of mice and washing the lungs with medium taking care not to burst lung tissue.

Alveolar macrophages. The resulting cell suspension was centrifuged at 300 *x* g for 10 minutes, the supernatant discarded and the pellet re-suspended in 2 ml medium containing antibiotics. The number of AM/ml was determined using a haemocytometer. Cells were then plated onto tissue culture treated 96-well reaction plates at a concentration of 5 x 10^4/well and incubated at 37°C (5% CO_2) for 3 hours. Following incubation, wells were gently washed twice with sterile, pre-warmed (37°C) PBS to remove non-adherent cells and antibiotic-free medium was added to the wells.

3.5.4 Establishing and maintaining J774.2 cell culture

J774.2 is a murine derived macrophage cell line. Frozen cell aliquots were quickly thawed in a 21°C water bath and centrifuged at 300 *x* g for 10 minutes to remove the potentially toxic DMSO from the cryopreservation solution. The supernatant was discarded and cells re-suspended in 10 ml sterile PBS for another wash (300 × g for 10 minutes). The supernatant was then discarded, the resulting cell pellet re-suspended in 15 ml of medium and this cell solution added to a 75 ml tissue culture flask (BD Biosciences, UK) for overnight incubation at 37°C (5% CO_2). The following day, viable cells had attached to the tissue culture treated flask. Medium was carefully removed from the flask and the cells washed once with 10 ml of RT Dulbecco's Phosphate Buffered Saline (DPBS [Gibco, UK]). 15 ml of fresh medium was added and the cell culture maintained by washing cells and replenishing medium every two days. The cell line was split as soon as macrophage attachment to the flask reached 50% confluence, judged by assessing the flask using an inverted microscope. Medium was removed and cells washed with 10 ml DPBS. 8 ml of medium was added to the flask and a cell suspension created by gently detaching cells using a cell scraper. 1 ml of the resulting cell suspension was added to 14 ml fresh medium, placed into a new 75 ml tissue culture flask and cell culture maintained as above. Similarly, cells were also

detached, counted and added to reaction wells for use in opsonophagocytosis killing assays.

3.6 Opsonophagocytosis using murine macrophages

Opsonophagocytosis was performed using *ex vivo* derived alveolar and peritoneal macrophages as well as J774 tissue culture macrophages.

3.6.1 Opsonisation

See General methods: Chapter II (p. 75)

3.6.2 Phagocytosis

See General methods: Chapter II. (p. 76)

3.6.3 Analysis

See General methods: Chapter II. (p. 77)

3.7 Statistical analysis

Groups of five mice per time point or condition were used with the exception of behavioral studies where groups of three mice were used. All *in vivo* experiments were performed in duplicates on separate occasions. Through the use of age, sex and strain matched, data from biological replicates were pooled and presented as means ± standard deviation. Each *in vitro* or *ex vivo* assay consisted of > three technical replicates and were performed on separate assay days. Data from separate assay days were pooled and shown as means ± standard deviation. Distribution of data was assessed using D'Agostino and Pearson omnibus normality test. For normally distributed data, an unpaired or paired T-test was used to test for significance. For non-normally distributed data, a Mann-Whitney T-test was used to test for significance. Differences between data sets were designated significant if $p<0.05$.

C. Results

3.7 Behavioral response to P4

Two groups of mice (n=3) receiving (A) the treatment dose and (B) a dose 10x higher were assessed for differences in behavioral signs compared to healthy mice from the same colony. Group A were treated with 100 µg P4 / 100 µl and observed at 8AM (immediately after injection), 2PM and 8PM. Group B were treated with 1 mg P4 / 100 µl and observed at 8AM (immediately after injection), 2PM and 8PM. The data regarding animal behavior are shown in Table 10. There was no difference between Group A, Group B and control mice at any timepoints.

Table 10 - Behavioral responses to peptide administration. Group A (n=3) were treated with 100 µg P4 / 100 µl while Group B (n=3) were treated with 1 mg P4 / 100 µl. Mice were checked visually immediately after injection and six and 12 hours following injection. Changes in behavior relating to their movement in the cages (activity), rate of breathing, condition of fur (grooming) and posture were assessed.

Behavior	Control		Group A		Group B	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Activity	baseline		No change		No change	
Breathing	baseline		No change		No change	
Grooming	baseline		No change		No change	
Posture	baseline		No change		No change	

3.8 Cell populations in blood: intravenous P4

Mice (n=10 per group) were injected with peptide solution and blood was collected at 1, 4 and 24 hours following injection for Flow Cytometry staining.

3.8.1 Neutrophil migration and FcγR

Twenty-four hours following IV injection of P4 solution, a significant increase in systemic neutrophil numbers was detected as compared to the DEPC water control administration (control 6.1% vs. treated 14.6%, p=0.02) (Figure 31A) No significant differences in neutrophil numbers were detected at 1 or 4 hours between groups. While neutrophil numbers did not increase until 24 hours, their relative Fc-γ RII & III expression was rapidly modulated and a significant increase was detected in P4 treated mice compared to control mice at 4 (control 11.2% vs. treated 46.7%, p=0.02) and 24 (control 11.1% vs. treated 75.1%, p<0.0001) hours following administration (Figure 31B).

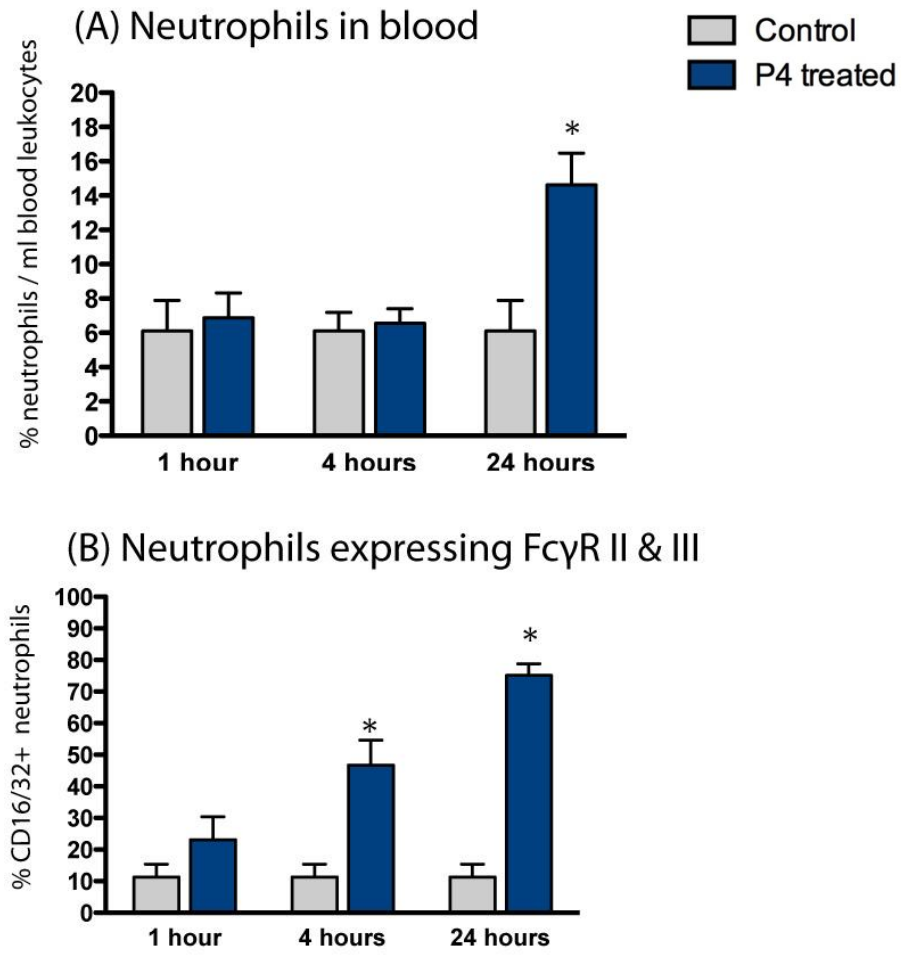


Figure 31 – Neutrophils detected per ml blood at 1, 4 and 24 hours following intravenous injection of P4 or DEPC treated water in naïve healthy mice (n=10 per group). (B) Percentage Fc-γ RII/III expression on neutrophils in blood. (*) denotes significant differences compared to control at time points using ANOVA where $p<0.05$.

3.8.2 Monocyte migration and FcγR

Differences in the proportion of circulating monocytes compared to control mice were seen at 1 (control 1.6% vs. treated 4.5%, p=0.06), 4 (control 1.7% vs. treated 4.3%, p=0.0005) and 24 hours (control 1.7% vs. treated 7.6%, p=0.0005) (Figure 32A). Fcγ RII & III expression was rapidly modulated in monocytes within the first hour and was significantly increased compared to control mice at 4 (control 22.6% vs. treated 51.6%, p=0.02) and 24 (control 22.4% vs. treated 56.4%, p=0.002) hours following injection (Figure 32B).

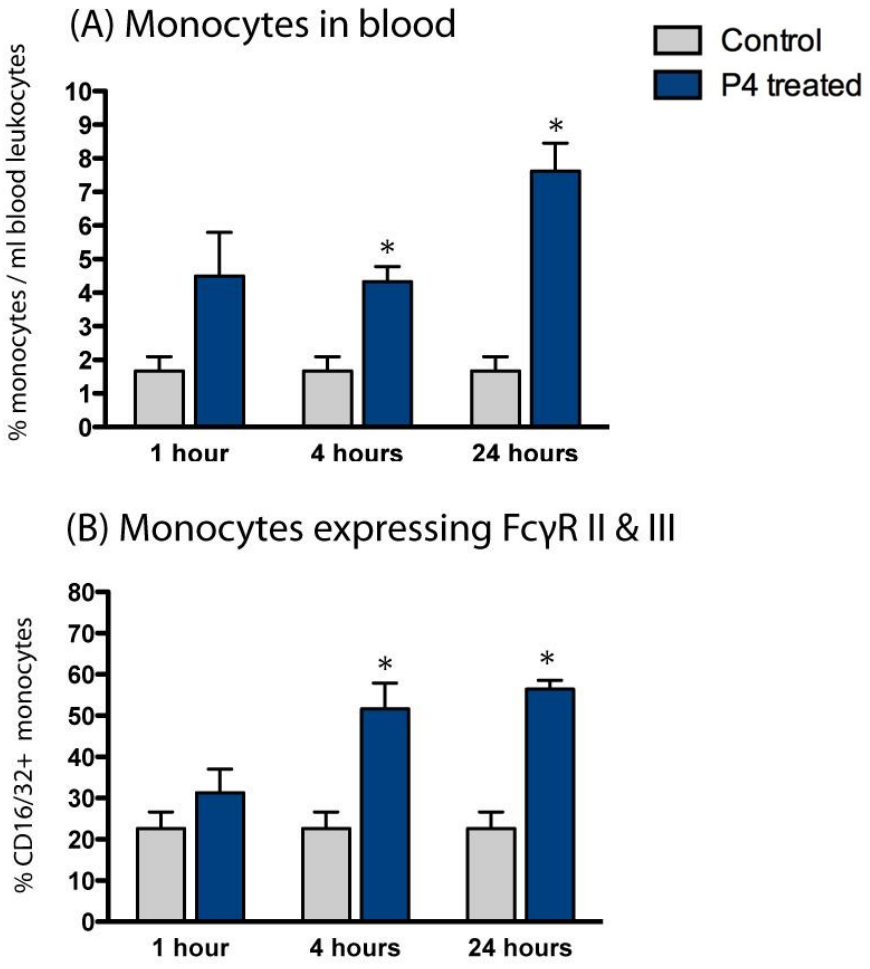


Figure 32 – (A) Monocytes detected per ml blood at 1, 4 and 24 hours following intravenous injection of P4 or DEPC treated water in naïve healthy mice (n=10 per group). (B) Percentage Fc-γ RII/III expression on monocytes in blood. (*) denotes significant differences at time points using ANOVA where p<0.05.

3.9 Cell populations in BAL: intranasal P4

3.9.1 Neutrophil migration and FcγR

There was no significant difference detected in the relative numbers of neutrophils present in BAL (Figure 33A). Fc-γ RII & III expression was however significantly up-regulated 4 hours following administration (control 11.6% vs. treated 26.6%, p=0.04) and remained elevated at 24 hours (but non significant to control at same time point) (Figure 33B).

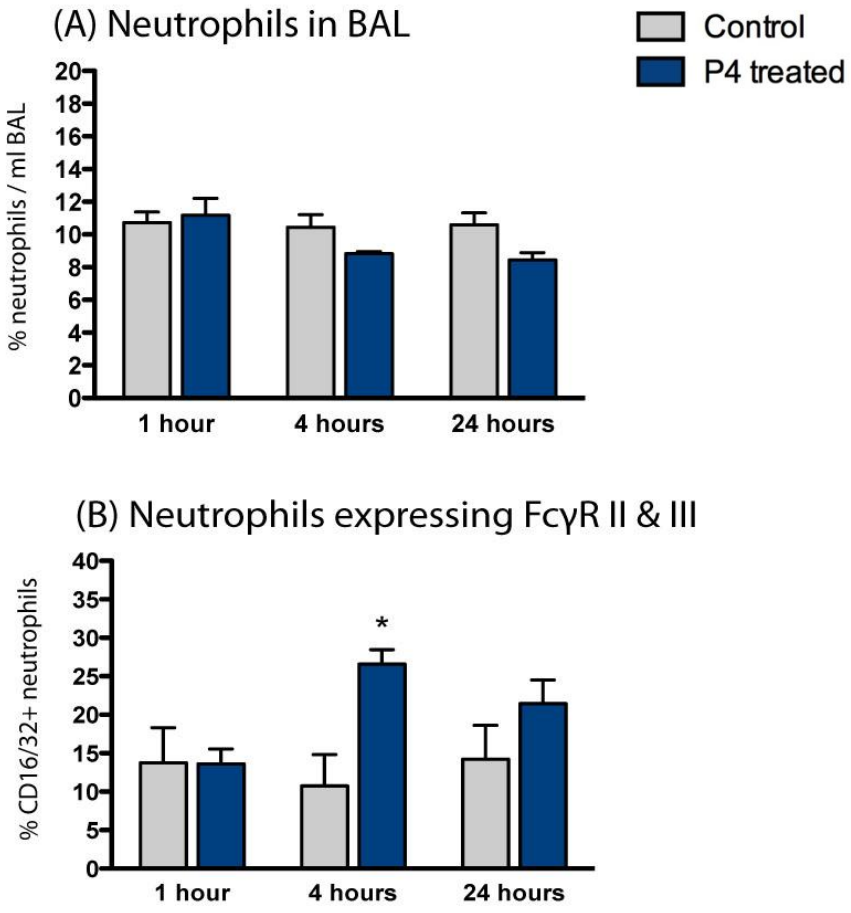


Figure 33 - Neutrophils detected per ml BAL at 1, 4 and 24 hours following intranasal administration of P4 or DEPC treated water in naïve healthy mice (n=10 per group). (B) Percentage Fc-γ RII/III expression on neutrophils in BAL. (*) denotes significant differences at time points using ANOVA where p<0.05.

3.8.1 BAL macrophages and FcγR

Relative numbers of macrophages in BAL did not increase following intranasal administration as shown in Figure 34A. The expression of Fcγ RII & III was up-regulated at 4 hours (control 24.7% vs. treated 39%, p=0.01) whereas at 1 and 24 hours Fc-expression was elevated but this did not reach statistical significance (Figure 34B).

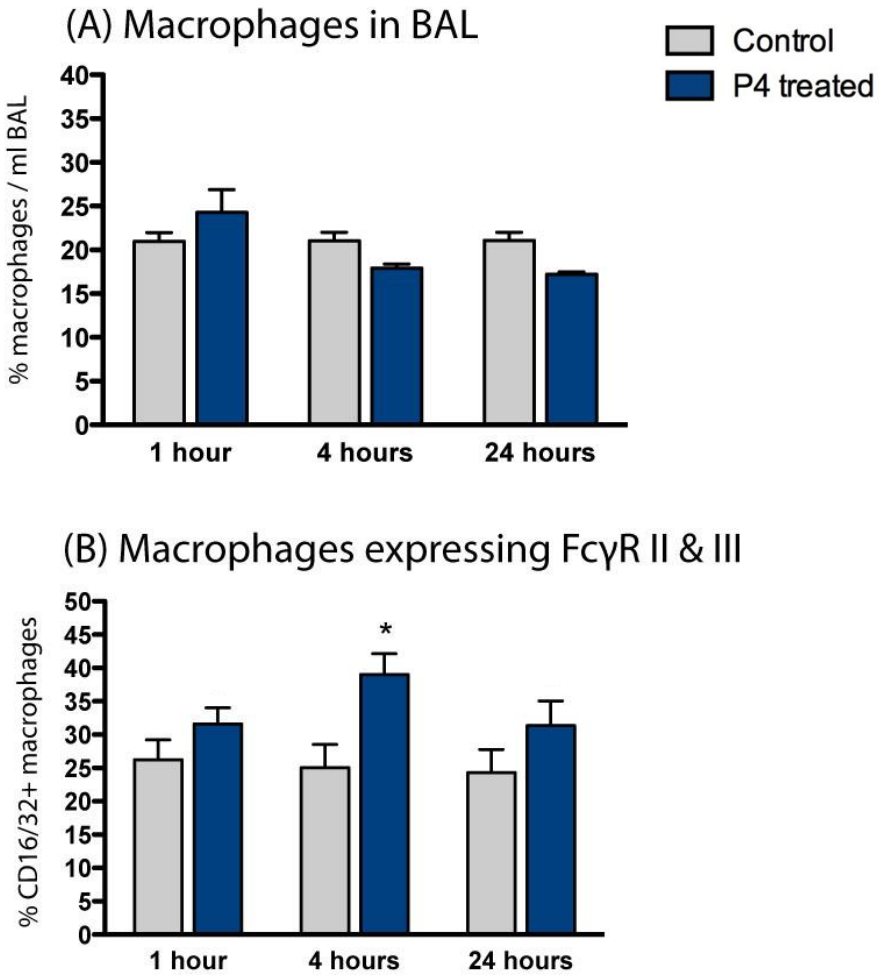


Figure 34 - Macrophages detected per ml BAL at 1, 4 and 24 hours following intranasal administration of P4 or DEPC treated water at 12 and 18 hours following infection in naïve healthy mice (n=10 per group). (B) Percentage Fc-γ RII/III expression on macrophages in BAL. (*) denotes significant differences at time points using ANOVA where p<0.05.

3.10 Immunogold staining of Fcγ receptors

Twenty-four hours after IV injection of P4 solution or DEPC water, peripheral blood leukocytes were stained and assessed for the presence of gold particle (Figure 35). A significant increase in Fcγ receptors (control 10 vs. treated 22, p=0.0009) was observed in monocytes (n=23 per group) while no significant difference was seen in neutrophils. Significant differences were also seen in the localization of FcγR: P4 stimulated cells had internalized more receptors than unstimulated controls (control 35% vs. treated 52%, p<0.005).

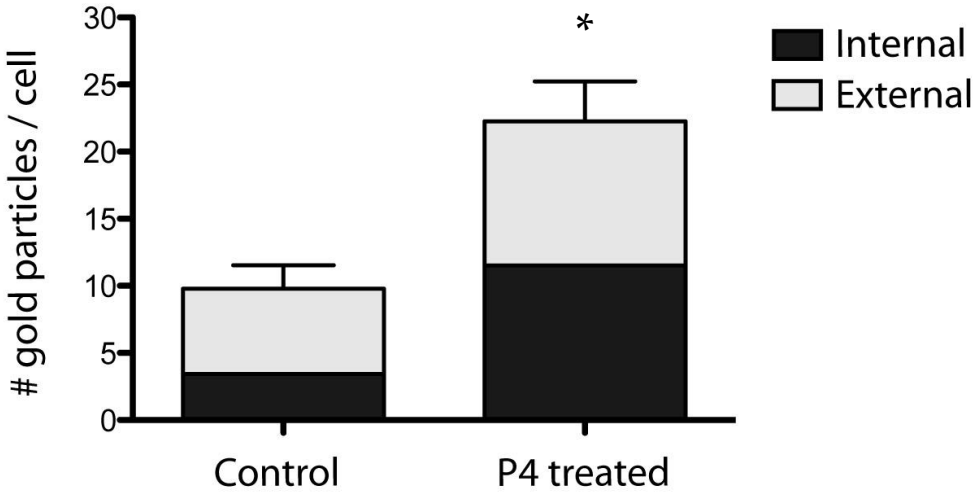


Figure 35 – Number of gold particles found on P4 treated and control monocytes (n=23 per group). Black shading shows number of internalized gold particles per cell, grey shading shows external binding of gold particles per cell. (*) denotes significant differences between groups using ANOVA where p<0.05.

3.11 Murine macrophage opsonophagocytosis

3.11.1 J774.2 OPKA

Following opsonisation of bacteria, J774.2 murine macrophages showed a significantly increased ability to kill capsulated ST2 D39 (control 15.7% vs. treated 33.28%, p=0.01) and unencapsulated ST2 D39 (control 50.4 % vs. treated 66%, p=0.04) pneumococci when treated with P4. Using a different serotype, non-stimulated cells were unable to induce detectable killing of opsonized ST14 pneumococci. Following P4 stimulation however, J774.2 macrophages were able to phagocytose 11% of the bacterial ST14 inoculum. Un-encapsulated pneumococci were more susceptible to phagocytosis than their capsulated homologues (capsulated 15.7% vs. unencapsulated 50.5%, p=0.0002) (Figure 36)

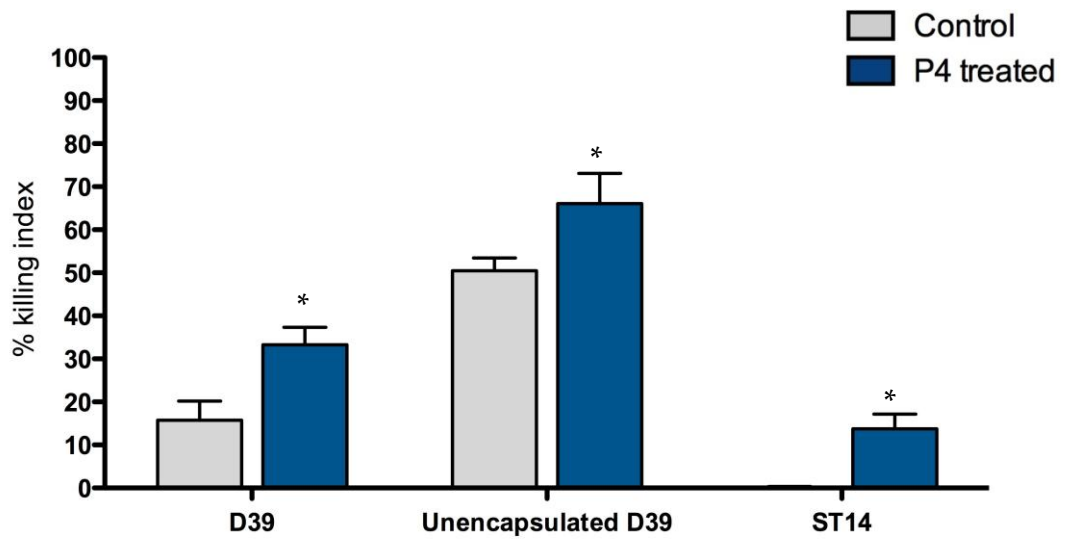


Figure 36 - P4 mediated opsonophagocytosis killing of capsulated and unencapsulated strains of D39-ST2 as well as ST14 pneumococi by J774.2 murine macrophages. N=3 per group per cell type. (*) denotes significant differences between groups using ANOVA where p<0.05

3.11.2 Alveolar and peritoneal macrophage OPKA

Peritoneal and alveolar macrophages were obtained from naïve MF1 mice. Following peptide stimulation, both peritoneal (control 44% vs. treated 68%, $p<0.03$) and alveolar (control 53% vs. treated 74%, $p=0.01$) macrophages were able to significantly increase their phagocytosis of opsonized ST2 D39 *S. pneumoniae* (Figure 37).

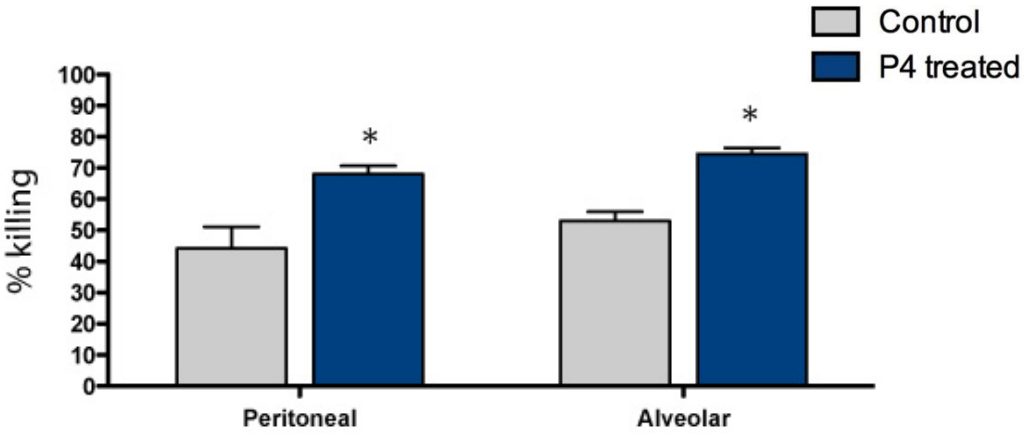


Figure 37 - *Ex vivo* opsonophagocytosis assay of serotype 2 (D39) *S. pneumoniae* by peritoneal and alveolar murine macrophages. (*) denotes significant differences at time points using ANOVA where p<0.05.

In addition, P4-mediated phagocytosis assays using alveolar and peritoneal macrophages were conducted in the absence of either antibody (i.e. non-opsonized bacteria) or functional complement. Functional complement was deactivated by placing an aliquot in a 56°C water-bath for 30 minutes. Non-opsonized *S. pneumoniae* and inactivated complement incubated cells had minimal levels of killing (<5% killing) using either peritoneal (Figure 38A, n=2) or alveolar (Figure 38B, n=2) macrophages. In the absence of antibody or complement, P4 stimulation of peritoneal or alveolar macrophages led to no significant increases in killing.

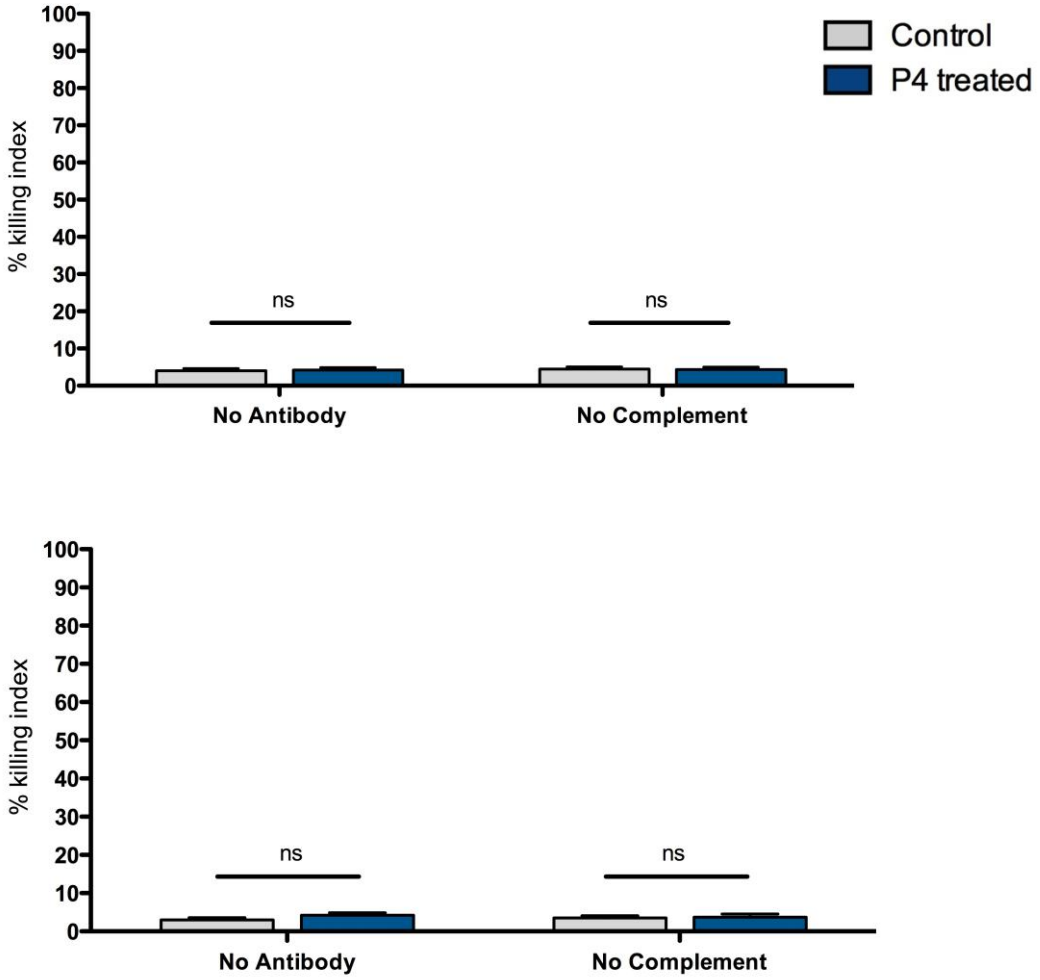


Figure 38 - *Ex vivo* opsonophagocytosis assay of serotype 2 (D39) pneumococci by stimulated and un-stimulated peritoneal (top panel) or alveolar (bottom panel) macrophages in the absence of antibody or complement.

D. Discussion

3.12 Discussion

This chapter aimed to assess the cellular responses to both intravenous and intranasal peptide administration in the absence of infection.

3.12.1 Behavioral responses

It is promising that no changes in behavioral responses were observed following peptide administration at the treatment dose and a dose 10 x higher. During infection, the host regulates neuronal responses to induce physical changes in order to assist in the immune response to infection [294]. Further modulation of these responses could disadvantage the host by negating the benefits of a therapeutical compound. Increasing the sample size and observing behavioral responses in a range of murine strains could strengthen these observations. In addition, behavioral responses could be correlated with inflammatory cytokines in the brain, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which are thought to induce sickness-like behavioral responses [297].

3.12.2 Cell populations

The responses of the main phagocytes to intravenous and intranasal peptide administration were measured in blood and BAL, respectively. Following intravenous administration of P4, a rapid increase of peripheral blood monocytes at 1, 4 and 24 hours was observed. In addition to bone marrow monocytes, a large proportion of monocytes reside in the spleen [133, 142, 298]. It is unclear from these data whether the increased monocyte populations are derived from bone marrow or spleen reservoirs, or whether

they are produced in the bone marrow in response to peptide stimulation. The release of monocytes from bone marrow is mediated by inflammation and is dependent on the presence of CCL2 while the release from spleen is mediated by tissue injury and is dependent on the presence of angiotensin II [133]. Studies inhibiting CCL2, angiotensin II or their respective receptors could elucidate whether the peptide induced monocyte population is derived from the spleen or bone marrow and therefore inform on the role of recruited monocytes.

In comparison to monocytes, neutrophil numbers did not increase until 24 hours following intravenous administration. At 24 hours, however, the proportion of circulating neutrophils more than doubled compared to control mice. The production of neutrophils in the bone marrow takes approximately 4-5 days, after which a small percentage are released into the blood stream while the majority are stored in the bone marrow [299]. The increased neutrophil population detected 24 hours following peptide administration is therefore likely to stem from the bone marrow reserve as opposed to freshly produced neutrophils. Release of neutrophils from the bone marrow is a complex process mediated by granulocyte-macrophage colony stimulating factor (GM-CSF), as well as granulocyte colony stimulating factor (G-CSF), produced by stromal cells in the bone marrow [300]. G-CSF reduces the secretion of the chemokine SDF-1α (CXCL12) by stromal cells leading to increased CXCR4 expression on neutrophils and their release into the blood stream [301]. It is unlikely that P4 acts on stromal cells to reduce SDF-1α expression in a G-CSF-like manner, as this results in rapid (within minutes) release of neutrophils [302], and P4 mediated neutrophil release was not detected until 24 hours. Instead, P4 may indirectly lead to the production of G-CSF via activation of macrophages (release G-CSF following endotoxin stimulation [303]) or endothelial cells (release G-CSF following TNF-α and IL-1 stimulation [304]) or stimulate the release of other neutrophil

chemoattractants such as IL-8 (released by macrophages and epithelial cells [305]).

In comparison to intravenous administration, intranasal administration of P4 did not lead to a significant recruitment of monocytes or neutrophils into the alveoli. Administration of P4 into the lungs is likely to stimulate alveolar macrophages, which are responsible for the recruitment of phagocytes. As no increase in phagocytes was detected in BAL, intranasal peptide administration does not induce the recruitment of phagocytes through secretion of pro-inflammatory cytokines by alveolar macrophages. The proportion of peripheral blood monocytes or neutrophils were not measured following intranasal administration of P4, so it is difficult to determine whether (a) monocytes and neutrophils were released similar to intravenous studies but did not migrate to the alveoli or (b) intranasal administration of P4 does not induce the release of phagocytes into the blood stream. A study looking at circulating blood phagocytes, lung tissue phagocytes and phagocytes present in BAL following intranasal peptide administration would help clarify this point.

3.12.3 Receptor expression

In addition to measuring the proportion of phagocytes following peptide administration, Fcγ membrane expression was measured using flow cytometry. Following intravenous P4 administration, monocytes and neutrophils rapidly upregulated their expression of Fcγ receptors by 1 hour, with further increases detected at 4 and 24 hours. Intranasal administration only led to significant up-regulation of Fcγ receptors at 4 hours, where expression levels were similar to control at 1 and 24 hours. The differences may arise in the cell types measured, as opposed to the route of administration chosen. Circulating blood monocytes and neutrophils are short-lived phagocytes that rapidly respond to stimulants, such as

chemokines secreted by cells present at the site of infection or P4 peptide. Alveolar macrophages, in comparison, are sentinel cells that orchestrate the immune response, including the recruitment of neutrophils and monocytes. As such, alveolar macrophages remain mostly anti-inflammatory to avoid unnecessary recruitment of phagocytes. P4 may therefore only transiently up-regulate alveolar macrophage Fcγ expression, where normal expression levels are re-instated in the absence of infection.

The use of FcγR immunogold staining and electron microscopy confirmed the increased FcγR expression seen using flow cytometry. In addition, it was observed that Fcγ receptors were frequently found internalized into phagosomes. This could suggest that phagocytes are primed following peptide exposure leading to rapid uptake following FcγR ligation. In the context of augmented passive immunotherapy this is beneficial as the administration of immunoglobulin leads to the formation of immunecomplexes that need to be rapidly cleared.

3.12.4 Phagocytosis

Peptide stimulation of *ex vivo* derived peritoneal and alveolar macrophages led to a 30% and 20% increase in phagocytic killing of opsonized pneumococci, respectively. Previous studies have shown a two-fold increase in phagocytic killing by murine neutrophils [280]. The modest, albeit significant, increase in phagocytic killing by murine macrophages correlates with the expression of FcγR seen on macrophages during FcγR expression studies. As peripheral blood neutrophils expressed higher amounts of FcγR than alveolar macrophages following peptide stimulation, their phagocytic killing ability is also greater.

The J774 murine macrophage cell line increased the killing of a range of IVIG opsonized pneumococci, independent of the presence of capsule. In the

absence of antibody or complement, however, phagocytosis was reduced and there were no detectable differences between stimulated and un-stimulated groups. This suggests that P4 enhances the natural process of phagocytosis as opposed to inducing unspecific killing mechanisms such as degranulation.

CHAPTER IV.

P4 peptide studies in disease

A. Introduction

4.1 Introduction

Studies in chapter III established that *in vivo* peptide administration led to the recruitment and activation of phagocytes in the absence of infection. This chapter assessed the efficacy of augmented passive immunotherapy (API) in invasive and acute invasive models of pneumococcal disease. Mice were infected with a virulent strain of *S. pneumoniae* and treated with augmented passive immunotherapy (API) *i.e.* the concomitant administration of pathogen specific immunoglobulin (IVIG) and P4 peptide.

4.1.1 Survival of young and aged mice in invasive models of pneumococcal disease (IPD)

In order to account for genetic variation and therefore differences in susceptibility to pneumococcal infection [306], two strains (Swiss Webster, BALB/c) of young, outbred mice were used to model and treat invasive pneumococcal disease. In addition, mice were purchased and aged in house until >50% of the initial colony died of natural causes, thereby providing an aged population . Aged mice were infected and treated with API to assess whether treatment would be effective in hosts affected by senescence.

4.1.2 Models of acute invasive pneumococcal disease (aIPD)

A highly susceptible mouse strain (MF1) was chosen to model rapidly progressing, fatal invasive pneumococcal disease. Using this model, API was administered during bacteraemic infection to assess whether treatment would be effective at the most severe stage of infection. To further elucidate the effect of API during infection, the bacterial burden in lungs and blood as well

as the expression of Fcγ receptors on phagocytes was measured following infection and treatment.

4.1.3 Intranasal administration

In the MF1 mouse model, pneumococcal infection of the lung leads to the activation and recruitment of phagocytes to affected tissue before pneumococci seed into the blood stream. Using intranasal administration of P4 during acute infection of the lungs, it was assessed whether API of pneumonic lungs would affect the outcome of infection. The bacterial burden in lungs and blood as well as the expression of Fcγ receptors on phagocytes were measured following infection and intranasal peptide administration.

4.1.4 Nasopharyngeal carriage

Pneumococcal disease is always preceded by pneumococcal carriage. The effect of API on nasopharyngeal carriage by *S. pneumoniae* during acute and asymptomatic infections was tested. Bacterial CFU numbers in nasopharyngeal tissue were enumerated following (a) intranasal peptide administration during asymptomatic carriage of *S. pneumoniae* and (b) intravenous peptide administration during invasive pneumococcal disease.

4.1.5 Peptide pre-treatment

In order to determine whether the effects of recruited and activated phagocytes seen in the previous chapter would translate to augmented phagocytic defense, mice were pre-treated with intravenous peptide prior to infection. The bacterial burden in lungs and blood as well as survival was measured following P4 pre-treatment and infection.

4.1.6 Publications

These studies form part of two studies published in the Journal of Infectious Diseases and Clinical and Vaccine Immunology:

Mathieu Bangert, Laura M. Bricio, Suzanna Gore, Gowrisankar Rajam, Edwin W. Ades, Stephen B. Gordon, Aras Kadioglu. "P4 mediated antibody therapy in an acute model of pneumococcal disease." *The Journal of Infectious Diseases* (2012):205(9):1399-1407.

Rajam Gowrisankar, **Mathieu Bangert**, Gabrielle M. Hammons, Nikkol Melnick, George M. Carlone, Jacquelyn S. Sampson, and Edwin W. Ades. "P4 Peptide Therapy Rescues Aged Mice from Fatal Pneumococcal Sepsis." *Clinical and Vaccine Immunology* 17.11 (2010): 1823-824.

B. Materials & Methods

4.2 Animal passage of pneumococci

4.2.1 Bacterial stock preparation

Non-passaged aliquots of pneumococci (chapter II) were thawed on ice and a loop full of pneumococci streaked on BAB culture plates for isolation followed by overnight incubation at 37°C (5% CO_2). A sweep of colonies from the culture plates was then inoculated into a universal tube containing 10 ml BHI medium and again incubated at 37°C (5% CO_2) overnight. 18-24 hours later, the BHI culture broth was centrifuged at 1500g for 15 minutes, the supernatant discarded and the resulting pellet re-suspended in 5 ml sterile PBS (to give an OD_{500} of 1.4-1.6). A 100 µl sample was taken for a viable cell count using the Miles & Misra technique.

4.2.2 *In vivo* passage

Using a 0.5 ml fine insulin syringe, 100 µl of pneumococci suspension was injected intraperitoneally into two MF1 mice (100 µl each). The mice were monitored to ensure that 22-28 hours following injection signs of disease characteristic of a systemic infection were visible, meaning that the passage was successful. Cardiac puncture was then performed to obtain blood samples.

4.2.3 Culturing and storing passaged stocks

50 µl of blood from the cardiac puncture was inoculated into a universal tube containing 10ml BHI medium and statically incubated overnight at 37°C (5% CO_2). Following incubation, cloudy suspension (bacteria) above the loose sediment (red blood cells) was carefully transferred to a sterile universal tube,

centrifuged at 1500g for 15 minutes, the supernatant discarded and the resulting pellet re-suspended into 1ml BHI serum broth. 700 µl of the re-suspended pellet was added to 10ml of BHI serum broth until OD_{500} 0.7 and incubated statically for 5 hours at 37°C (5% CO_2) until $OD_{500} \geq 1.6$. Following incubation, the culture broth was centrifuged at 1500 *x* g for 15 minutes, the supernatant discarded and the pellet re-suspended in 10 ml fresh BHI Serum Broth. The culture was then divided into 500 µl single use aliquots in sterile cryotubes and immediately stored at -80°C. After > 48 hours in the freezer, aliquots were thawed to determine the number of pneumococci present in each aliquot using the Miles and Misra technique.

4.3 Intranasal infection

The intranasal route of infection can be used to instill pneumococci into the lungs to establish invasive pneumonia [306] or to the nasopharynx to establish carriage [307], depending on the bacterial and murine strains. A summary of murine bacterial strains used to establish different infection models is detailed in Table 11.

Table 11 - Mouse strain, bacterial dose and volume used for different models of infection.

Model	Mouse Strain	Bacterial Strain	Dose	Volume
IPD	Swiss Webster BALB/c	WU2	1 x 10^7 CFU	40 µl
Acute IPD	MF1	D39	1 x 10^6 CFU	50 µl
Carriage	MF1	D39	1 x 10^6 CFU	10 µl

4.3.1 Preparing pneumococcal inoculum

Once stocks were prepared, passaged and the viable number of pneumococci / ml in each aliquot was determined, dosing for infection studies could be prepared. Aliquots of frozen passaged stocks were quickly thawed in a RT water bath and centrifuged at 900 *x* g for 3 minutes. The supernatant was discarded and the resulting cell pellet re-suspended in 1ml sterile PBS for another centrifugation at 900 *x* g for 3 minutes, following which the supernatant was discarded and the cell pellet re-suspended in 400 µl sterile PBS. The bacterial suspension was then diluted in sterile PBS to establish the desired infection dose (Table 11).

4.3.2 Intranasal dose administration

Mice were first lightly anaesthetized using 2.5% v/v inhaled Isofluorane (1.6-1.8 L O_2/min) in an anaesthetic box. The absence of reflex reactions and twitching of whiskers confirmed anesthesia. Once anaesthetized, the mice were scruffed and the bacterial suspension (see Table 11 for volume and dose) administered equally into both nostrils in a drop-wise fashion, allowing each drop to be inhaled before the next was administered. Following infection, the dose was measured for viable count as described using the Miles and Misra technique.

4.4 Virulence testing passaged pnemococci

In order to assure reproducible and effective modeling of acute invasive pneumococcal disease using the MF1 mouse strain as a host, animal passaged pneumococci were tested for virulence in a small number of mice.

4.4.1 Infection and monitoring

An aliquot was thawed and prepared for intransasl infection as described in section 4.3. Five eight-week old female MF1 mice received a dose to establish the acute IPD model (see Table 11). Following infection, mice were monitored for signs of disease. If mice reached "lethargic ++" (see Chapter II) stage then it was assumed they would progress to moribund and so they were humanely culled before this stage was reached after 44-52 hours.

4.5 Augmented Passive Immunotherapy (API)

Augmented passive immunotherapy is the combined treatment of pooled Immunoglobulin and the immunomodulating peptide. Infection is established in a mouse host prior to the administration of augmented passive immunotherapy. For peptide solution, two routes of administration were chosen. The peptide concentration and volume for each route of administration is detailed in Table 12.

Table 12 – Route, volume and concentration of P4 peptide solution administration (per mouse) used for augmented passive immunotherapy in models of pneumococcal disease.

Administration	Treatment	DEPC Water	P4 Peptide
Intravenous	Sepsis secondary to acute pneumonia	100 µl	100 µg
Intranasal	Acute pneumonia	40 µl	100 µg
Intranasal	Asymptomatic carriage	15 µl	100 µg

4.5.1 Intraperitoneal immunotherapy

IVIG was used as a source of pathogen specific immunoglobulin. Gamunex (Talecris, Lot 26NG042) was used for all *in vivo* studies in this thesis. IVIG was always administered intraperitoneally, 20 minutes before peptide administration to allow for opsonisation to occur *in vivo*. Mice were scruffed and held at a slight downward angle. Using an insulin syringe, 100 µl of IVIG solution (equivalent to 10 µg pooled IgG) was injected into the lower right quadrant of the abdomen as shown in Figure 39. Care was taken to insert only the tip of the syringe into the abdomen in order to avoid accidental damage of vital organs.

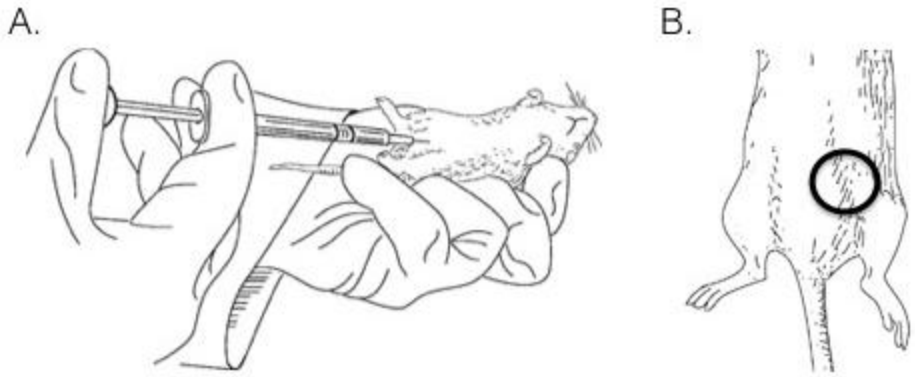


Figure 39 - Intraperitoneal injection of IVIG into mice. (A) Mice were scruffed and held at a slight angle for administration of 100 µl IVIG using an insulin syringe. (B) Circle indicates the injection site in the lower right quadrant of the mouse abdomen. This site helps avoids damage of internal organs through accidental puncture. Adapted from [308].

4.5.2 Intravenous peptide administration

Solubilized peptide was administered intravenously (IV) following infection and IVIG administration. Mice were placed in a cage-warmer (37°C) for >2minutes to allow for vasodilation of veins to occur. Mice were then placed in a restrainer allowing access to the tail veins. The tails were sterilized using 70% ethanol solution. Using an insulin syringe, peptide solution (see Table 12

for concentration and volume) was injected into dorsal tail veins as shown in Figure 40.

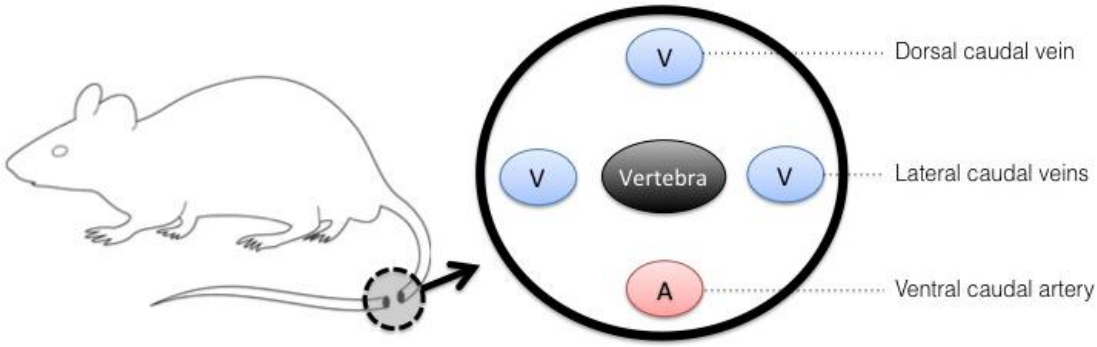


Figure 40 - Schematic diagram of a transverse section of a mouse-tail indicating the various veins available for intravenous administrations. Mouse-tails were pre-warmed to allow for vasodilation to occur and facilitate injections. P4 solution was injected into the dorsal caudal vein.

4.5.3 Intranasal administration

Solubilized peptide was administered intranasally (IN) following infection and IVIG administration. Mice were lightly anaesthetized using 2.5% v/v inhaled isofluorane, (1.6-1.8 L O_2/min) in an anaesthetic box. The absence of reflex reactions and twitching of whiskers confirmed anesthesia. Once anaesthetized, mice were scruffed and P4 solution (see Table 12 for concentration and volume) was administered equally into both nostrils in a drop-wise fashion, allowing each drop to be inhaled before the next was administered.

4.6 Invasive pneumococcal disease (IPD)

In the IPD model, untreated outbred Swiss Webster or BALB/c mice become moribund 72-96 hours following infection as shown in Figure 41. Ageing in both humans and mice causes a general reduction of biological function including in the immune system. At very young ages, the immune system of

both mice and humans has not fully developed. As a result, pneumonia is common in young (<5 years) and aged (>65 years) populations where infections can develop acutely. As such, we assessed whether augmented passive immunotherapy would be effective in these populations.

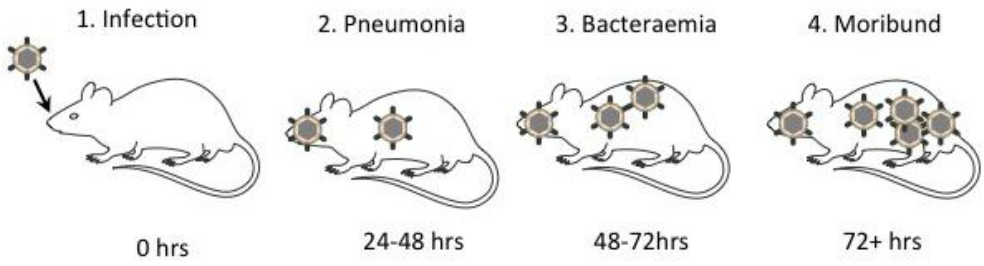


Figure 41 - Schematic diagram showing the course of infection during the invasive pneumococcal disease model using Swiss Webster or BALB/c mice and intranasal administration of WU2 ST-3 pneumococci. 24-48 hours following infection, mice establish pneumonia, which develops to bacteraemia between 48-72 hours. 72-96 hours following infection, 80-100% of untreated mice are expected to be dead or moribund due to sepsis.

4.6.1 IPD in young mice

Four groups of eight-week old female Swiss Webster mice were infected with 1 x 10^7 CFU of WU2 ST-3 pneumococci as described in Chapter II. Augmented passive immunotherapy was administered intravenously at 48 and 72 hours following infection.

- Group 1 (n=5) received DEPC-treated water alone
- Group 2 (n=5) received IVIG alone
- Group 3 (n=5) received P4 peptide alone
- Group 4 (n=5) received P4 peptide and IVIG

Mice were monitored for signs of disease for up to 96 hours following infection. These experiments were performed in duplicate on separate days.

4.6.2 IPD in aged mice

Female Swiss Webster and female BALB/c mice were purchased and aged in-house at the Centres for Disease Control and Prevention Animal Research Facility until >50% of the initial colony died of natural causes (15 months for Swiss Webster and 11 months for BALB/c mice). This in effect provided an "aged population" which can be used to model disease in mice affected by senescence [309]. Eleven-month-old BALB/c (n=20) and 15-month-old Swiss Webster mice (n=20) were infected intranasally with WU2 ST-3 pneumococci and split into treatment and control groups (n=10 per group). Mice were monitored and visually scored twice daily for moribund characteristics. P4 therapy was administered intravenously at 48 and 72 hours post infection. Treated and untreated animals were monitored for 168 hours.

4.7 Acute invasive pneumococcal disease (aIPD)

In the aIPD model, MF1 mice develop pneumonia by 12 hours, bacteraemia by 24 hours and become moribund by 48 hours following infection (Figure 42). This process is highly reproducible and more rapid than in the IPD model. The aIPD model reflects infections that in a clinical setting rapidly progress to fatal sepsis and where antibiotic administration does little to affect mortality. Two treatment methods were tested in both pneumococcal pneumonia and sepsis secondary to pneumococcal pneumonia.

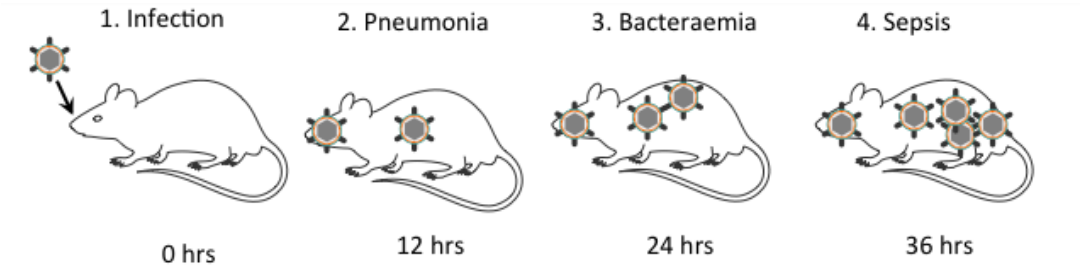


Figure 42 – Schematic diagram showing the course of infection during the acute invasive pneumococcal disease model using MF1 mice and intranasal administration of *in vivo* passaged D39 ST-2 pneumococci. Twelve hours following infection, mice develop pneumonia, which develops to bacteraemia and sepsis at 24 and 36 hours following infection, respectively. At 48 hours (not shown) 100% of untreated mice are expected to be dead or moribund.

4.7.1 Intravenous P4 and pneumococcal septicemia

In an attempt to treat the most severe cases of acute infection, augmented passive immunotherapy was administered intravenously at 24 and 30 hours following infection where pneumonia and bacteraemia was already established. Three studies were done using this model: **(A)** Survival study to establish the P4 treatment model in the MF1 mouse and to confirm the lack of protection using IVIG or peptide alone **(B)** Tissue CFU study to assess bacterial growth during infection and treatment and **(C)** cell population study to describe cellular responses during infection and treatment. The time points in which blood, lung and nasopharyngeal tissue were extracted in this treatment model were at 0, 24, 36 and 48 hours following infection.

4.7.2 Intranasal P4 and pneumococcal pneumonia

In these studies, augmented passive immunotherapy was given intranasally at 12 and 18 hours following infection, when pneumonia was established but had not yet progressed to bacteraemia. Three studies were done using this model: **(A)** Survival study to test whether IN peptide administration treatment is effective compared to control administrations **(B)** CFU study to

assess bacterial load in the lungs and blood during infection and treatment and **(C)** Cell population study to describe cellular responses to infection and treatment. The time points in which blood and lung tissue were extracted in this treatment model were at 0, 24 and 48 hours following infection.

4.7.3 *In vivo* study design for intravenous and intranasal P4

(A) Survival studies

Four groups of five mice were infected intranasally:

- Group 1 (n=5) received DEPC-treated water alone
- Group 2 (n=5) received IVIG alone
- Group 3 (n=5) received P4 peptide alone
- Group 4 (n=5) received P4 peptide and IVIG.

Mice were monitored for signs of disease for up to 48 hours following infection and survival recorded. These experiments were performed in duplicate on separate days.

(B) Infection studies

Two groups of mice were infected intranasally:

- Group 1 (n=5 per time point) received DEPC-treated water alone
- Group 2 (n=5 per time point) received P4 peptide and IVIG.

Mice were monitored for signs of disease for up to 48 hours following infection. Lung, blood and nasopharyngeal tissue was collected at 24, 36 and 48 hours following infection. These experiments were performed in duplicate on separate days.

(C) Cell population studies

Two groups of mice were infected intranasally:

- Group 1 (n=5 per time point) received DEPC-treated water alone
- Group 2 (n=5 per time point) received P4 peptide and IVIG.

Mice were monitored for signs of disease for up to 48 hours following infection. These experiments were performed in duplicate on separate days.

4.8 Pneumococcal Carriage

A pre-requisite for invasive pneumococcal disease is the successful colonization of the nasopharnyx. From there, pneumococci can be aspirated into the lungs causing pneumonia. In order to understand the immunological events that occur during pneumococcal colonization, animal models were established in which pneumococci are stably carried in the nasopharynx for up to 28 days without causing disease [307]. Preventing, reducing or blocking carriage is of great interest.

4.8.1 Infection to establish pneumococcal carriage

This study assessed whether peptide treatment of the nasopharynx alongside IVIG would alter carriage in a mouse model. 20 mice were infected with 1 x 10^6 CFU / 10 µl to establish pneumococcal carriage in the nasopharync. Three time points were chosen at which nasopharyngeal tissue was collected and level of colonization determined: Day 0 (n=3) to establish whether the correct dose was administered, Day 3 (n=3) to confirm stable colonization and Day 7 (n=7 per group) as a final timepoint. 100 µg of P4 solubilized in 15 µl of DEPC treated water was administered intranasally and 100 µl of IVIG peritoneally on days 5 and 6 (48 and 24 hours before final time point, respectively).

4.9 Pre-treating before aIPD

To assess whether the effect seen when administering P4 solution in the absence of infection would benefit the host during pneumococcal infection, peptide solution only (i.e. no IVIG) was intravenously administered 24 hours prior to an aIPD infection. A survival study and infection study was done. For the survival study, two groups (pre-treatment, no pre-treatment, n=5 per group) were infected and their survival rate recorded. For the infection studies two groups (pre-treatment, no pre-treatment, n=10 per group) were infected and blood and lung tissue collected at 24 and 48 hours following infection (n=5 per time point per group).

4.10 Statistical analysis

Groups of five mice per time point or treatment were used. All *in vivo* experiments were performed in duplicates on separate occasions with the exception of the "aged mice" and "pneumococcal carriage" studies, where experiments were performed once with groups of eight and seven mice, respectively. CFU, Flow Cytometry and survival data from the primary and repeat *in vivo* studies were pooled resulting in groups of n=10. Data is presented as means ± standard deviation. Distribution of data was first assessed using D'Agostino and Pearson omnibus normality test. For normally distributed data, an unpaired or paired T-test was used to test for statistical differences between treated and control groups. For non-normally distributed data, a Mann-Whitney T-test was used to test for statistical differences between treated and control groups. For survival studies, data were plotted into a Kaplan-Meier estimator and analyzed for statistical difference compared to control groups using a logrank test. Differences between data sets were designated significant if $p<0.05$.

B. Results

A summary of infection studies in Chapter IV showing the infection model, bacterial and murine strain, route of API administration and outcomes measured is shown in Table 13.

Table 13 - Summary of infection studies in Chapter IV showing the infection model, bacterial and murine strain, route of API administration and outcomes measured.

Expt	Model	SPN	Mouse	API administration	Outcomes	Notes	Page
1	IPD (young)	ST3	Swiss Webster	Intravenous	Survival	Invasive model, Increased survival following API compared to control groups (peptide or IVIG alone)	123
2	IPD (aged)	ST3	Swiss Webster	Intravenous	Survival	Model of senescence, Increased survival following API	124
3	IPD (aged)	ST3	BALB/c	Intravenous	Survival	Model of senescence, Increased survival following API	125
4	aIPID (young)	ST2	MF1	Intravenous	Survival CFU Phagocytes	Susceptible model, Increased survival, decreased CFU, increased phagocyte recruitment and activation following API during bacteraemia	126
5	aIPD (young)	ST2	MF1	Intranasal	Survival CFU Phagocytes	Susceptible model, no bacteraemia and mortality, decreased lung CFU, increased phagocyte recruitment and activation following API during pneumonia	132
6	Carriage	ST2	MF1	Intranasal	CFU	Asymptomatic carriage, increased CFU following API	137
7	Carriage	ST2	MF1	Intravenous	CFU	Carriage during invasive disease, no significant changes in CFU following API	138
8	aIPD	ST2	MF1	Intravenous (Pre-treatment)	Survival CFU	Peptide alone (no IVIG) prior to infection, increased survival, decreased CFU	139

4.11 Invasive pneumococcal disease (IPD)

Using a model of invasive pneumococcal disease (IPD), the ability of combined administration of P4 peptide & IVIG to rescue moribund mice was compared to that of P4 peptide administration alone, IVIG or DEPC treated water alone. Mice were infected with 2 x 10^7 ST3 pneumococci and treated at 24 and 48 hours post infection with either DEPC treated water, P4 solution, IVIG or a combination of P4 and IVIG.

4.11.1 Survival in young Swiss Webster mice

Survival of 8-12 week old mice in different groups following infection and treatment is shown in Figure 43. 96 hours following infection 0% (0/10) mice in the control group survived, 20% (2/10) in the IgG alone and P4 alone groups survived whereas there was 80% survival (8/10) in the P4-IVIG treatment group (p=0.01).

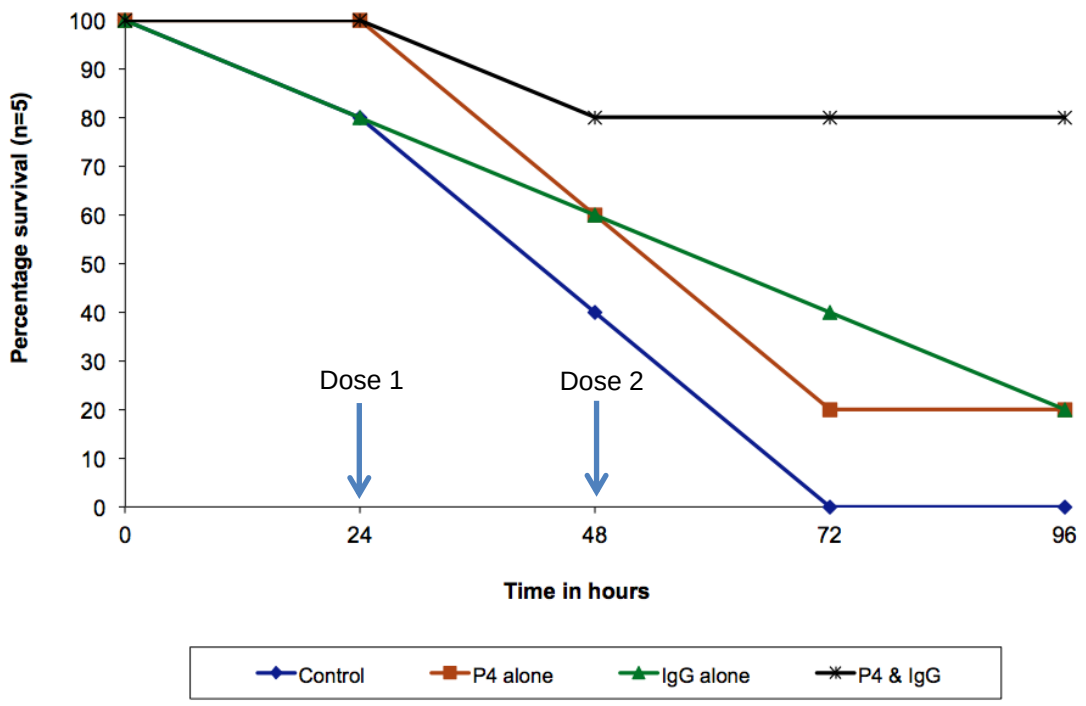


Figure 43 – Survival in a model of invasive pneumococcal disease using young Swiss Webster mice and ST3-WU2 pneumococci. Mice were infected and treated with either DEPC treated water (blue), P4 solution alone (orange), IVIG alone (green) or P4 solution and IVIG (black) (n=10 per group). Treatment was administered at 24 and 48 hours following infection.

Aging is known to reduce the effectiveness of the immune system and the elderly population is highly susceptible to pneumococcal infection. P4-IVIG treatment was assessed in a host whose immune system is weakened as a result of ageing.

4.11.2 Survival in aged Swiss Webster mice

15 month old Swiss Webster mice were infected with 2 x 10^7 ST3 pneumococci and monitored for signs of disease. Survival of treated and untreated mice following infection is shown in Figure 44. At 48 h post challenge, 80% (16/20) were moribund. The moribund mice were randomly divided into a control (n = 8) and a treatment group (n = 8). Following intra-nasal infection, 73% of treated 15-month-old Swiss Webster mice survived with complete remission of symptoms, compared to 20% survival in the control group (p=0.0002).

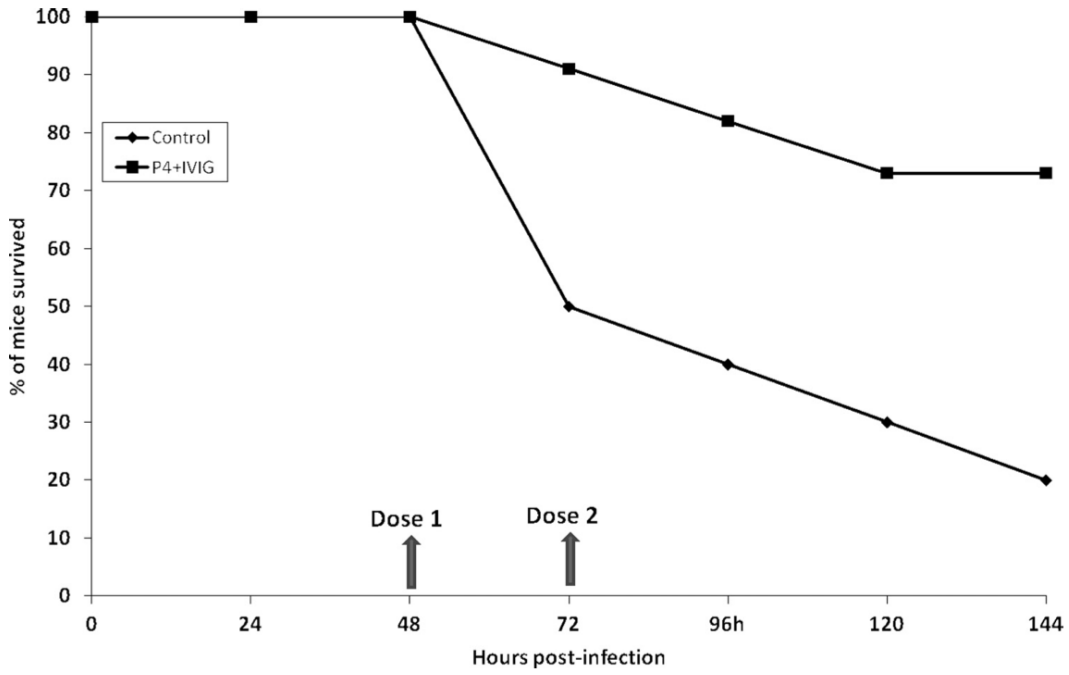


Figure 44 - P4 peptide alongside IVIG confers protection to 15-month-old Swiss Webster mice against intranasal *S. pneumoniae* serotype 3 (WU2) challenge. Intravenous injection of P4 (100 μg/mouse) with IVIG (100 μl/mouse) at 48 and 72 h after challenge provided highly significant protection (73%; P = 0.0002) from *S. pneumoniae* WU2 infection. Published figure [283].

4.11.3 Survival in aged BALB/c mice

BALB/c mice are naturally more resistant to pneumococcal infection than Swiss Webster or MF1 mice [310]. 11 months old BALB/c mice were infected with 2 x 10^7 ST3 pneumococci and monitored for signs of disease. Survival of treated and untreated mice following infection is shown in Figure 45. At 48 h post challenge, 80% (16/20) were moribund. The moribund mice were randomly divided into a control (n = 8) and a treatment group (n = 8). Similar to Swiss-Webster aged mice, 95% of the treated 11-month-old BALB/c mice survived, while only 45% of the mice from the control group survived following infection (p=0.02).

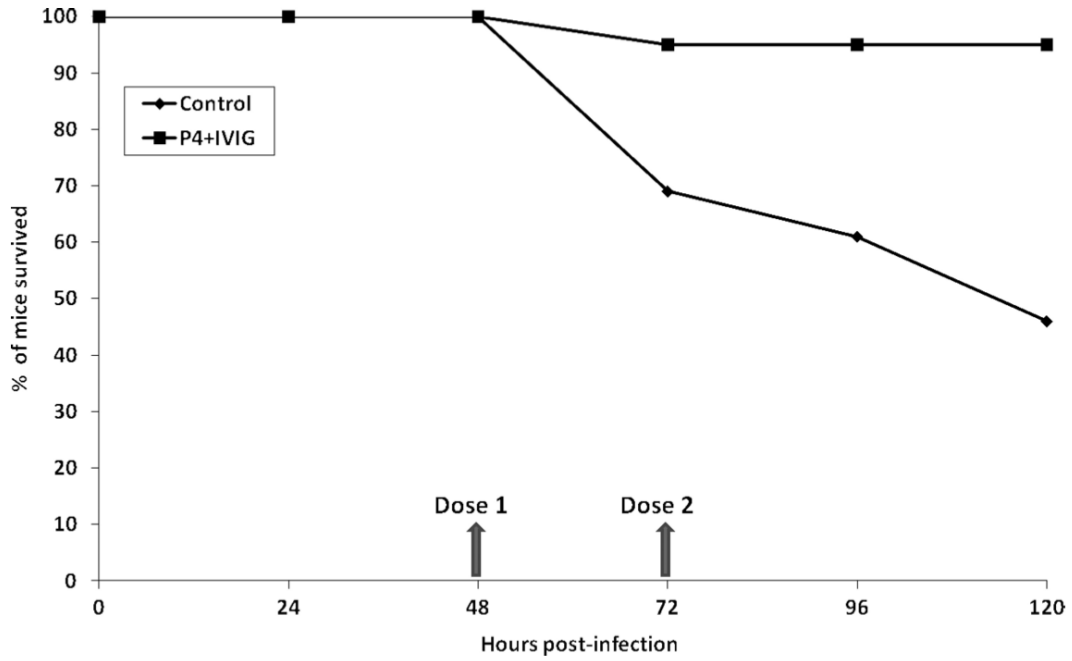


Figure 45 - P4 peptide alongside IVIG confers protection to 11-month-old BALB/c mice against intranasal *S. pneumoniae* serotype 3 (WU2) challenge. Intravenous injection of P4 (100 μg/mouse) with IVIG (100 μl/mouse) at 48 and 72 h after challenge provided highly significant protection (95%; P=0.02) from *S. pneumoniae* WU2 infection. Published figure [283].

4.12 Acute invasive pneumococcal disease (aIPD) - intravenous P4 during pneumococcal septicemia

4.12.1 Survival studies

8-12 week old mice were infected intranasally using an acute dose (1 x 10^6 CFU) of D39 pneumococci and treated intravenously with P4-IVIG or DEPC solution at 24 and 30 hrs post-infection. Figure 46 shows a Kaplan-Meier survival plot of control and P4-IVIG treated mice. 48 hours following infection, mice in the P4-IVIG group exhibited 60% survival as compared to 0% survival (p=0.03) in the control group.

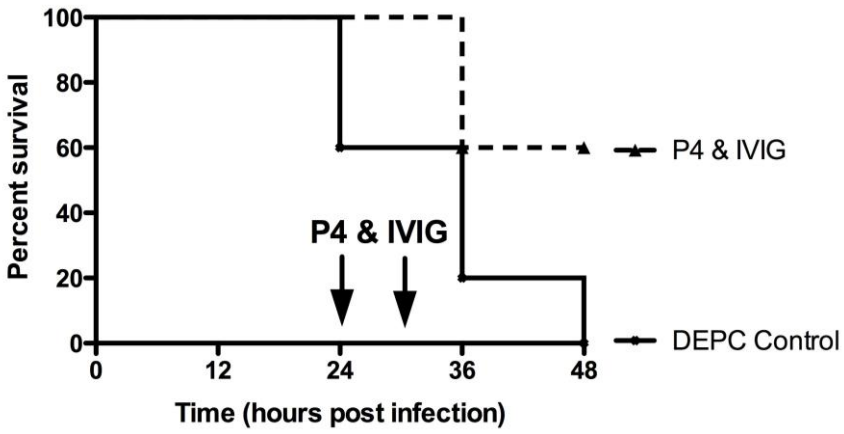


Figure 46 – Kaplan-Meier survival plot of control of P4-IVIG treated groups in a model of acute pneumococcal disease using MF1 mice and ST2-D39 pneumococci. Mice were infected and (A) DEPC treated water or (B) P4 and IVIG was administered at 24 and 30 hours following infection. Published figure [285].

Survival of control groups, where mice were treated with P4 peptide alone or IVIG is shown in Figure 47. Following treatment at 24 and 30 hours post infection, neither P4 alone nor IVIG alone treated mice showed significant differences (p=0.1 and p=0.3, respectively) in augmenting survival compared to the control group, which received DEPC treated water alone.

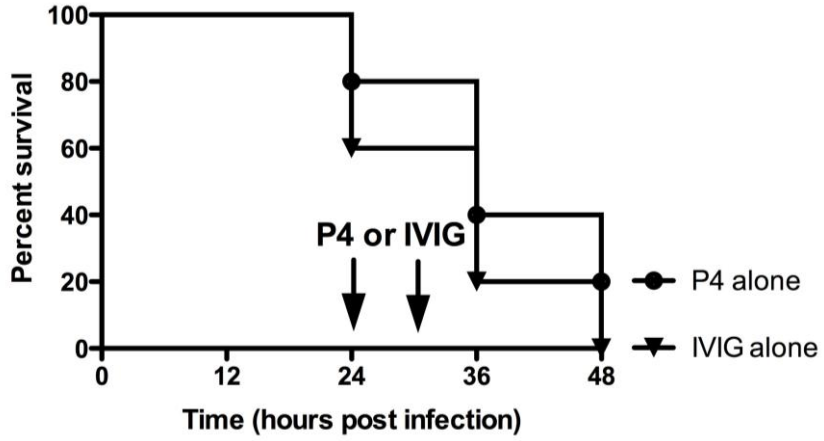


Figure 47 – Kaplan-Meier survival plot of control groups in a model of acute pneumococcal disease using MF1 mice and ST2-D39 pneumococci. Mice were infected and (A) DEPC treated water or (B) P4 and IVIG was administered at 24 and 30 hours following infection.

4.12.2 Infection studies

The CFU count in treated and untreated mice are compared in Figure 48. Panel (A) shows CFU/mg of lung tissue and panel (B) shows CFU/ml of blood. P4-IVIg mice had significantly fewer bacteria in their lungs at 36 hrs (control Log_{10} 6 CFU vs. treated Log_{10} 4.5 CFU, p=0.04) and 48 hrs (control Log_{10} 6.9 CFU vs. treated Log_{10} 3.9 CFU, p=0.004), 12 and 24 hours following the first treatment respectively, as compared to the control group (Figure 48A). Bacteremia levels, which in control mice steadily increased as a result of uncontrolled lung infection, was reduced in P4-IVIG treated mice where a significant decreased amount of bacteria in the blood was detected at 48 hrs (control Log_{10} 8.8 CFU vs. Log_{10} 4 CFU, p=0.01) (Figure 48B).

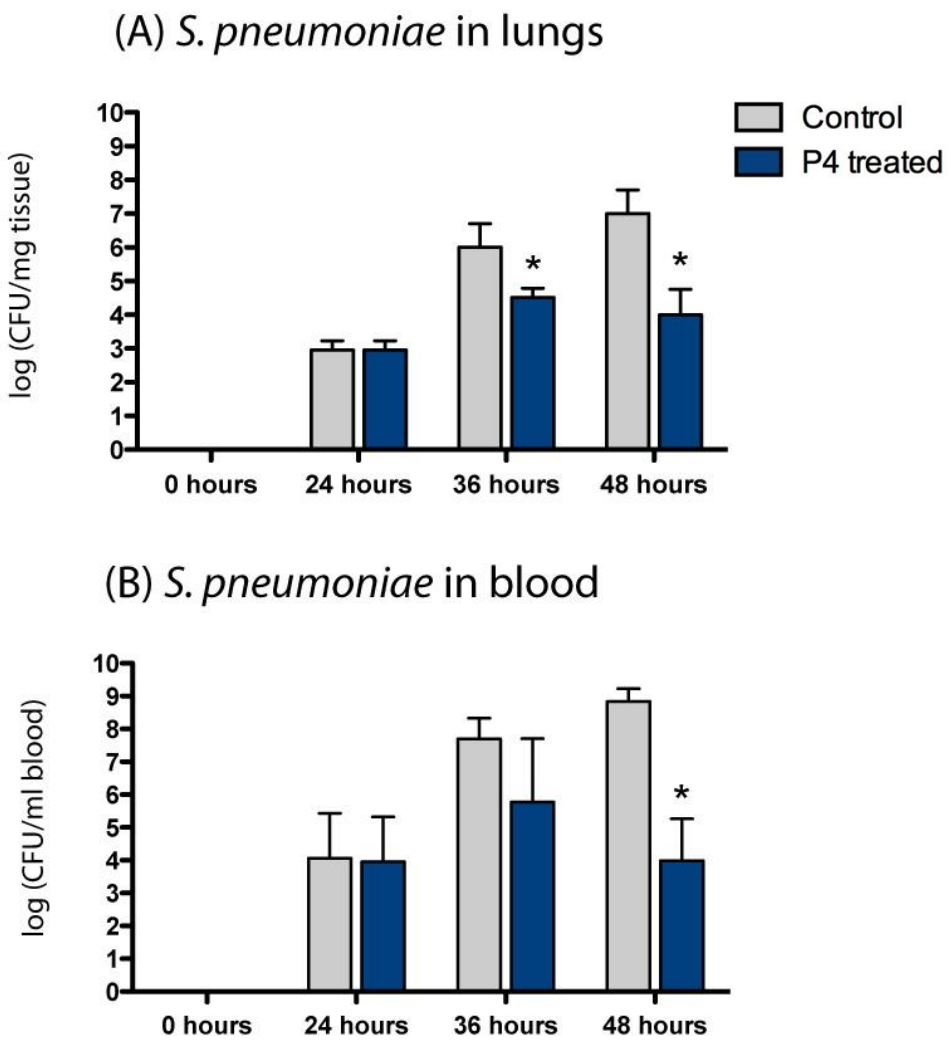


Figure 48 - Log10 *S. pneumoniae* colony forming units (CFU) recovered per (A) mg of lung tissue and (B) ml of blood at 0, 24, 36 and 48 hours following infection in treated and non-treated mice. Mice were treated with either DEPC water or P4-IVIG at 24 and 30 hours following infection. (*) denotes significant differences at time points using ANOVA where $p<0.05$. Published figure [285].

4.12.3 Cell population studies

Neutrophil numbers and activation are compared between treated and untreated mice in Figure 49. Panel (A) shows cells/mg of tissue and panel (B) shows FcγR expression. Throughout infection and treatment, neutrophil numbers remained similar between both groups following the same pattern of early increase peaking at 36 hrs, followed by a decline in numbers by 48 hrs (Figure 49A). Neutrophil Fcγ expression levels were increased in both groups by 36 hours, where neutrophils from P4 treated mice had elevated, albeit non-significant levels of Fc expression compared to the DEPC treated mice (control 38.2 % FcγR$^+$ vs. treated 72.4% FcγR$^+$, p=0.07). At 48 hours, FcγR expression decreased in control mice but remained higher and significantly different in P4-IVIG treated mice (control 15.7 % vs. treated 66.9 %, p=0.02) in comparison (Figure 49B).

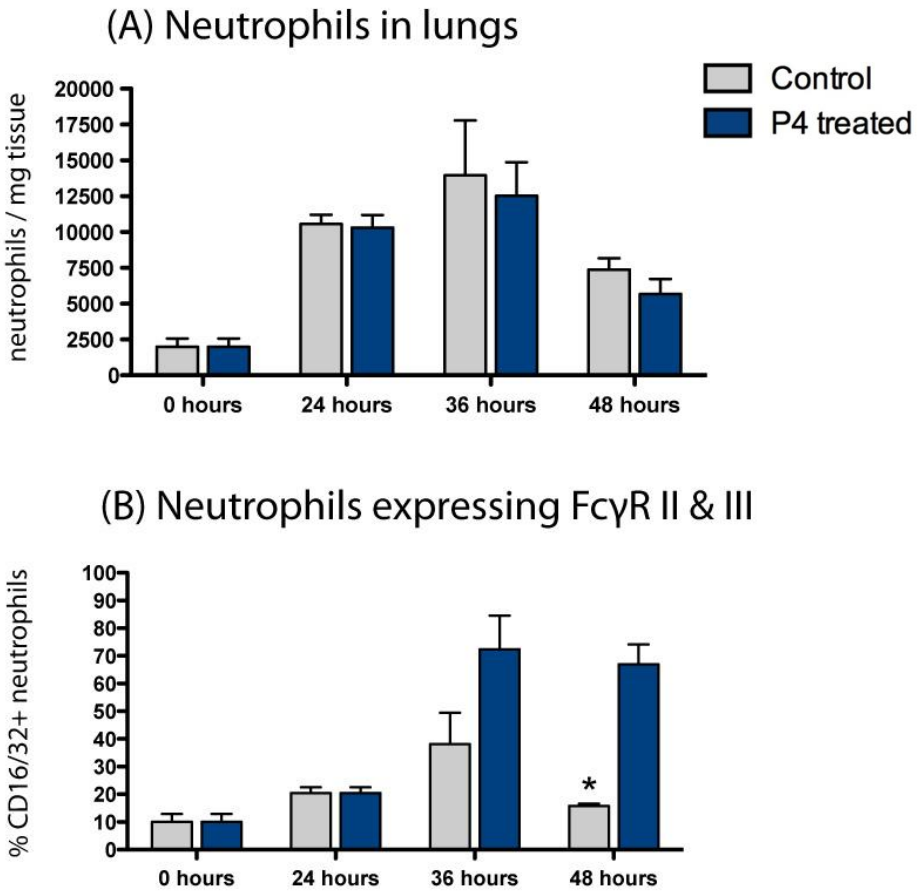


Figure 49 – (A) Neutrophils detected per mg lung tissue at 0, 24, 36 and 48 hours following infection in treated and non-treated mice. (B) Fc-γ RII/III expression in neutrophils detected in lungs at 0, 24, 36 and 48 hours following infection in treated and non-treated mice. Mice were treated with either DEPC water or P4-IVIG at 24 and 30 hours following infection (*) denotes significant differences at time points using ANOVA where $p<0.05$. Published figure [285].

While neutrophil numbers remained similar in the lungs of treated and control mice, macrophages in lungs of P4-IVIG treated mice were significantly higher at 36 hrs (control 1,069/mg vs. treated 3,850/mg, p=0.001) (Figure 50A). By 48 hours, macrophages in the control group also increased while numbers in the P4-IVIG group remained at the same level. There was no significant difference in Fc-γ expression levels in macrophages as was observed for neutrophils (Figure 50B).

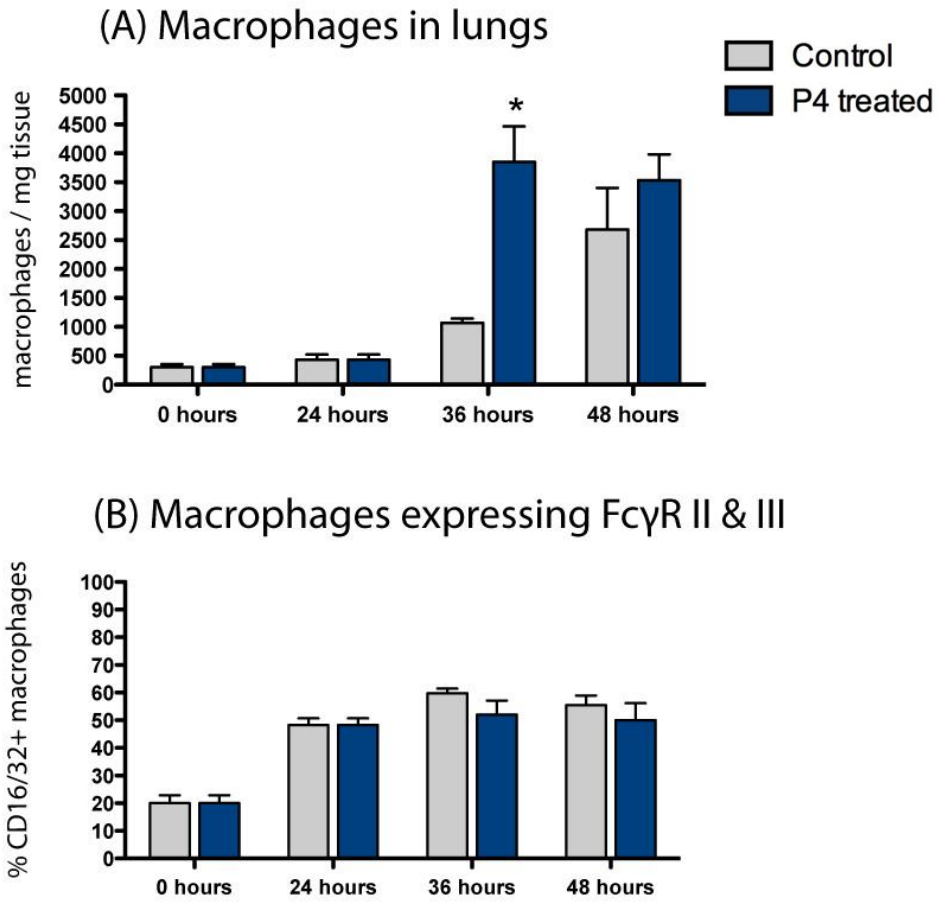


Figure 50 - (A) Macrophages detected per mg lung tissue at 0, 24, 36 and 48 hours following infection in treated and non-treated mice. (B) Fc-γ RII/III expression in macrophages detected in lungs at 0, 24, 36 and 48 hours following infection in treated and non-treated mice. (*) denotes significant differences at time points using ANOVA where p<0.05. Published figure [285].

4.13 Acute invasive pneumococcal disease (aIPD) - intranasal P4 during pneumococcal pneumonia

4.13.1 Survival studies

Intranasal infection was used to test whether very early pre-emptive P4-IVIG treatment of lungs during invasive pneumonia, prior to the development of bacteremia, would alter the course of infection. Mice were infected and intranasally treated with P4 and IVIG at 12 and 18 hrs post-infection and their survival is shown in Figure 51. By 48 hrs following infection, 100% (10/10) of P4-IVIG mice survived compared to 80% moribund mice in the control group (p=0.01).

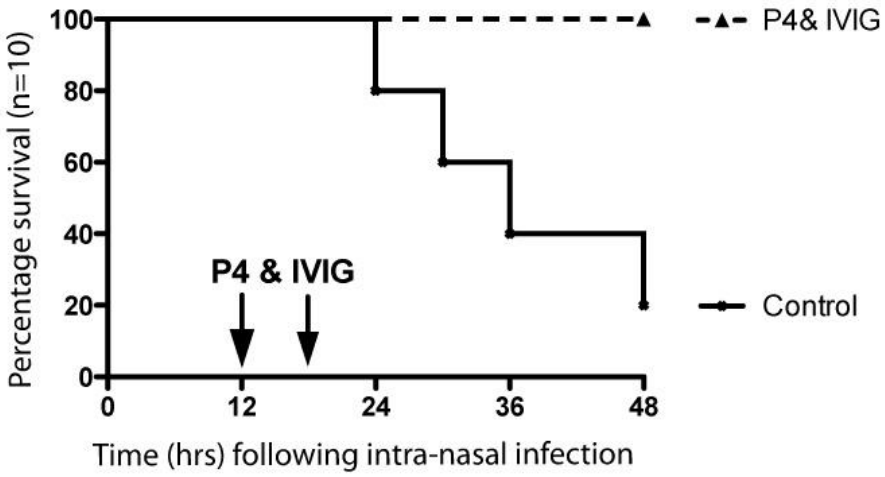


Figure 51 - Survival of MF1 mice intranasally infected with 10^6 *S. pneumoniae* (D39) and treated with intranasal P4 and intraperitoneal IVIG at 12 and 18 hours. Treatment groups: P4-IVIG (100 μl IVIG, 100 μg P4) and DEPC water (100 μl) control (n=10 per group). Published figure [285].

No significant differences were seen when P4 or IVIG was administered alone and compared to the DEPC treated water control group as shown in Figure 52. Mice intranasally treated with P4 solution alone showed 40% survival (4/10) while IVIG alone treated mice showed 30% (3/10) survival. Both control groups (P4 alone and IVIG alone) in the IN study had slightly higher survival rates as compared to control groups in the IV administration studies.

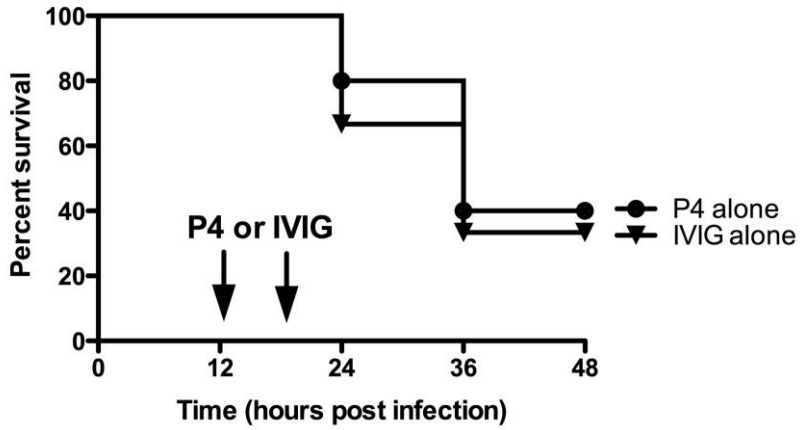


Figure 52 – Kaplan-Meier survival plot of MF1 mice intranasally infected with 10^6 *S. pneumoniae* (D39) and treated with intranasal P4 and intraperitoneal IVIG at 12 and 18 hours. Treatment groups: P4 (100 μl IVIG, 100μg P4) and IVIG (100 μl) controls (n=10 per group). Published figure [285].

4.13.2 Infection studies

Lung and blood tissue was collected during the course of infection and treatment in order to assess bacterial load. At 24 hrs following infection, there was no significant difference (p=0.09) in bacterial lung CFUs between control and P4-IVIG treated groups (Figure 53A). By 48 hrs however, bacterial loads in control mice significantly increased compared to 24 hrs levels while pneumococcal loads in P4-IVIG treated mice remained the same as 24 hrs and were significantly less compared to the control group (control Log_{10} 4.9 CFU vs. treated Log_{10} 2 CFU, p=0.002). The presence of bacteria in the blood of control mice significantly increased by 48 hrs while none of the P4-IVIG treated mice had any bacteria in blood at either 24 or 48 hrs (Figure 53B).

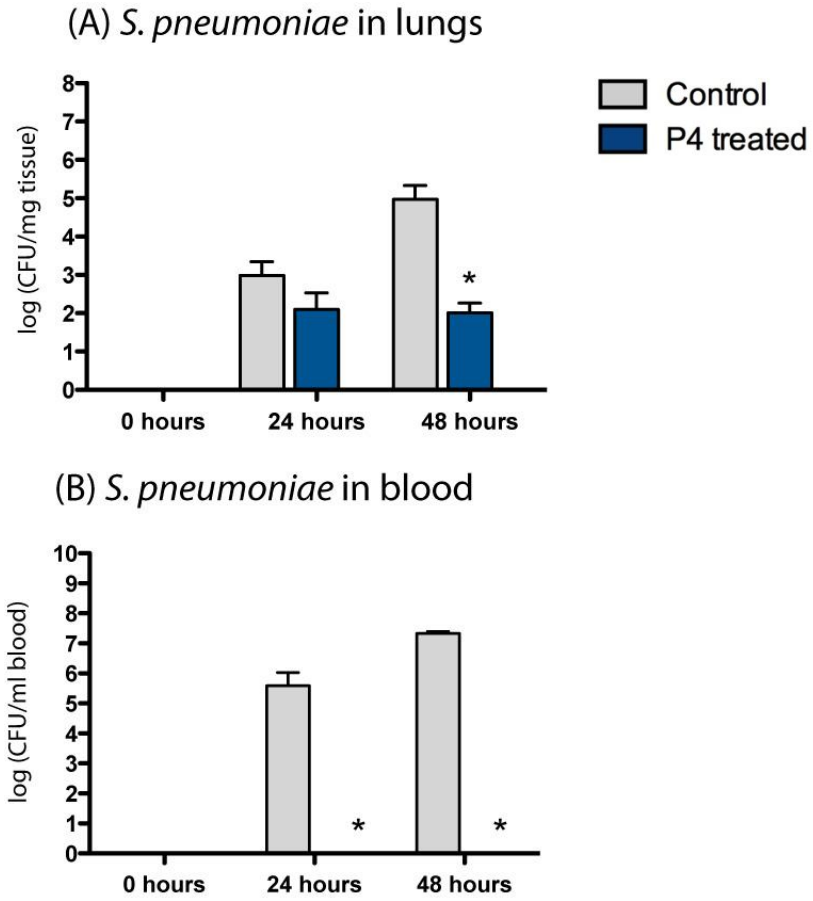


Figure 53 - Log_{10} *S. pneumoniae* colony forming units (CFU) recovered per (A) mg of lung tissue and (B) ml of blood at 0, 24 and 48 hours following infection in treated and non-treated mice. (*) denotes significant differences at time points using ANOVA where p<0.05. Published figure [285].

4.13.3 Cell population studies

An influx of neutrophils by 24 hrs was detected in both control and P4-IVIG treated groups. However, by 48 hrs, P4-IVIG treated mice had significantly lower numbers of neutrophils in their lungs (control 13,496/mg vs. treated 4,638/mg, p=0.03), while neutrophil numbers remained constant in control mice (Figure 54A). Fc-γ RII/III expression on neutrophils was significantly higher at 48 hours in P4-IVIG treated mice than in control mice (control 10.2% vs. treated 45%, p=0.03) (Figure 54B).

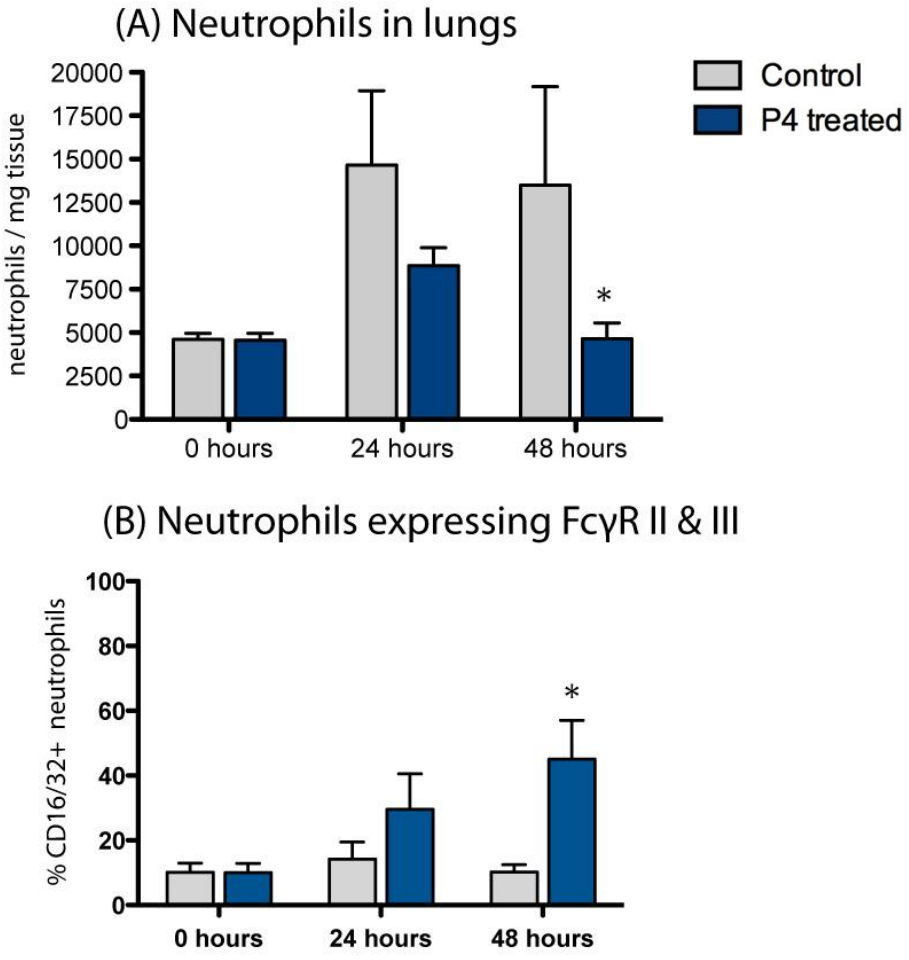


Figure 54 – (A) Neutrophils detected per mg lung tissue at 0, 24 and 48 hours following infection in treated and non-treated mice. (B) Fc-γ RII/III expression on neutrophils detected in lungs at 0, 24 and 48 hours following infection in treated and non-treated mice. (*) denotes significant differences at time points using ANOVA where p<0.05. Published figure [285].

Macrophage numbers in lungs remained similar between all groups throughout infection except at 48 hrs, where a substantial decrease (albeit non-significant, control 1,604/mg vs. treated 2,742/mg, p=0.06) in macrophages was detected in P4-IVIG treated mice (Figure 55A). Throughout the course of infection and treatment, Fc-γ RII/III expression on macrophages remained statistically similar (Figure 55B) but elevated levels of Fc-γ RII/III were detected on macrophages in the P4 treated group at 24 hours (control 42 % vs. treated 73%, p=0.08).

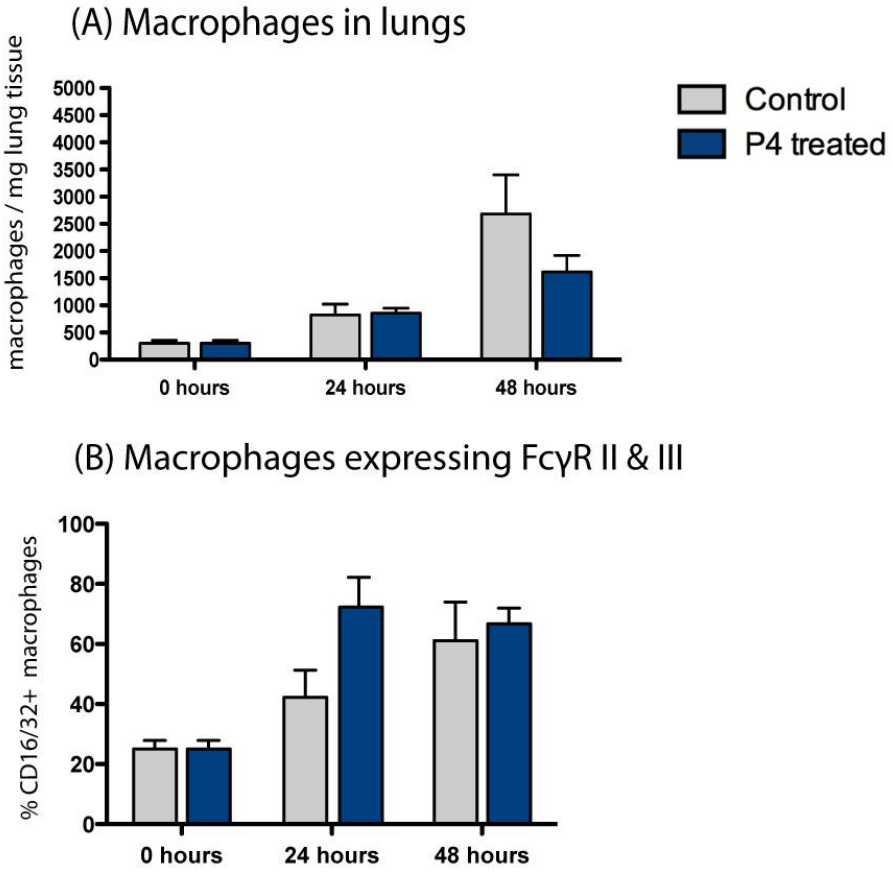


Figure 55 - (A) Macrophages detected per mg lung tissue at 0, 24 and 48 hours following infection in treated and non-treated mice. (B) Fc-γ RII/III expression on marcophages detected in lungs at 0, 24 and 48 hours following infection in treated and non-treated mice. (*) denotes significant differences at time points using ANOVA where p<0.05. Published figure [285].

4.14 Pneumococcal carriage

4.14.1 Nasopharyngeal carriage (asymptomatic infection)

Pneumococci were instilled intranasally to murine nasopharynx to establish long-term nasopharyngeal carriage. P4 was administered to the nasopharynx alongside IVIG on days 5 and 6. The CFU levels in nasopharyngeal tissue of treated and control mice are shown in Figure 56. At days 0, 3 and 7, CFU levels detected in the nasopharynx of control mice were similar (Log_{10} means of 2.73, 2.59 and 2.49 CFU respectively), with no significant changes detected. P4-IVIG treated mice had significantly higher levels of CFU (control Log_{10} 2.59 vs. treated Log_{10} 3.1, p=0.03) in their nasopharyngeal tissue at day 7. No CFU were detected in the lungs of control or P4-IVIG treated mice at any time-points.

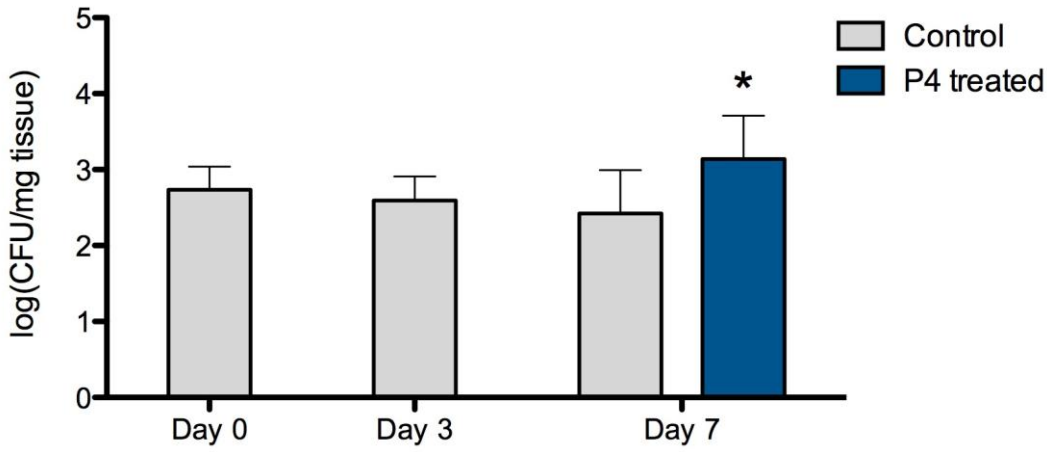


Figure 56 – Intranasal administration of P4 peptide during nasopharyngeal carriage of D39 pneumococci in MF1 mice. 20 mice were infected intranasally and placed into Day 0 (n=3), Day 3 (n=3), Day 7-Control (n=7) and Day 7-P4-IVIG (n=7) groups. Mice were treated intranasally with P4 on days 5 and 6. Nasopharyngeal tissue was harvested at time-points and assessed for bacterial loads. (*) denotes statistical significance using ANOVA.

4.14.2 Nasopharyngeal carriage (acute invasive infection)

Colonization of the nasopharynx was measured during the course of infection and treatment in an acute model of invasive pneumococcal disease. The CFU levels in nasopharyngeal tissue of treated and control mice are shown in Figure 57. Following infection at time 0, nasopharyngeal colonization levels dropped at 24 hours post infection but began increasing in both the control and P4-IVIG treated mice at 36 and 48 hours. At time point 0, 24 and 36 hours following infection, colonization levels were similar between Control and P4-IVIG treated groups. P4-IVIG treated mice at 48 hours however showed less, albeit non-significant (control Log_{10} 6.2 vs. treated Log_{10} 4.8, p=0.28), levels of pneumococci in the nasopharynx as compared to control mice.

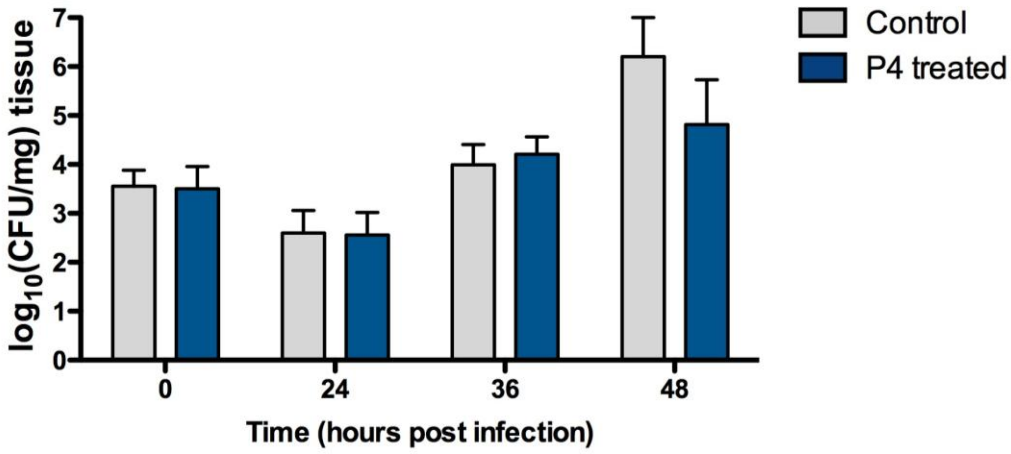


Figure 57 – Nasopharyngeal carriage of D39 pneumococci in MF1 mice during an acute invasive pneumococcal disease model. Mice were infected (n=5 per group per time-point), treated intravenously with P4 and IVIG at 24 and 30 hours following infection and nasopharyngeal tissue extracted for CFU analysis. Statistical analysis was done using ANOVA.

4.15 Peptide pre-treatment

It was tested whether recruited and activated phagocytes seen following peptide administration in the absence of infection would benefit the host during subsequent pneumococcal infection. Mice were first injected intravenously with P4 solution, and then infected intranasally with an acute dose of D39 pneumococci 24 hours later. The survival and bacterial loads in blood and lung was assessed following infection and are shown in Figure 58 and Figure 59, respectively.

4.15.1 Survival studies

Control mice that were pre-treated with DEPC solution succumbed to infection 48 hours following infection. In comparison, mice that received P4 solution 24 hours prior to infection showed significant improvements in survival (p=0.007) where no mortality was observed at 48 hours. Mortality was however delayed as by 72 hours, 80% of pre-treated mice had died.

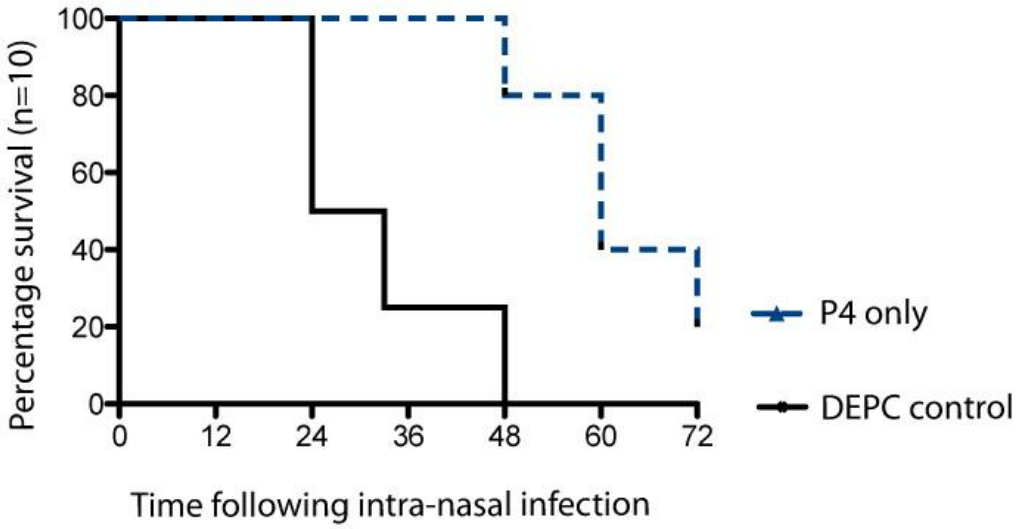


Figure 58 - Survival of mice pre-treated intravenously 24 hours prior to infection with either P4 solution (100 μg P4 peptide / 100 μl DEPC treated water) or control DEPC treated water. n=10 per group.

4.15.2 Infection studies

In addition to survival, bacterial loads were measured in mice pre-treated with either P4 solution or DEPC treated water. At 24 hours, both the lung CFU (control Log_{10} 3.7 vs. treated Log_{10} 0.8, p=0.002) and blood CFU (control Log_{10} 4.6 CFU vs. treated Log_{10} 1.2 CFU, p=0.04) levels were significantly lower in P4 pre-treated mice than in the control groups. By 48 hours however, CFU levels were statistically similar between both groups.

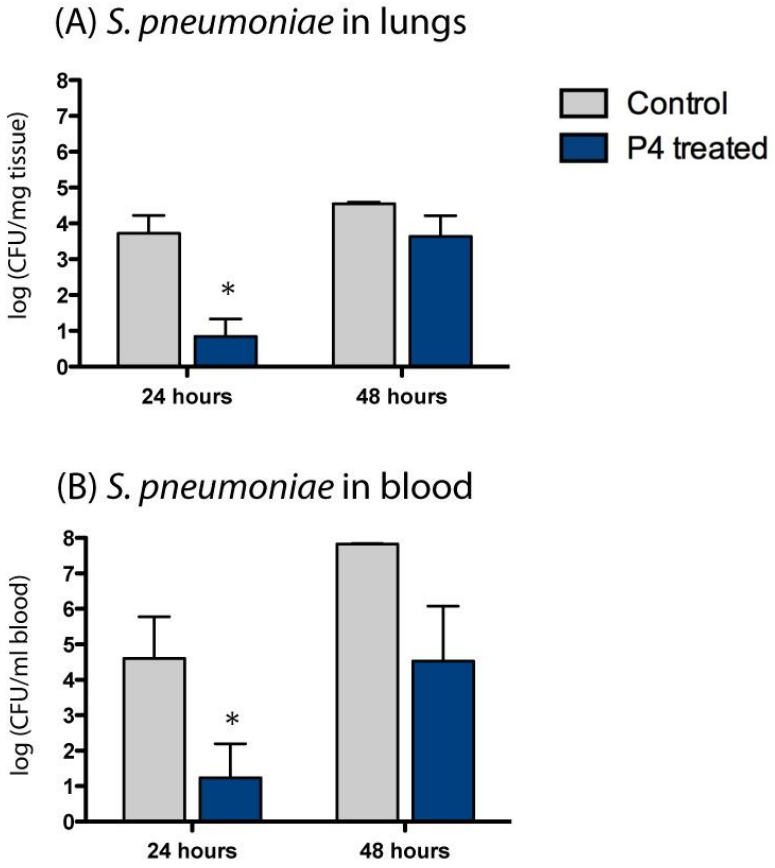


Figure 59 - CFU recovered in lungs and blood of mice pre-treated 24 hours prior to infection with either (a) 100 μg P4 peptide / 100 μl DEPC treated water or (b) DEPC treated water alone. n=10 per group. (*) denotes significant differences at time points using ANOVA where p<0.05

C. Discussion

4.16 Discussion

In this chapter, the efficacy of API using murine models of invasive pneumococcal disease was assessed. Measurement of treatment efficacy included survival, bacterial burden, phagocyte recruitment and activation following both intravenous and intranasal administration of P4 and IVIG using a range of murine models. Furthermore, the effect of API on nasopharyngeal carriage of *S. pneumoniae* during symptomatic and asymptomatic infection, as well as peptide pre-treatment was assessed.

4.16.1 Invasive pneumococcal disease (IPD)

Survival of infected mice was significantly increased in young and aged Swiss Webster and BALB/c mice. It is well established that young (<5 years) and aged (>65 years) populations are more susceptible to infection [311, 312]. Factors influencing susceptibility include an underdeveloped immune system in the young [313], and dysregulated immune responses in aged populations [314], leading to poor recognition and killing of pathogens [315]. The efficacy of API in both young and aged mice is therefore promising. Studies have shown that neutrophils in aged populations [316] and in children >1-year of age [317] exhibit reduced phagocytic function. Assessing whether P4 peptide could augment phagocytosis of human blood neutrophils from young and aged populations would strengthen these data.

Enhanced survival in all models was seen during concomitant administration of P4 and IVIG but not when P4 or IVIG was administered on their own. These results confirm previous results where mice infected with *S. pneumoniae, S.pneumoniae*-Influenza co-infection and *S. aureus* were rescued

only when P4 was administered in the presence of pathogen-specific immunoglobulin [280-282, 284]. An augmented phagocytic therapy, where phagocytes are stimulated for enhanced uptake, relies on the presence of opsonised pathogens. Similarly, passive immunotherapy, where pathogens are opsonised with exogenous immunoglobulin, relies on effective phagocytosis. The necessity for both P4 and IVIG to be present for effective treatment underlines the synergistic mechanism of API proposed in previous studies [274].

4.16.2 Acute invasive pneumococcal disease (aIPD) – intravenous peptide

The susceptible MF1 host was chosen to model rapidly progressing pneumococcal infections. In a clinical setting, acute infections are responsible for the majority of deaths in intensive care units due to the time taken for antimicrobial compounds to take effect [318]. To challenge the treatment model, API was administered intravenously at 24 hours, when pneumonia had already progressed to bacteraemia.

Intravenous API increased survival by reducing the bacterial burden in lungs and blood as well as recruiting monocytes and activating both neutrophils and monocytes in the lung. Comparable to the cell population studies in the absence of infection in chapter III, monocyte/macrophages were heavily recruited to the lungs following IV peptide administration during infection. It was suggested in chapter III that, in the absence of infection, P4 induces monocyte release from either the bone marrow or spleen monocyte reservoir. As monocyte/macrophage recruitment to the lung is correlated with a halt in the progression of pneumonia, it is possible that P4 enhances the release of monocytes from their reservoir during infection, which migrate to the lungs (due to chemotactic cytokines produced by alveolar macrophages and epithelial cells during infection) and actively participate in clearance of

bacteria. An increase of activated monocytes during infection is beneficial as it has previously been shown that monocytes are essential in containing septic infections [319]. Studies looking at the expression of junctional adhesion molecule-like protein (JAML), an important adhesin for trensendothelial migration, would help understand whether increased monocyte populations in the lungs are due to chemotactic cytokines in the blood or surface proteins on monocytes [141].

Blood CFU levels in treated mice remained similar to control mice until 48 hours following infection, where a significant reduction of CFU was detected in treated mice. The delayed reduction in blood CFU numbers during infection correlates with the delayed recruitment of neutrophils following IV peptide administration in the absence of infection (Chapter III). It is possible that P4 rapidly releases monocytes, which help clear infection in the lungs, and induces a delayed release of neutrophils, which contribute to the phagocytic clearance of bacteria in the blood. Further studies are necessary, however, to confirm the phagocyte recruitment and migration dynamics following infection and peptide administration.

4.16.3 Acute invasive pneumococcal disease (aIPD) – intranasal peptide

Intranasal administration of P4 alongside IVIG during acute pneumonia prevented the onset of bacteraemia and subsequent host mortality. P4 was administered into the lungs at 12 and 18 hours following infection leading to a halt in the progression of pneumonia, similar to the IV administration studies. Importantly, no CFU were detected in the blood of P4 treated mice meaning that host pulmonary defense was augmented sufficiently to contain the spread of infection. In the MF1 host, neutrophils infiltrate to infected lung tissue by 12 hours post pneumococcal infection and assist resident lung macrophages in clearing pathogens [306]. As such, it is likely that intranasal

peptide administration leads to augmented phagocytosis of recruited neutrophils and resident macrophages. This is supported by data in this thesis, and in previous studies [280], where P4 was able to augment murine neutrophil and alveolar macrophage phagocytosis *ex vivo*. Similar to intranasal administration in the absence of infection, intranasal P4 did not lead to enhanced recruitment of phagocytes despite the presence of infection and therefore chemotactic cytokines. P4 administration actually led to a reduction of both neutrophils and macrophages in lungs. This is most likely due a decrease of bacterial numbers and therefore apoptotic debris in the lungs, leading to reduced cytokine mediated recruitment of phagocytes [320]. Other intranasal studies, using TLR activation or pro-inflammatory cytokines to augment host defence, administered their stimulant >24 hours prior to infection [321, 322]. Administering treatments before infection, however, does not reflect the application of a therapeutic in a clinical setting and therefore still raises the question whether treatment during infection would also be effective.

4.16.4 FcγR expression

FcγR expression was measured on neutrophils, monocytes and macrophages in lungs following both intravenous and intranasal peptide administration. IV peptide administration during bacteraemia up-regulated FcγR expression on neutrophils at 36 hours post infection (12 and 6 hours following peptide administration) and significantly up-regulated FcγR expression by 48 hours. Control mice, in comparison, up-regulated their FcγR expression on neutrophils by 36 hours, but expression decreased by 48 hours. Macrophages and recruited monocytes in the lungs did not alter their FcγR expression following IV peptide administration. Similarly, expression of FcγR on neutrophils at 12 and 6 hours following peptide administration led to enhanced expression, which was statistically significant by 48 hours post-infection (12 and 18 hours following peptide administration). Macrophage

FcγR expression was up-regulated at 24 hours (not significant) but leveled at 48 hours following infection when compared to control mice. It is important to place FcγR expression in context to phagocytosis. In the absence of infection, there was a sharp increase of circulating blood monocytes expressing significantly higher FcγR than in the control group. During infection, however, a sharp increase of recruited monocytes/macrophages was detected in the lungs but their FcγR expression was similar to control mice. As the increase of monocytes is correlated with a decrease in bacterial burden, it is likely that available FcγR internalized through ligation of opsonised pneumococci. Similarly, Weeks *et al* found that FcγR expression on neutrophils was significantly down-regulated following peptide treatment in a model of *S. pneumoniae*-influenza co-infection [284]. Down-regulated expression correlated with reduced viral and bacterial loads. As this study looked at FcγR expression in 24 hours intervals following treatment, the initial up-regulation may not have been detected.

4.16.5 Nasopharyngeal carriage

Administration of P4 to the nasopharynx during asymptomatic carriage of pneumococci led to significantly increased carriage levels but no invasive disease. These results are not surprising when put into the context of the role of pneumococcal surface adhesin A (PsaA), the protein from which P4 is derived. Bacterial surface adhesins have long been recognized for their ability to modulate the activities of various host cells [220, 279]. *Yersinia enterocolitica*, for example, uses its adhesin A to invade epithelial cells through interaction with various integrins [323]. Similarly, the meningococcal adhesin NadA augments bacterial binding and invasion of epithelial cells *in vitro* [324]. S*treptococcus pneumoniae* possesses a range of adhesins that facilitate the adhesion and invasion of epithelial cells. PavA, for example binds to epithelial cells and facilitates meningeal infection in a murine model of meningitis [224, 325] while CbpA modulates epithelial cytokine secretion and

invades through binding to human polymeric immunoglobulin receptor (hpIgR) [326]. Similarly, the functional epitope of pneumococcal surface adhesin A (P4) has been shown to induce cytological changes in epithelial cells resulting in enhanced invasion by pneumococci [279].

Concentrated administration of P4 to the nasopharynx of mice would therefore facilitate pneumococcal invasion of epithelial cells leading to the increased bacterial CFU detected in this chapter. In addition, IVIG is made up of >95% IgG which is unable to enter mucosal areas and therefore unable to assist in augmented phagocytosis of recruited neutrophils. The role of P4 mediated pneumococcal invasion in the nasopharynx is supported by studies of nasal carriage during API treatment of invasive pneumococcal disease models in this chapter. Intravenous administration did not alter nasal carriage suggesting that direct contact of P4 to nasal epithelial cells is necessary to facilitate pneumococcal invasion.

4.16.6 Pre-treatment

The *in vivo* cell population studies in chapter III showed significant recruitment and activation of monocytes and neutrophils in the blood stream following intravenous peptide administration in the absence of infection. To determine whether this would translate to host protection during infection, mice were pre-treated with intravenous peptide 24 hours prior to infection. Pre-treatment with P4 peptide led to enhanced survival, with bacterial loads in blood and lungs significantly lower in pre-treated mice at 24 hours following infection. Despite this, 80% of pre-treated mice succumbed to infection at 72 hours. As IVIG was not administered in these studies, it is possible that pre-treated mice succumbed due to pathogen specific immunoglobulin being spent by 24 hours. Pre-treatment studies with additional IVIG only and P4-IVIG control groups could help better support the role of IVIG in API.

CHAPTER V.

In vitro peptide studies

A. Introduction

5.1 Introduction

Studies in chapter IV confirmed that API treatment rescued mice from sepsis and that this involved neutrophils, monocytes and macrophages. In addition, flow cytometry studies showed that FcγR expression was modulated on neutrophils and monocytes in the presence and absence of infection. In this chapter, the HL-60 neutrophil-like cell line was used to determine (a) the effectiveness of API across a range of pneumococcal serotypes and (b) the involvement of phagocytic Fcγ receptors in P4 mediated opsonophagocytic killing.

5.1.1 Opsonophagocytosis killing assay

The HL-60 opsonophagocytosis killing assay (OPKA) is a method developed at the Centres for Disease Control and Prevention (CDC) to determine the opsonising efficacy of pneumococcal vaccine induced antibodies [327]. This robust assay [328] was modified to measure the ability of granulocytes to kill opsonised pathogens in the presence of stimulants [281]. The OPKA was used in this chapter to determine whether P4 could augment killing of a range of pneumococcal serotypes by HL-60 granulocytes. These included capsulated and unencapsulated D39 (ST2) and TIGR4 (ST4) laboratory pneumococcal strains as well as ST14, 6B and 19F strains derived from invasive clinical isolates. In addition, the monocytic THP-1 cells were used to determine whether P4 could augment the phagocytic killing of opsonised pneumococci in this cell line.

5.1.2 Fcγ receptor function

In previous chapters the expression of Fcγ receptors was correlated with peptide administration and survival *in vivo*. To confirm that Fcγ receptors are necessary for P4 enhanced opsonophagocytic killing of bacteria, functional studies using the OPK assay were performed by (a) occupying Fcγ receptors and (b) blocking Fcγ receptors prior to OPK assays.

5.1.3 Fcγ receptor expression

In addition to function, the ability of P4 to modulate expression of Fcγ receptors was assessed in the HL-60 cell line. FcγR studies in the previous chapters investigated expression of FcγRII and FcγRIII using an antibody that binds shared epitopes on both receptors. In this chapter, membrane expression of three phagocytic Fcγ receptors (FcγRI, FcγRII and FcγRIII) was measured using individual antibodies and flow cytometry. Expression was measured in the presence and absence of infection.

B. Materials & Methods

5.3 HL-60 cell culture

The HL-60 cell line was originated from a patient with promyelocytic leukemia in 1977 [329]. The main characteristic of this cell line is that it is composed of undifferentiated haemopoeitic cells that can undergo differentiation into functional polymorphonuclear-like (PMN) cells upon chemical induction. The granulocytic differentiation induced by DMF (N,N-dimethylformamide) yields 44% myelocytes/metamyelocytes and 53% PMNs .

5.3.1 HL-60 media

RPMI 1640 (RPMI, Sigma, UK]) was used for all HL-60 cell culture. Media was supplemented with 20% FBS and 5% antibiotic/mycotic (10,000 units penicillin, 10mg streptomycin + 25mg amphotericin B/ml [Sigma, UK]) solutions to maintain growth and prevent contamination.

5.3.2 Establishing HL-60 cell culture

1ml aliquots of frozen HL-60 cells were kindly donated by the Immunobiology Laboratory at the Centres for Disease Control and Prevention (Atlanta, GA, USA). Vials were quick-thawed in a RT water bath and immediately diluted in 15 ml of RT sterile PBS for a 10 min centrifugation step at 300 *x* g. The supernatant was discarded and the resulting cell pellet re-suspended in 15 ml RT media for another 10 min centrifugation step at 300 *x* g. The supernatant was discarded and the cell pellet re-suspended in 10 ml media to make up a cell concentration of ~$5x10^5$ cells / ml. The cell solution was transferred to a T-75 tissue culture flask and placed upright in a tissue culture incubator at 37°C (5% CO_2). 24 and 48 hours later, 10 ml of media was

added to the flask. At 72 hours, the flask was observed for turbidity (indication of cell growth) and 20 ml of fresh medium was added to make a working volume of 50 ml. Cell suspension in the flask was then tested for cell viability using Trypan Blue and a haemocytometer to assure >80% cell viability and maintained as described below.

5.3.3 Maintaining HL-60 cell culture

To ensure high viability during maintenance and differentiation, growing flasks of cells were maintained on a strict 24 hour schedule, 7-days a week. At the same time each day (±2 hours), flasks were taken out of the incubator while making sure not to disrupt the sediment (growing cells) and 25 ml of medium was removed from the top down. 25 ml of fresh medium was then added to the flask, thereby re-suspending the cells, and a sample taken to assess cell number and viability. A specific amount of cell suspension (depending on cell concentration) was then removed and replaced with fresh medium to make a concentration of 5×10^5 cells/ml and the flask returned to the incubator. One frozen aliquot was maintained for <3 months in order to avoid changes in cell morphology and function as a result of passaging.

5.3.4 Differentiating HL-60 cells

DMF was used to differentiate HL-60 cells into PMN-like phagocytic cells. 750 µl of DMF (100 mM, Fisher) was carefully added to a T-75 tissue culture flask containing 80 ml of fresh media. Growing, undifferentiated cells (Figure 60A) were tested for cell number and viability, and 20 ml of cell suspension at a concentration of 2×10^5 cells/ml was carefully transferred to the flask containing media and DMF. Cell viability of >90% only was used for differentiation. 4 days following chemical induction, cell morphology of undifferentiated cells changed towards PMN-like cells as demonstrated by

increased granularity and a lobe like shape (Figure 60B), indicating that the cells were ready for use.

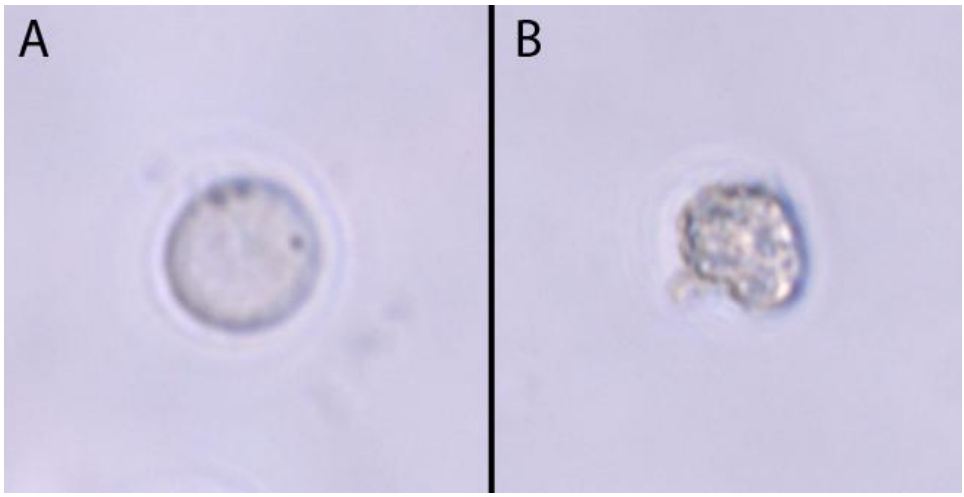


Figure 60 - HL-60 cells can be differentiated into PMN-like cells. (A) Undifferentiated, growing HL-60 cells and (B) PMN-like cells, 4 days following chemical differentiation using DMF and stained with Trypan Blue for measurement of viability.

5.4 Establishing and maintaining THP-1 tissue culture

THP-1 cells are functional monocytic cells that can be differentiated into macrophages. In their undifferentiated state, THP-1 cells are monocyte-like phagocytes and express opsonic Fcγ and C3 receptors [330].

5.4.1 THP-1 media

RPMI 1640 medium (Sigma) supplemented with 10% FCS, 2 mmol/L L-glutamine, p-mercaptoethanol and 5% antibiotic/mycotic (10,000 units penicillin, 10mg streptomycin + 25mg amphotericin B/ml [Sigma, UK]) was used.

5.4.2 Establishing THP-1 cell culture

1ml aliquots of frozen THP-1 cells were quick-thawed in a 37°C water bath and immediately diluted in 15 ml of RT sterile PBS for a 10 min centrifugation step at 300 *x* g. The supernatant was discarded and the resulting cell pellet re-suspended in 15 ml RT medium for another 10 min centrifugation step at 300 *x* g. The supernatant was discarded and the cell pellet re-suspended in 10 ml medium to make up a cell concentration of ~$2x10^5$ cells / ml. The cell solution was transferred to a T-75 tissue culture flask and placed upright in a tissue culture incubator at 37°C (5% CO_2). 24 and 48 hours later, 10 ml of medium was added to the flask. At 72 hours, the flask was observed for turbidity (indication of cell growth) and 20 ml of fresh medium added to make a working volume of 50 ml. The flask was then tested for cell viability using Trypan Blue and a haemocytometer to assure >80% cell viability and maintained as described below.

5.4.3 Maintaining THP-1 cell culture

Cells were maintained every 2-3 days or until cell concentration reached >1 x 10^6 cells/ml. Flasks were taken out of the incubator while making sure not to disrupt the sediment (growing cells) and 20 ml of medium removed from the top down. 20 ml of fresh medium was then added to the flask, thereby re-suspending the cells, and a sample taken to assess cell number and viability. A specific amount of cell suspension (depending on cell concentration) was then removed and replaced with fresh medium to make a concentration of 2 x 10^5 cells/ml and the flask returned to the incubator.

5.5 Opsonophagocytosis using HL-60 and THP-1 cells

Opsonophagocytosis assays were performed using HL-60 neutrophil-like cells and THP-1 monocyte-like cells. Additional experiments using Fc fragments or

blocking antibodies to occupy or block Fc-receptors prior to phagocytosis assays were also performed.

5.5.1 Opsonisation

See General methods in chapter II.

5.5.2 Preparation of HL-60 or THP-1 cells

Two flasks of differentiated HL60 cells **or** two flasks of growing THP-1 cells were pooled and centrifuged at 300 *x* g for 10 minutes. In order to remove any residues of antibiotics in the media, cells were washed twice by centrifuging at 300 *x* g for 10 minutes, discarding the supernatant and re-suspending the cells in Hank's Balanced Salt Solution without Calcium or Magnesium ($HBSS^{-/-}$) to minimize adherence. Cells were finally re-suspended in Hank's Balanced Salt Solution with Calcium and Magnesium ($HBSS^{+/+}$) supplemented with 10% FBS at a cell concentration of 1 x 10^7 cells/ml and placed in an incubator at 37°C (5% CO_2) until use.

5.5.3 Phagocytosis

See General Methods: Chapter II, section 2.9.2.

5.5.4 Analysis

See General Methods: Chapter II, section 2.9.3.

5.6 HL-60 OPKA and Fcγ receptors

5.6.1 Fcγ receptor occupation

Fc-receptor occupation relies on the binding of the Fc portion of Immunoglobulin to unoccupied Fc-receptors on the surface on HL-60 cells thereby inhibiting any further interaction until the receptor is recycled or

freed. Fc fragments were generated and kindly donated by the Immunobiology laboratory at the CDC Atlanta. Polyclonal Immunoglobulin G was enzymatically digested using papain to create Fc and Fab fragments after which Fc fragments were used for OPK blocking assays (Figure 61).

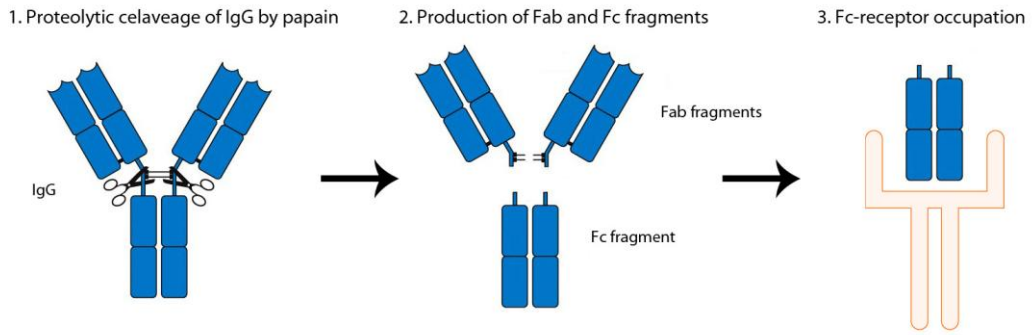


Figure 61 - Graphical representation of Fc receptor occupation method. (1) Whole IgG fragment is enzymatically digested (2) Fab fragments and Fc fragments are produced (3) Fc fragments naturally bind to available Fc-receptors thereby occupying them.

The Opsonophagocytosis assay with Fc receptor occupation was performed using the same method as in section 5.5 with one modification. Following the washing steps in (B.), neutrophils were incubated in 1.5 ml tubes containing 100 μg of polyclonal IgG Fc fragments / 10^6 cells or PBS alone for 20 minutes at 4°C. Following incubation, cells were centrifuged and resuspended in medium to a final cell concentration of 1 x 10^7 cells / ml. 20 μl of this cell suspension were then added to wells containing opsonized pneumococci, complement and P4 solution or DEPC-treated water.

5.6.2 Fcγ receptor blocking

Fc-receptor blocking relies on antibodies binding to receptors via the variable fragment of the antibody thereby preventing any further interaction with the receptor (
Figure 62). Anti-CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI) antibodies were purchased from Biolegend, USA.

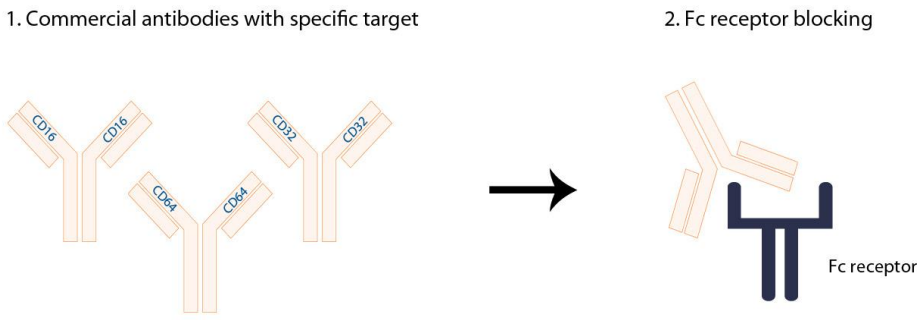


Figure 62 - Graphical representation of Fc receptor blocking method. (1) Commercial antibodies against several Fc-receptors (2) binding of antibody to active site of Fc-receptor thereby blocking further interaction.

The Opsonophagocytosis assay with Fc receptor blocking was performed using the same method as in 4.4.1 with one modification. Following the washing steps in 4.4.1 (B.), neutrophils were incubated in 1.5 ml tubes containing 10 µl of antibody (either CD16, CD32 and CD64 alone or all together) / 10^6 cells or PBS alone for 20 minutes at 4°C. Following incubation, cells were centrifuged and re-suspended in medium to a final cell concentration of 1 x 10^7 cells / ml. 20 µl of this cell suspension was then added to wells containing opsonized pneumococci, complement and P4 solution or DEPC-treated water as in section 4.4.1.

5.7 HL-60 Fcγ I, II and III expression

We measured peptide stimulation of HL-60 cells in the absence of immune complexes and complement in order to determine the effect of P4 on Fcγ receptor expression. This was compared to the receptor expression following an opsonophagocytosis assay.

5.7.1 Cell preparations

Cell suspensions from two differentiated flasks of HL60 cells were pooled and centrifuged at 300 *x* g for 10 minutes. The supernatant was discarded and the resulting cell pellet re-suspended in 20 ml sterile HBSS for another washing step at 300 *x* g for 10 minutes. The cell pellet was then re-suspended in 10 ml RPMI medium supplemented with 20% FBS and samples taken from the cell suspension for counting and testing cell viability using a haemocytometer. Cell viability of >90% was used. The cell suspension was then diluted and aliquoted into 1.5 ml tubes at a concentration of 5×10^5 cells / 400 μl. The tubes were divided into two groups, un-stimulated and P4-stimulated (n=10 per group).

5.7.2 Cell stimulation and OPKA

Test wells received 100 μl of P4 solution (1 mg of peptide / ml DEPC treated water) whereas the un-stimulated wells received 100 μl of DEPC treated water alone and were placed in an incubator at 37°C (5% CO_2). At 15 and 60 minutes following the addition of peptide or control, tubes were removed from the incubator, centrifuged in a bench top centrifuge at 300 *x* g for 10 minutes and the supernatant discarded. The resulting cell pellet was then taken for antibody staining. In addition, differentiated HL60 cells were allowed to phagocytose pneumococci for 60 minutes in the presence of antibody and complement with and without P4 stimulation. Following

phagocytosis, cells were centrifuged at 300 *x* g for 10 minutes, the supernatant discarded and the resulting cell pellet stained as below.

5.7.3 Cell staining and acquisition

APC-conjugated primary and isotype antibodies were purchased from Biolegend (USA) and used for all staining. Cells were stained using anti-FcγR I (CD64), II (CD32) and III (CD16) antibodies or their corresponding isotype control antibodies (IgG1 for CD16 and CD64, IgG2b for CD32). Large volumes of each diluted antibody (ratio of 1:4 in PBS) were prepared on the day of experiment. The cell pellet from 5.7.3.2 was stained using 50 µl of diluted antibodies at 4°C for 20 minutes after which the tubes were centrifuged and excess antibody removed by discarding the supernatant and re-suspending the cell pellet in 500 µl PBS for another washing step. The cell pellet was then finally re-suspended in 500 µl PBS and acquired using a BD LSR II Flow Cytometer and the supplied FACS DIVA acquisition software. Results from three separate assay days were pooled and presented as percentage increase in mean fluorescence intensity (MFI) compared to MFI of isotype controls.

5.8 Statistical analysis

All statistical tests were performed using GraphPad Prism 5.0 for Macintosh (TreeStar Inc., USA). Different statistical tests were used depending on the data set and these are described below and defined in the figure legends. Each *in vitro* assay consisted of > three technical replicates performed on separate assay days. Data from separate assay days were pooled and shown as means ± standard deviation. Distribution of data was assessed using D'Agostino and Pearson omnibus normality test. For normally distributed data, an Unpaired T-test was used to test for significance. For non-normally distributed data, a Mann-Whitney unpaired T-test was used to test for significance. Differences between data were designated significant if $p<0.05$.

C. Results

5.9 HL-60 opsonophagocytosis killing assay

Using the HL-60 opsonophagocytosis assay we tested if P4 treatment of neutrophil-like cells would lead to greater internalization of opsonized pneumococci as compared to the un-stimulated controls independent of capsule. Specifically, we tested whether P4 mediated phagocytosis was dependent on the presence or absence of capsule. All bacteria were opsonized using pathogen specific pooled human immunoglobulin G (IVIG).

5.9.1 Capsulated and unencapsulated D39 pneumococci

Following peptide treatment, HL-60 cells were significantly better at phagocytosing both capsulated (control 14% vs. treated 73%, $p<0.0001$) and unencapsulated (control 18% vs. treated 68%, $p<0.0001$) strains of D39 as compared to the untreated control cells (Figure 63, n=3 per strain).

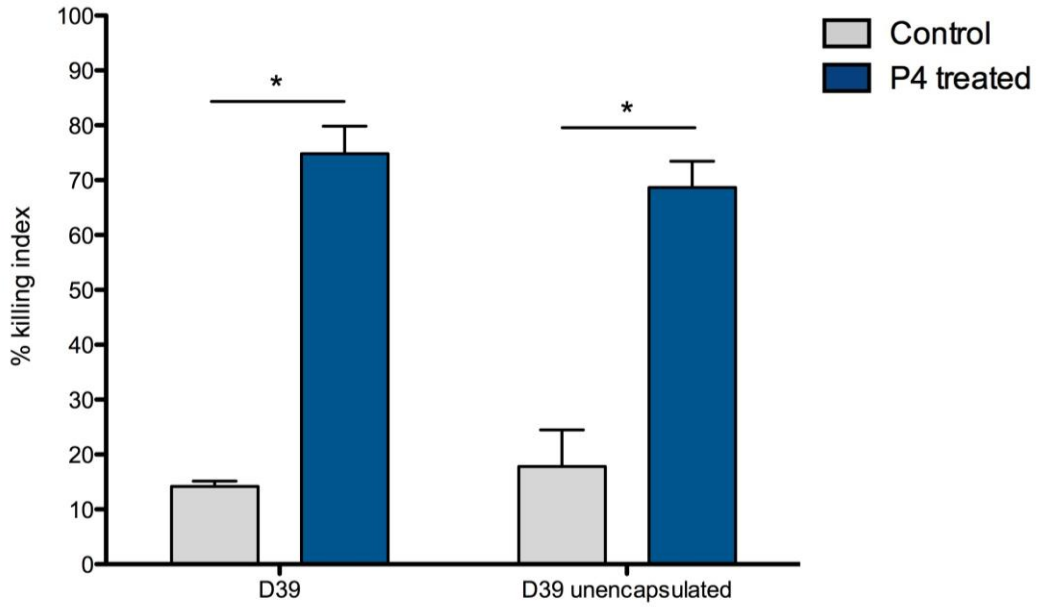


Figure 63 – Opsonophagocytosis using differentiated HL-60 cells and IVIG-opsonized capsulated or unencapsulated D39 (Serotype 2) pneumococci (n=3 per serotype). (*) denotes statistical significance using a paired t-test where p<0.001.

5.9.2 Capsulated and unencapsulated TIGR4 pneumococci

In addition to D39, we tested if HL-60 cells would be better at phagocytosing another pneumococcal laboratory strain, TIGR4 (Serotype 4), and the unencapsulated TIGR4 mutant following peptide stimulation (Figure 64, n=3 per strain). Peptide stimulation significantly increased the opsonophagocytosis of TIGR4 (control 12% vs. 58%, $p<0.0001$) and unencapsulated TIGR4 (control 34% vs. treated 59%, $p>0.05$) by HL-60 cells. The unencapsulated strain of TIGR4 was more susceptible to opsonophagocytosis by control cells compared to its capsulated parent strain (control capsulated 12% vs. control unencapsulated 34%, $p<0.05$) but phagocytosis of capsulated and unencapsulated TIGR4 remained similar in peptide treated cells (treated capsulated 58% vs. treated unencapsulated 59%, $p>0.05$).

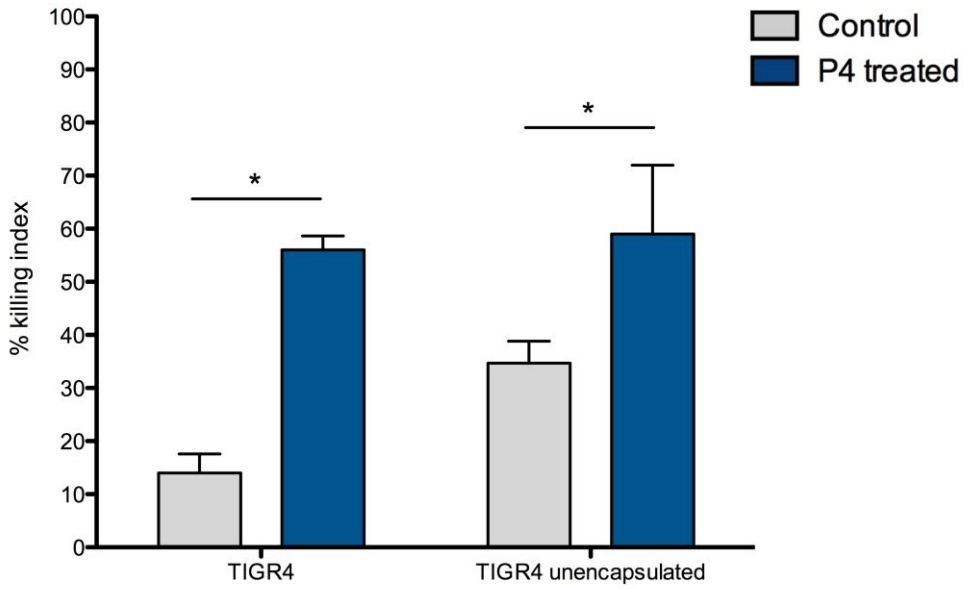


Figure 64 - Opsonophagocytosis using differentiated HL-60 cells and IVIG-opsonized capsulated or unencapsulated TIGR4 (Serotype 4) pneumococci (n=3 per serotype). (*) denotes statistical significance using a paired t-test where p<0.0001.

5.9.3 Clinical strains of *S. pneumoniae*

As D39 and TIGR4 are heavily passaged laboratory strains of pneumococci, we tested whether peptide treatment of HL-60 cells would also be effective at increasing the phagocytosis of clinically relevant strains of pneumococci (Figure 65). ST14, ST6B and ST19F were isolated from clinical samples obtained from patients with invasive pneumococcal disease (n=3 per serotype). Following peptide stimulation, HL-60 cells were significantly better at phagocytosing IVIG opsonized ST14 (control 10% vs. treated 72%, $p<0.0001$), ST6B (control 14% vs. treated 82%, $p<0.0001$) and ST19F (control 18% vs. treated 68%, $p<0.0001$). No statistical difference in the rate of phagocytosis between serotypes in stimulated and non-stimulated cells was detected ($p>0.05$).

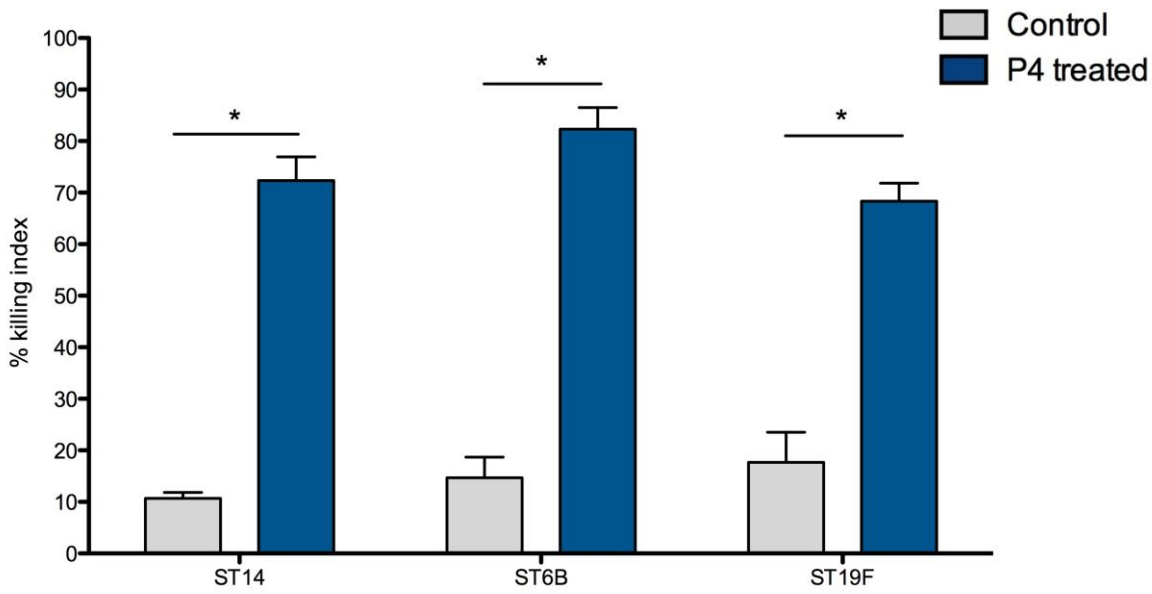


Figure 65 - Opsonophagocytosis using differentiated HL-60 cells and IVIG-opsonized ST14, ST6B or ST19F strains of clinically isolated pneumococci (n=3 per serotype). (*) denotes statistical significance using a paired t-test where p<0.0001.

5.10 Fcγ receptors, HL-60 & OPKA

These experiments assessed whether blocking Fcγ receptors would influence the effect of peptide stimulation using HL-60 cells in an opsonophagocytosis killing assay. Two strategies were used: Occupying receptors by adding IgG derived Fc fragments and blocking receptors by adding FcγR specific blocking antibodies.

5.10.1 Occupying Fcγ receptors with Fc fragments

HL-60 cells were stimulated with P4 peptide after which cells were incubated with an excess of IgG Fc fragments to occupy receptors. In control reactions (n=3), where no receptors were occupied, cells treated with P4 peptide were significantly better at phagocytosing opsonized D39 pneumococci than the untreated control (control 21% vs. treated 68%, p=0.001). No difference between stimulated and non-stimulated cells was detected where Fcγ receptors were occupied prior to phagocytosis (control 19% vs. treated 12%, p>0.05). Results from these assays are shown in Figure 66.

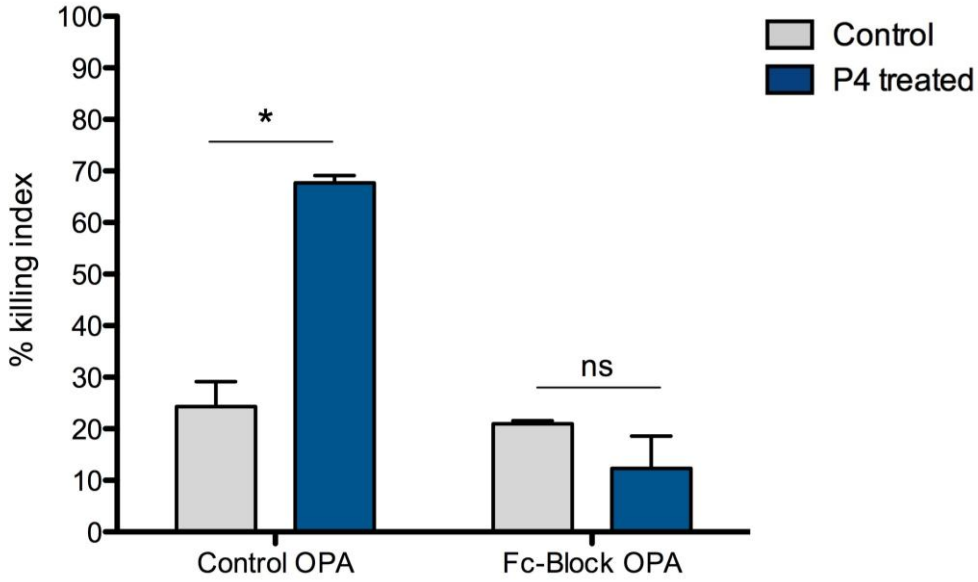


Figure 66 – Opsonophagocytosis with and without Fcγ-receptor occupation using differentiated HL-60 cells and IVIG-opsonized TIGR4 strains of clinically isolated pneumococci (n=3 per group). (*) denotes statistical significance using a paired t-test where p<0.0001.

5.10.2 Blocking Fcγ receptors with antibodies

HL-60 cells were stimulated and incubated with either anti-CD16, CD32, CD64 or a combination of all three antibodies in order to block specific Fcγ receptors prior to phagocytosis (n=3 per antibody). In addition, control OPKA reactions were also performed (n=3) without any blocking. The level of phagocytosis was significantly increased following P4 stimulation in cells that received no antibody blocking (control 20% vs. treated 81%, p=0.003). The results from these experiments are shown in Figure 67.

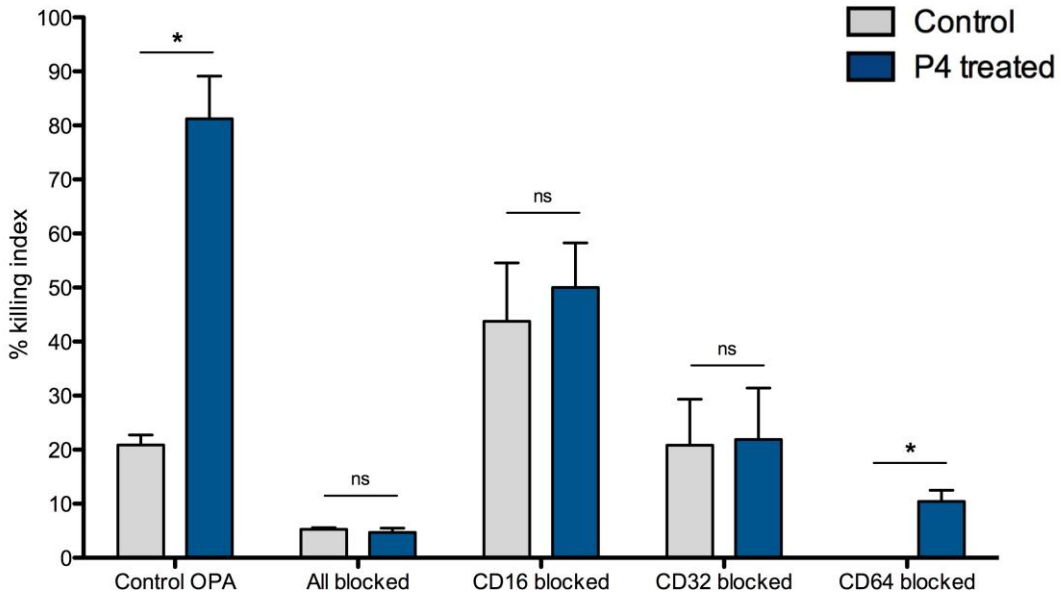


Figure 67 – Opsonophagocytosis assay with and without Fcγ-receptor blocking using differentiated HL-60 cells and IVIG-opsonized D39 strains of pneumococci (n=3 per group). Cells were incubated with antibodies 20 minutes prior to the phagocytosis assay. "Control OPA" are reactions without any blocking, "All blocked" reactions with all three antibodies added, "CD16 blocked" with the addition of anti-CD16 antibody, "CD32 blocked" with the addition of anti-CD32 antibody and "CD64 blocked" with the addition of anti-CD64 antibody. (*) denotes statistical significance using a paired t-test where p<0.0001.

Stimulated and un-stimulated HL-60 cells incubated with all three antibodies prior to phagocytosis took up significantly less bacteria than in the control group (no blocking 20% vs. blocking 6%, $p<0.05$) and no significant difference was detected between peptide treated and control cells in this instance (control all blocked 6% vs. treated all blocked 6%, $p>0.1$). HL-60 cells incubated with anti-CD16 antibodies prior to phagocytosis did not increase the rate of phagocytosis in response to peptide treatment (control CD16 blocked 43% vs. treated CD16 blocked 49%, $p>0.05$). Phagocytosis was significantly higher in non-stimulated cells incubated with anti-CD16 antibodies than in the non-stimulated cells with no blocking (no blocking control 21% vs. CD16 blocked control 42%, $p=0.04$). Similarly, peptide treatment of HL-60 cells did not significantly change phagocytosis levels following CD32 blocking (control CD32 blocked 20% vs. treated CD32 blocked 21%, $p>0.05$). Finally, there was no detectable phagocytosis in control cells incubated with anti-CD64 antibodies whereas phagocytosis was detected, and significantly higher, following peptide stimulation in CD64 blocked cells (control CD64 blocked 0% vs. treated CD64 blocked 11%, $p=0.04$).

5.11 HL60 Fcγ Flow Cytometry

We tested whether P4 stimulation of HL-60 cells would lead to Fc-receptor modulation in the absence of bacteria. Cells were stimulated with P4 peptide alone and stained with CD16 (FcγRIII), CD32 (FcγRII) or CD64 (FcγRI) antibodies 15 and 60 minutes later. In addition, it was tested whether Fc-receptor expression was modulated in an HL-60 opsonophagocytosis assay in response to peptide treatment.

5.11.1 HL-60 FcγRIII expression

CD16 expression at 60 minutes in peptide treated cells in the absence of bacteria was significantly higher than in control cells (control 120 MFI vs. treated 179 MFI, p=0.03). No statistical difference in CD16 expression was detected at 15 minutes following stimulation (control 125 MFI vs. treated 112 MFI, p>0.05) (Figure 68).

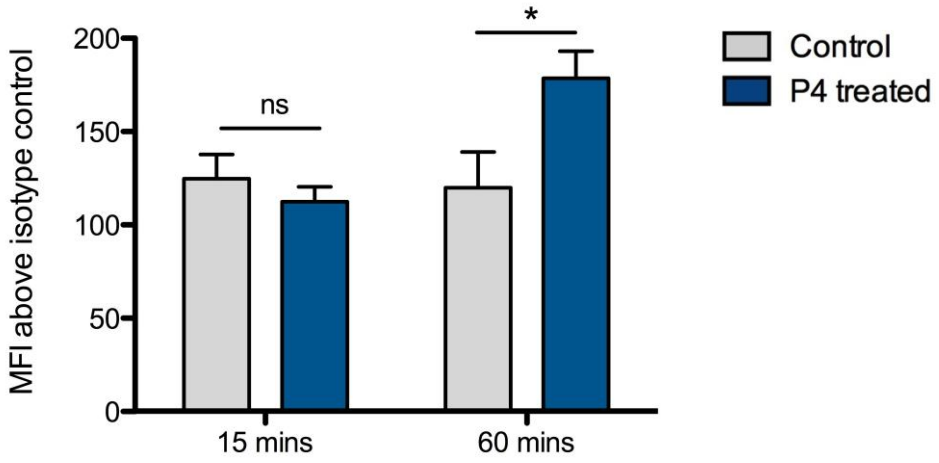


Figure 68 – Mean fluorescent intensity (MFI) above isotype control of CD16. HL-60 cells were stained with anti-CD16 antibodies at 15 and 60 minutes following incubation with either control or peptide solution (n=3). (*) denotes statistical significance using a paired t-test where p<0.05.

5.11.2 HL-60 FcγRII expression

CD32 expression remained similar in both control and peptide treated cells at either 15 (control 3128 MFI vs. treated 2840 MFI, p>0.05) or 60 (control 3211 MFI vs. treated 3356 MFI, p>0.05) minutes where no statistical difference was detected between groups (Figure 69).

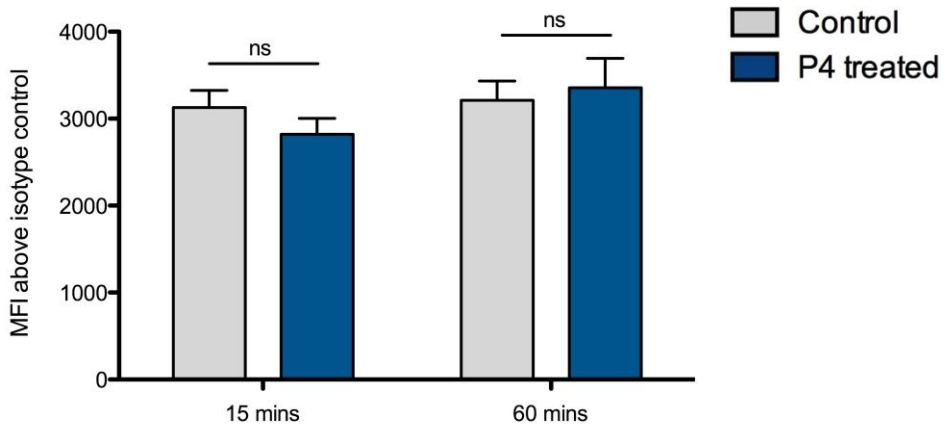


Figure 69 - Mean fluorescent intensity (MFI) above isotype control of CD32. HL-60 cells were stained with anti-CD32 antibodies at 15 and 60 minutes following incubation with either control or peptide solution (n=3). (*) denotes statistical significance using a paired t-test where p<0.05.

5.11.3 HL-60 FcγRI expression

In comparison, CD64 expression was significantly lowered at 60 minutes in HL-60 cells treated with P4 peptide (control 4078 MFI vs. treated 3298 MFI, p=0.03) but no detectable difference was measured at 15 minutes following stimulation (control 4151 MFI vs. treated 3772 MFI, p>0.05) (Figure 70).

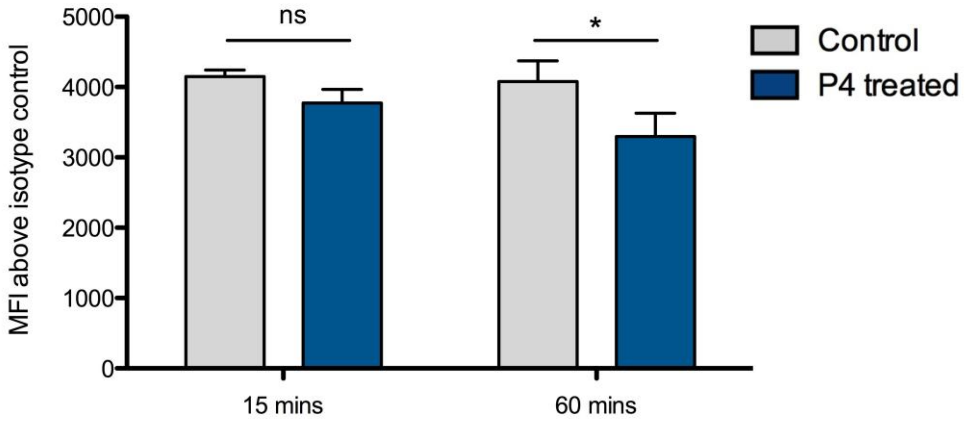


Figure 70 - Mean fluorescent intensity (MFI) above isotype control of CD64. HL-60 cells were stained with anti-CD64 antibodies at 15 and 60 minutes following incubation with either control or peptide solution (n=3). (*) denotes statistical significance using a paired t-test where p<0.05.

5.11.4 HL-60 FcγR expression following OPKA

Finally, it was measured whether P4 treatment during phagocytosis of opsonized pneumococci by HL-60 cells would alter the expression of CD16, CD32 or CD64. Following an opsonophagocytosis assay, HL-60 cells were stained using anti-FcR I, II & III antibodies. CD16 expression remained similar between stimulated and non-stimulated control (control 533 MFI vs. treated 542 MFI, p>0.05). Both CD32 and CD64 expression was modulated, however, where CD32 expression increased (control 5742 MFI vs. treated 6001 MFI, p=0.03) and CD64 expression decreased (control 15077 MFI vs. treated 14209 MFI, p=0.02) in response to peptide treatment (Figure 71).

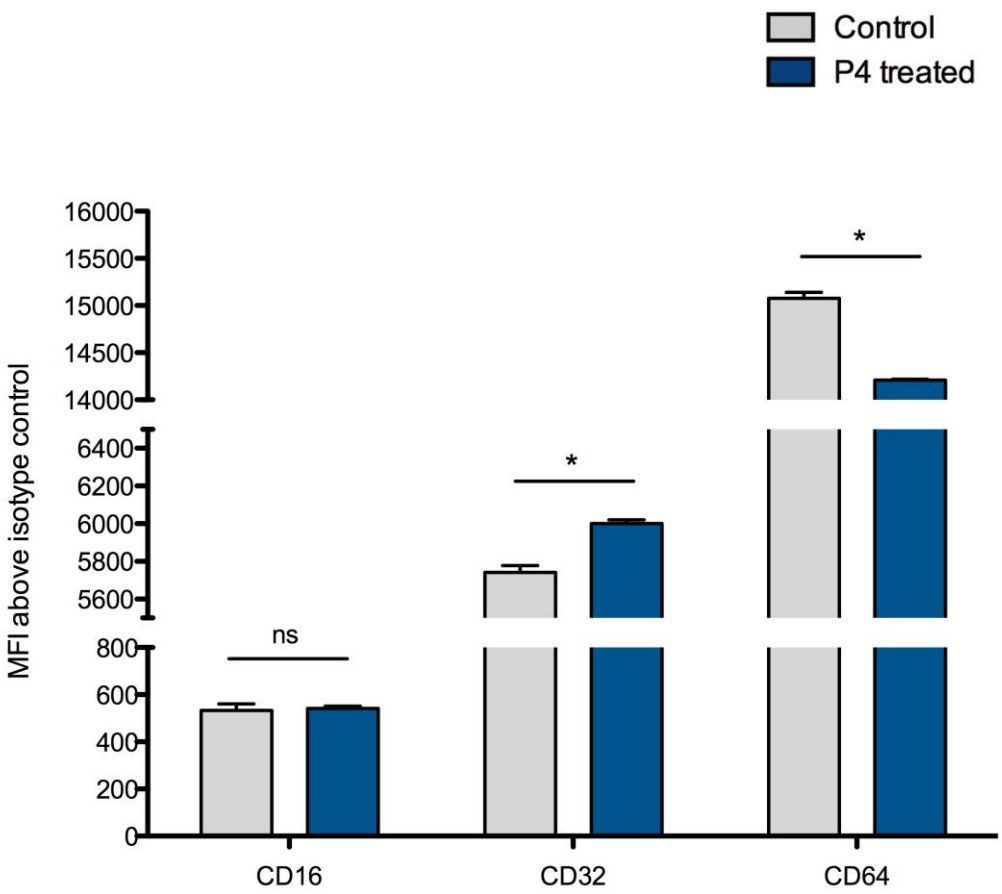


Figure 71 - Mean fluorescent intensity (MFI) above isotype control of CD16, CD32 or CD64. Stimulated or non-stimulated HL-60 cells (n=3) were stained following a 45 min opsonophagocytosis assay using IVIG-opsonized D39 pneumococci. (*) denotes statistical significance using a paired t-test where $p<0.05$.

5.12 Opsonophagocytosis using THP-1 monocytes

Using undifferentiated THP-1 cells and the standardized HL-60 opsonophagocytosis assay, it was tested whether peptide treatment of monocyte-like cells would up-regulate the phagocytosis of IVIG-opsonized D39 pneumococci (Figure 72, n=3). 45 minutes following opsonophagocytosis, there was no significant difference in the rate of phagocytosis between stimulated and non-stimulated THP-1 cells (control 56% vs. treated 63%, p=0.054).

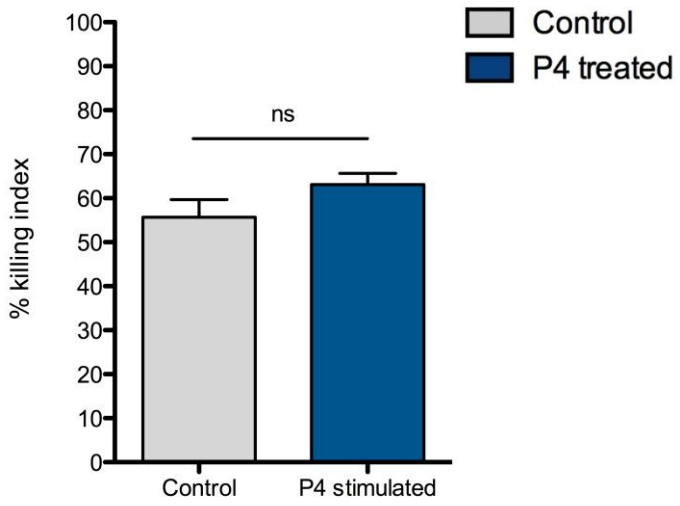


Figure 72 - Opsonophagocytosis using undifferentiated THP-1 monocyte like cells and IVIG-opsonized D39 (Serotype 2) pneumococci (n=3). (*) denotes statistical significance using a paired t-test where p<0.001.

D. Discussion

5.13 Discussion

In this chapter, the HL-60 cell line was used to determine the ability of P4 to upregulate the phagocytic killing of a range of IVIG opsonised pneumococcal serotypes. In addition, using flow cytometry, the effect of P4 to modulate Fcγ receptor expression was assessed. Specifically, FcγRI, FcγRII and FcγRIII expression was measured in the presence and absence of immunecomplexes and P4.

5.13.1 Opsonophagocytosis assay

The opsonophagocytosis killing assay (OPKA) was used to measure the efficacy of pneumococcal killing by P4 stimulated HL-60 cells. Two laboratory strains, ST2 (D39) and ST4 (TIGR4), as well as their unencapsulated mutants showed significantly enhanced susceptibility to phagocytic killing by P4 stimulated cells. Unencapsulated strains were more susceptible to killing than capsulated strains in the absence of P4. This is not surprising as the capsule has long been shown to inhibit phagocytosis [331]. In the presence of P4, however, phagocytic killing of unencapsulated strains was similar to capsulated strains suggesting that P4 up-regulates phagocytic killing independent of the presence of capsule. This is most likely facilitated through the use of IVIG, as serum typically contains antibodies against membrane proteins as well as pneumococcal capsule [332, 333]. In addition, P4 stimulated cells also killed pneumococcal strains isolated from patients with invasive pneumococcal disease at significantly higher rates. These results are important as the invasiveness of pneumococcal serotypes differs between geographical locations [334], and serotype replacement and switching (previously non-invasive strains becoming invasive) has been reported as a result of widespread vaccination [335, 336]. These data strengthen the case of

using IVIG as an opsonin for passive immunotherapy and further demonstrate the ability of P4 to upregulate the phagocytic killing of *S. pneumoniae*.

In addition to HL-60 cells, the OPK assay was adopted to assess the ability of the THP-1 monocytic cell line to up-regulate phagocytic killing in response to peptide stimulation. No differences in phagocytic killing were detected in stimulated and control cells in this chapter. THP-1 cell lines are mostly used for their ability to differentiate into macrophages and dendritic cells [337] which lead to better responses to stimuli than undifferentiated THP-1 cells [338]. Some studies have looked at undifferentiated THP-1 cell phagocytosis but the outcome was measured using fluorescent beads and flow cytometry and not viable bacteria [339]. While it is possible that undifferentiated THP-1 cells do not respond to peptide treatment, these data cannot rule out the ability of P4 to upregulate monocyte phagocytosis, particularly as monocyte migration played key role in API during *in vivo* infection studies. Further studies, using peripheral blood derived monocytes as well as other monocyte cells lines (i.e. HL-60 cells differentiated into monocytes), could help understand the role of monocyte phagocytosis in API.

5.13.2 Fcγ receptor function and expression

By using two techniques, receptor occupation and receptor blocking, the role of FcγR in P4 mediated opsonophagocytosis was assessed. Exposing whole IgG or Fcγ fragments to granulocytes leads to the occupation of Fcγ receptors [340], which are then unable to ligate opsonised pathogens until the receptor is internalized and recycled to the cell membrane [341]. The occupation of Fcγ receptors did not affect the ability of control cells to kill opsonised pneumococci. In contrast, FcγR occupation of P4 stimulated cells prevented the augmented killing of pneumococci seen in the absence of FcγR occupation. It is likely that the effect of P4 mediated FcγR up-regulation and

priming of cells was reversed through Fc fragment occupation. Ligation and internalization of Fc fragment bound FcγR would not stimulate TLR or NOD like receptors, which are important for downstream activation signaling and may be necessary for P4 stimulation to be effective [342, 343]. Alternatively, it is possible that Fc fragments ligated inhibitory FcγR leading to the reversal of P4 mediated activation of HL-60 cells [344]. Measuring Fc fragment internalization in P4 stimulated and control cells would help understand the role of FcγR occupation in these assays.

Blocking Fcγ receptors de-activates the receptor's function by preventing ligation with IgG [345, 346]. Blocking specific Fcγ receptors, namely FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16), led to a range of phagocytic killing responses in this chapter. When all Fcγ receptors were blocked, phagocytic killing of stimulated and control cells was significantly reduced compared to control assays. Blocking of FcγRIII led to enhanced killing by both stimulated and control cells while blocking of FcγRII resulted in phagocytosis similar to control cells in the absence of receptor blocking. There was no detectable phagocytic killing following FcγRI blocking in control cells, but P4 stimulation resulted in some phagocytic killing in this instance. In the absence of infection, peptide stimulation increased expression of FcγRIII, decreased expression of FcγRI but did not alter expression of FcγRII.

The use of anti-FcγRIII antibodies may have resulted in activation of receptor as well as preventing further Fc-FcγRIII interactions. As FcγRIII activation leads to increased neutrophil phagocytosis through the generation of phosphatidylinositol-3 kinase (PI-3K) [347, 348], it is possible that the enhanced phagocytic killing seen by both control and P4 stimulated cells is mediated by FcγRIII activation. In the absence of infection P4 upregulated the expression of FcγRIII. It is therefore likely that P4 mediated phagocytic killing is enhanced through the upregulation of FcγRIII leading to internalization of immune complexes and subsequent enhanced PI-3K activity. Assays

measuring FcγRIII and PI-3K activity during P4 mediated OPK assays would assist in confirming these observations.

The affinity of specific FcγR to IgG may also have contributed to the observed differences. FcγRIII, for example, is a low-affinity IgG receptor [349] meaning that its absence would affect immune complex ligation to a lesser extent than the high affinity FcγRI receptor. This is supported by data in this chapter where blocking of the high affinity FcγRI receptor resulted in complete cessation of phagocytic killing in control cells. In the absence of infection, P4 stimulation led to a decrease of FcγRI. High expression of FcγRI is correlated with activation of cells [350] and used as a measure of the severity of infection during sepsis [351] while decreased expression is observed following hydrocortisone administration during sepsis [352].

CHAPTER VI.

Ex vivo peptide studies

A. Introduction

6.1 Introduction

In previous chapters, P4 peptide treatment of pneumococcus-infected lungs prevented the onset of sepsis and subsequent host mortality. In addition, P4 upregulated the phagocytic killing of opsonised pneumococci by murine alveolar macrophages and the HL-60 neutrophil like cell line. This chapter assessed whether P4 treatment of human phagocytes *ex vivo* would lead to enhanced phagocytic function with no or limited generalized inflammation.

6.1.1 Opsonophagocytosis killing assays

Similar to HL60 phagocytic killing assays in chapter V, the CDC opsonophagocytosis killing assay (OPKA) was used to determine the ability of alveolar macrophages and neutrophils to kill IVIG opsonised pneumococci following peptide stimulation. Healthy adult volunteers from two populations (Liverpool, UK and Blantyre, Malawi) with different experience of pneumococcal disease were invited to consent to bronchoscopy and lavage in order to isolate alveolar macrophages. Healthy adult volunteers from Liverpool (UK) were invited to consent to venesection to isolate peripheral blood neutrophils.

6.1.2 Intracellular oxidation

The effect of P4 treatment on human alveolar intracellular oxidation was assessed using DCFH oxidation assays. Specifically, it was tested whether P4 mediated OPK assays would lead to increased intracellular oxidation. In addition, intracellular oxidation by P4 stimulated alveolar macrophages in the

absence of infection was measured and compared to LPS stimulated alveolar macrophages.

6.1.3 Cell surface markers and cytokine secretions following OPKA

Phagocytes are activated and alter their inflammatory mediators, including cytokine secretion and expression of markers of inflammation, following phagocytosis of pathogens. To determine whether P4 would modulate inflammatory mediators on alveolar macrophages following OPK assays, cell surface markers and cytokine secretions of control and P4 stimulated cells were measured using flow cytometry.

B. Materials & Methods

6.2 Volunteer recruitment for blood and BAL

Volunteers were invited to give informed consent to a study in Liverpool (UK) and Blantyre (Malawi) involving venesection, bronchoscopy and lavage (BAL). Inclusion and exclusion criteria were identical for recruitment in both countries.

6.2.1 Inclusion criteria

i. Adults aged 18-65 years.
   Age chosen to minimise risk of bronchoscopy.
ii. Fluent spoken English.
   To ensure a comprehensive understanding of the research project and their proposed involvement.

6.2.2 Exclusion criteria

Individuals who are at increased risk during bronchoscopy. Patients in whom clinical features might affect the interpretation on the results will be excluded. Exclusion criteria are therefore:

i. Asthma or pre-existing lung disease requiring regular immunoregulatory treatment or having any recent ill health
ii. Cigarette smoking of greater than 10 pack years (20 cigarettes per day for 10 years)
iii. Chronic illness
iv. Pregnancy

6.2.3 Ethics

Ethical approval was obtained from the National Health Service Research Ethics Committee, Sefton, Liverpool (11/NW/0011 - sponsored by the Royal Liverpool and Broadgreen University Hospitals Trust) and the Malawi College of Medicine Ethics committee (COMREC, P.03/11/1063 - sponsored by Liverpool School of Tropical Medicine).

6.3 Alveolar macrophage and peripheral blood neutrophil isolation

6.3.1 Cell culture medium

For all alveolar macrophage and peripheral blood neutrophil cell culture in this chapter, RPMI 1640 medium (2mM L-glutamine (Sigma, UK), 10% fetal bovine serum (Invitrogen, UK), with or without antibiotics (penicillin 40U/ml, streptomycin 40μg/ml, neomycin 80μg/ml) and fungicide (amphotericin B 0.5μg/ml) (Sigma, UK)) was used.

6.3.1 Alveolar macrophage isolation

Macrophages were extracted from lungs by gently placing a bronchoscope in the middle-lobe subsegmental bronchus and lavaging with 200 ml of warm sterile saline [353]. BAL (bronchoalveolar lavage) fluid collected during bronchoscopy (variation of 30ml – 140 ml) was strained through sterile gauze into 50ml Falcon centrifuge tubes to remove any large debris and mucous. The tubes were centrifuged at 300 *x* g for 10 minutes, the supernatant stored and the cells re-suspended in 15 ml media with antibiotics. A sample was taken from the cell suspension to enumerate number and viability of cells. Alveolar macrophages were plated onto 96-well culture plates (Greiner Bio, UK) at a concentration of 1 x 10^5 cells / well and incubated at 37°C (5% CO_2)

for three hours in antibiotic supplemented media to allow for macrophages to adhere. Following incubation, non-adherent cells were carefully removed by washing wells twice with PBS and fresh RPMI media + 10% FBS without antibiotics was added to the wells. Cells were then kept in an incubator at 37°C (5% CO_2) for > 2 hours until use.

6.3.2 Peripheral blood neutrophil isolation

50 ml peripheral blood was obtained from healthy volunteers and collected in heparin containing tubes (10 units / ml, Sigma). Neutrophils from peripheral blood were isolated using gradient density centrifugation, which relies on components of blood being separated by their ability to pass through differing densities of solutions (Histopaque). First, 12 ml of Histopaque 1119 (Sigma) was added to a 50ml tube followed by carefully layering 12 ml of Histopaque 1077 (Sigma) on top. Whole blood, diluted 1:2 with sterile PBS, was then carefully layered on top of both Histopaque solutions (Figure 73A). The tube was then centrifuged at RT for 30 minutes at 700 *x* g to allow for separation to occur. Following centrifugation, two clear white bands were seen containing the monocyte and neutrophil populations (Figure 73B).

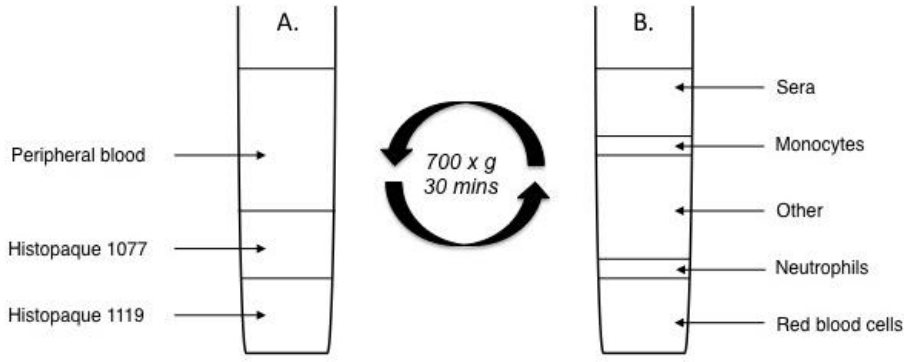


Figure 73 - Density gradient separation of peripheral blood leukocytes. (A.) Peripheral blood is layered on top of two histopaque solutions with differing densities. Following centrifugation, (B.) monocytes and neutrophils are separated into two distinct bands.

The layers above the neutrophil population were carefully removed using a Pasteur pipette and the neutrophil layer then transferred to a fresh 50ml tube, taking care not to carry over red blood cells. The tube containing neutrophils was brought up to 50 ml using PBS and washed by centrifuging the tube at 300 *x* g for 10 minutes after which the supernatant was discarded and the cell pellet re-suspended in PBS for another washing step. Finally, the cell pellet was re-suspended in 10ml RPMI 1640 media and the cell number and viability assessed using Trypan Blue and a haemocytometer. Freshly isolated cells were used within two hours of density separation to ensure maximum viability.

6.4 Alveolar macrophage OPKA

6.4.1 Opsonisation

Pneumococcal strains were opsonised using IVIG as described in chapter II.

6.4.2 Phagocytosis

IVIG-opsonised pneumococci were used to infect alveolar macrophages as described in chapter II.

6.4.3 Analysis

A killing index was established as described in chapter II.

6.5 Fcγ receptor occupation

Fcγ-receptors are frequently occupied using pooled IgG prior to Flow Cytometry assays to avoid non-specific binding during staining. This technique was adapted to occupy Fcγ-receptors following peptide stimulation, and prior to phagocytosis, in order to assess whether FcγR occupation influences the ability of P4 to up-regulate phagocytosis.

6.5.1 Opsonisation

Pneumococcal strains were opsonised using IVIG as described in chapter II.

6.5.2 Fcγ-receptor occupation and phagocytosis

Adhered alveolar macrophages were pre-treated with DEPC treated water or P4 solution for 20 minutes followed by a 20 minute incubation with 100 μl of 1 mg/ml human IgG (Sigma I4506). Following incubation, wells were washed twice with pre-warmed (37°C) PBS to remove unbound IgG. The phagocytosis assay was then performed without additional P4 solution (DEPC treated water for control and treated wells).

6.5.3 Analysis

A killing index was established as described in chapter II.

6.6 Intracellular killing of pneumococci

The adhesion and invasion assay was originally developed to test the ability of bacteria to adhere and invade cultured epithelial cells [354]. It was used to determine whether P4 mediated opsonophagocytosis also mediated intracellular killing (Figure 74).

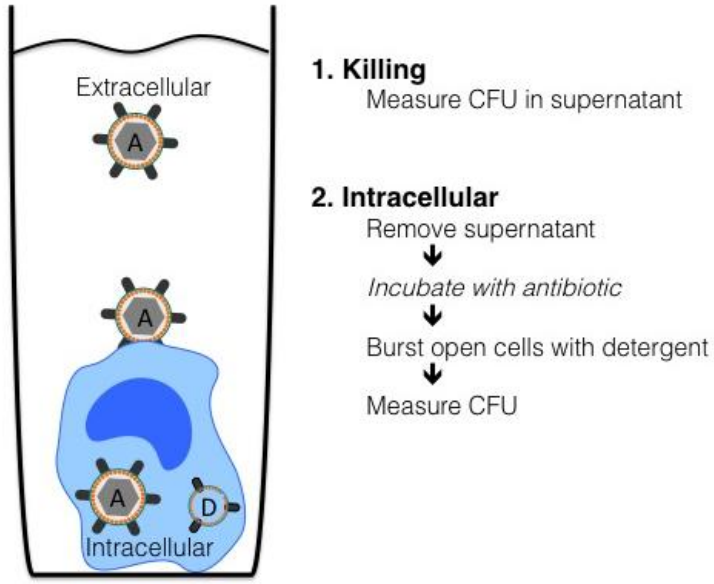


Figure 74 - Schematic diagram of the opsonophagocytosis assay and how to determine bacterial killing and presence intracellularly. Alveolar macrophages are incubated with opsonized pneumococci and complement for two hours with and without peptide stimulation in duplicate wells. (1) Uptake of pneumococci is measured by sampling the supernatant. (2) Intracellular killing is measured by removing the supernatant, incubating wells with bactericidal antibiotics that do not penetrate cells to remove adhered pneumococci. Cells are then burst open using a detergeant and viable intracellular pneumococci cultured. A=alive bacteria, D=dead bacteria.

6.6.1 Opsonisation

Pneumococcal strains were opsonised using IVIG as described in chapter II.

6.6.2 Phagocytosis

IVIG-opsonised pneumococci were used to infect alveolar macrophages as described in chapter II.

6.6.3 Intracellular pneumococci and killing

Following OPKA, a sample from the supernatant was taken for Miles & Misra enumeration. The supernatant was then removed and wells gently washed 5 x with pre-warmed (37°C) sterile PBS. Once the supernatant was washed off, 200 µl of antibiotic solution (100 µg ampicillin/200 µl) was added to wells for a 2 hour incubation at 37°C (5% CO_2). This killed any adherent bacteria while not affecting internalized pneumococci. Following incubation, wells were washed gently 5 x using sterile pre-warmed (37°C) PBS to remove traces of antibiotic and then incubated with 1% saponin solution to burst cells and release intracellular pneumococci.

6.6.4 Analysis

A killing index was established as in chapter II. Percentage internalized pneumococci was calculated by comparing the number of CFU recovered from the intracellular compartment and relating it to the original dose (opsonized bacteria in the presence of complement added to the reaction).

$$\%\, Internalized\ bacteria = \frac{CFU\ internalized}{CFU\ in\ Dose} \times 100$$

6.7 Neutrophil OPKA

6.7.1. Opsonisation

Pneumococcal strains were opsonised using IVIG as described in chapter II.

6.7.2 Phagocytosis

IVIG-opsonised pneumococci were used to infect alveolar macrophages as described in chapter II.

6.7.3 Analysis

A killing index was established as described in chapter II.

6.8 Cytokines following OPKA

In addition to phagocytosis, alveolar macrophages orchestrate immune responses by secreting cytokines following stimulation. Excessive cytokine secretion, however, is a hallmark of inflammation. We measured cytokine secretion following P4–mediated opsonophagocytosis.

6.8.1 Cytokine collection

Supernatants from the OPKAs were collected and stored at -80°C. Using the BD Cytokine Bead Array kits for soluble proteins (BD-CBA Flex Sets, Becton Dickinson, UK) we determined levels of MIP-1α, MIP-1β, MCP-1, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α and RANTES (CCL5) in the supernatants. Figure 75 gives an overview of the method used.

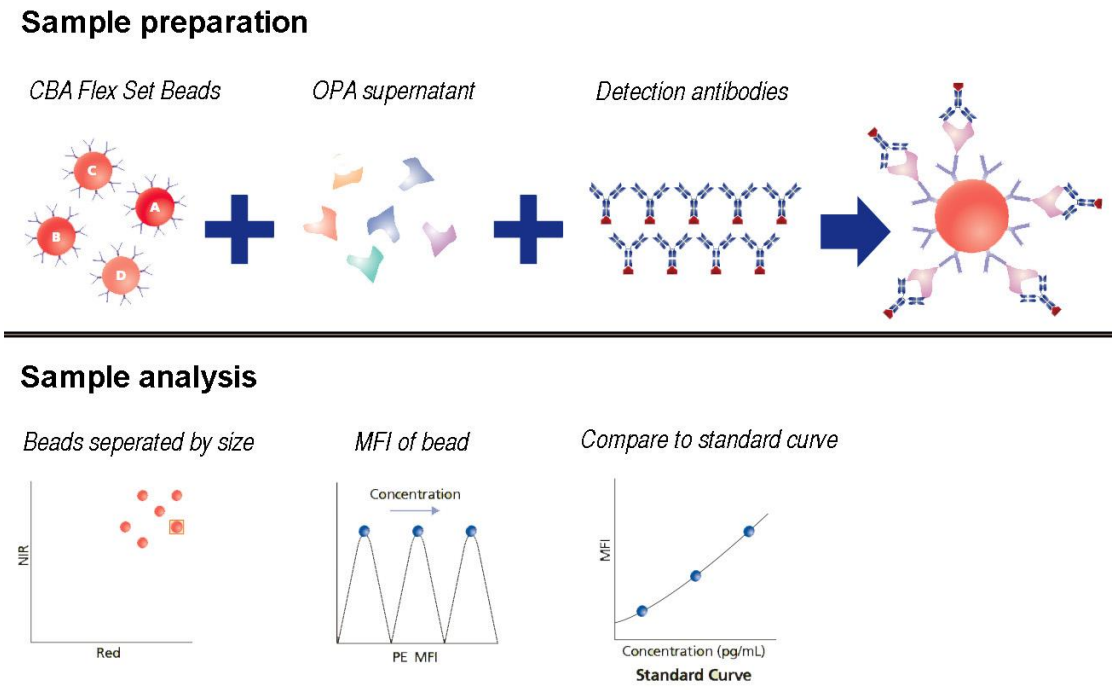


Figure 75 - Diagram of multiplex cytokine detection in opsonophagocytosis supernatants using CBA FlexSet cytokine detection kit. Cytokine specific beads of varying size and fluorescence are incubated with cytokines present in OPKA supernatant. The beads are washed and then further incubated with a cocktail of cytokine specific antibodies conjugated to a fluorescent (PE) reporter. The Antibody-Cytokine-Bead complex is then analyzed by flow cytometry (lower panel). Bead populations are identified using a light scatter plot and gated onto a bivariate dot plot to separate bead populations corresponding to each cytokine analysed. To measure cytokine concentration each bead population is analysed in the PE channel to determine relative brightness of the reporter and thus the degree of bound cytokine. The fluorescence intensity of the reporter in the test samples is compared to a standard curve of known cytokine concentration from which the concentration in the test samples can be determined.

6.8.2 Capturing and measuring cytokines

Protein specific capture beads were incubated with dilutions of OPKA supernatant (neat, 1:2, 1:10, 1:100) and supplied standards followed by incubation with detection antibodies. Once excess detection antibodies were washed off, stained samples were acquired using a BD LSR II Flow Cytometer using FACS Diva software. Gating and comparison of each bead's MFI to the

standard curve was done using the supplied FCAP Array 2.0 software. Data was presented as pg cytokine/ml.

6.9 Phenotyping following OPKA

Expression of cell surface markers were used to establish the activation status of cells following peptide treatment. Following opsonophagocytosis assay, wells were carefully washed twice using PBS. Alveolar macrophages were then detached from culture plates by incubating them for 10 minutes (37°C, shaking at 200RPM) in citric saline (0.15M Na citrate, 0.135M KCl).

6.9.1 Alveolar macrophage staining and aquistion

Once detached, cells were transferred to 1.5ml eppendorf tubes and washed twice by centrifuging them at 300 *x* g for 10 minutes. Following the washing steps, cells were stained using mouse anti-human HLA-DR (MHC-Class II, 560743), CD206 (Mannose Receptor, 55089), CD11b (Adhesion molecule, 347557), CCR7 (Chemokine receptor, 557648), CD163 (Activation marker, 556018), CD86 (Activation marker, 555657) purchased from BD Biosciences. Acquisition was done using a BD LSR II Flow Cytometer using FACS Diva software and results presented as median fluorescence intensity (MFI) corrected for background fluorescence.

6.10 Intracellular Oxidation

Macrophages are able to produce an intracellular oxidative burst in order to degrade phagocytosed bacteria [355]. Intracellular oxidation is also required for the initiation of inflammatory cytokine production [355]. The classical inflammatory molecules IFNγ and LPS have been shown to act as an inducer of intracellular oxidation [356]. In order to measure oxidation, 5-6-carboxy-

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) can be used as a probe. The addition of DCFH-DA to macrophages will lead to internalization of the probe and enzymatic digestion to convert DCFH-DA to DCFH [357]. DCFH is naturally non-fluorescent but becomes fluorescent in response to oxidation and as such can be used to quantify intracellular oxidation [358]. We measured the intracellular oxidation of DCFH inside alveolar macrophages in response to P4 and LPS, and also during an opsonophagocytosis assay with and without peptide stimulation.

6.10.1 Preparing alveolar macrophages

Alveolar macrophages were isolated from BAL fluid as described in Chapter II and plated into a 96-well plate at a concentration of 1 x 10^5 cells / well. Each condition was performed in triplicate. Wells were first incubated with 100 µl of 25 µM DCFH-DA solution (Invitrogen 10086129, UK) for 30 minutes at 37°C (5% CO_2). Control wells consisted of alveolar macrophages incubated with 100 µl RPMI alone and was used as base-line fluorescence during analysis to account for auto-fluorescence. Following incubation, wells were washed twice with pre-warmed (37°C) PBS to remove extracellular DCFH-DA and used for the measurement of either intracellular oxidation in the absence of immune complexes or intracellular oxidation during opsono-phagocytosis.

6.10.2 Intracellular oxidation in the absence of immune complexes

After the DCFH-DA incubation and washing steps, wells were replenished with 150 µl media. 50 µl of stimulant was then added to the reaction tubes to make up a final volume of 200 µl / well. The stimulants were: LPS solution (100 µg / ml DEPC treated water) or P4 solution (1 mg / ml DEPC treated water). Control wells received 50 µl DEPC treated water.

6.10.3 Intracellular oxidation during opsono-phagocytosis

After the DCFH-DA incubation and washing steps, wells were replenished with 30 µl media and components for an opsono-phagocytosis was added to each well. Briefly, 20 µl IVIG-opsonized ST23F pneumococci, 10 µl baby rabbit complement and 20 µl P4 or control solution was added to each well.

6.10.4 Acquisition and Analysis

Immediately after the addition of either stimulants (6.12.2-A) or OPKA components (6.12.2-B) plates were added to a MARS fluorescent plate reader(BMG Labtech, UK) for measurement of fluorescence every two minutes for 200 minutes. The excitation wavelength was set at 485nm and the emission wavelength was set at 520nm. Following acquisition, data was analysed using MARS software version 1.20 (BMG Labtech, UK). Results are presented as the percentage increase in emission intensity corrected against control wells (auto-fluorescence).

6.11 Statistic analysis

Each *ex vivo* assay consisted of > six biological replicates and were performed on separate assay days. Data from separate assay days were pooled and shown as means ± standard deviation. Distribution of data was assessed using D'Agostino and Pearson omnibus normality test. For normally distributed data, an Unpaired T-test was used to test for significance. For non-normally distributed data, a Mann-Whitney unpaired T-test was used to test for significance. Differences between data were designated significant if $p<0.05$.

C. Results

6.12 Volunteer recruitment

Informed consent and bronchoalveolar lavage samples were obtained from 14 subjects in Liverpool (UK) and 13 subjects in Blantyre (Malawi). Informed consent and peripheral blood samples were obtained from 5 subjects in Liverpool (UK).

6.13 Neutrophil OPKA

Neutrophils were isolated from the peripheral blood of healthy adult volunteers (n=5) in Liverpool (UK). We tested whether P4 stimulation of freshly derived neutrophils would lead to increased killing of opsonised pneumococci (Figure 76). Killing by freshly derived peripheral blood neutrophils was significantly increased following peptide stimulation (control 18% vs. treated 43%, p=0.0005) as compared to non-stimulated control cells.

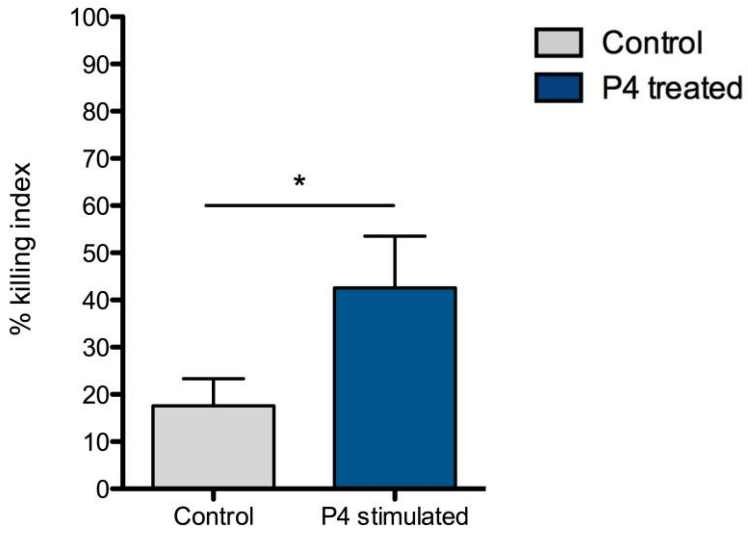


Figure 76 – Opsonophagocytosis of IVIG-opsonized D39 pneumococci using freshly derived peripheral blood neutrophils with and without peptide stimulation (n=5). Data is represented as

mean of replicates ± standard deviation. (*) denotes significant differences using a paired student's T-test where $p<0.05$.

6.14 UK and Malawian healthy HAM OPKA

Human alveolar macrophages were isolated from healthy adult volunteers in the UK and Malawi to determine whether both populations would be able to up-regulate phagocytic killing in response to peptide stimulation.

6.14.1 HAM OPKA from UK population

Non-stimulated alveolar macrophages from the UK volunteers (n=14) killed an average of 40.1% and this was significantly up regulated following P4 stimulation (control 40.1% vs. treated 68.2%, $p<0.0001$) (Figure 77).

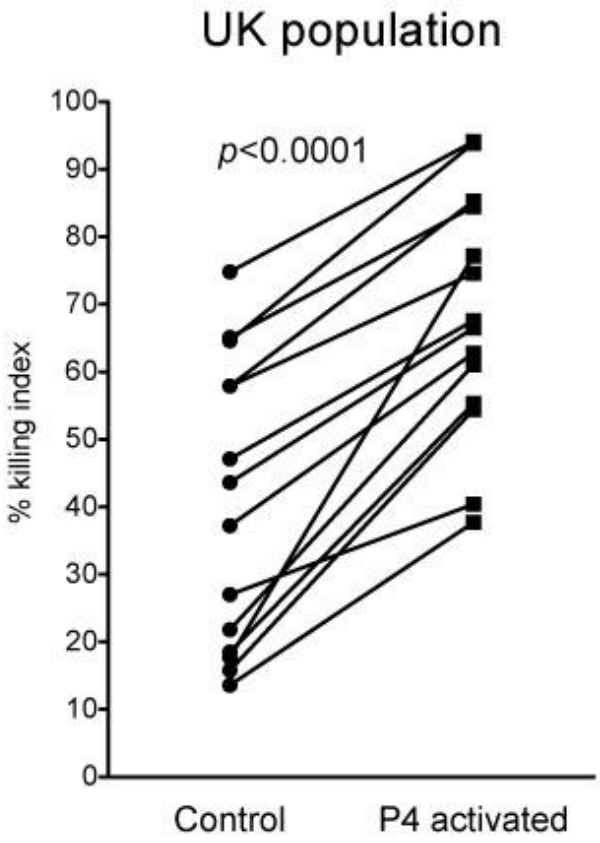


Figure 77 – Opsonophagocytosis of IVIG-opsonized D39 pneumococci by human alveolar macrophages isolated from UK adult volunteers in the presence or absence of peptide stimulation (n=14). (*) denotes statistical significance where $p<0.0001$ using a paired student's t-test.

6.14.2 HAM OPKA from Malawian population

Healthy adult volunteers were recruited in Malawi using identical inclusion and exclusion criteria as for UK volunteers. Non-stimulated Malawian alveolar macrophages killed an average of 35.8% of the IVIG opsonised D39 bacterial inoculum. P4 stimulation significantly enhanced the killing to an average of 47.6% (control 35.8% vs. treated 47.6%, $p<0.01$) (Figure 78).

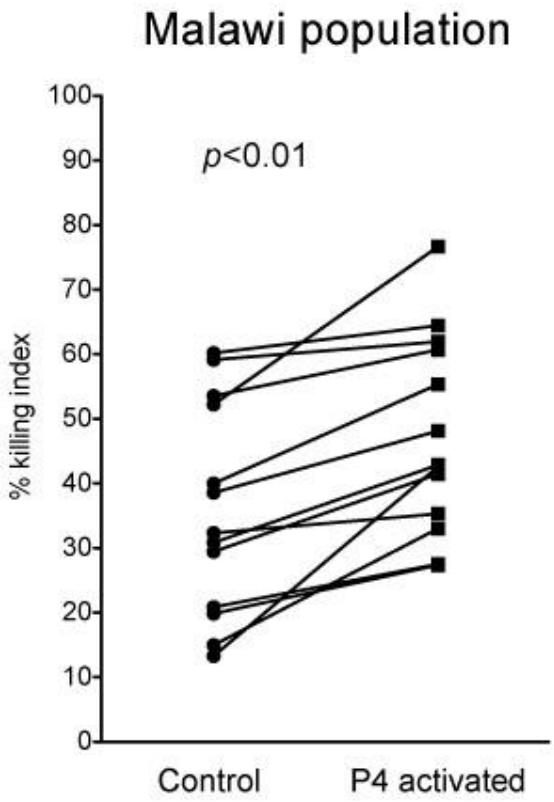


Figure 78 - Opsonophagocytosis of IVIG-opsonized D39 pneumococci by human alveolar macrophages isolated from Malawian adult volunteers in the presence or absence of peptide stimulation (n=13). (*) denotes statistical significance where $p<0.01$ using a paired student's t-test.

6.14.3. HAM OPKA control reactions

In the absence of antibody or complement, opsonophagocytosis was reduced to 13.4% and 19.2%, respectively (Figure 79). Following peptide stimulation, no detectable differences (n=6) could be seen in the absence of antibody (control 13.4 % vs. treated 14.5 %, $p>0.05$) or complement (control 19.2% vs. treated 18.3, $p>0.05$). Similarly, macrophages where Fcγ receptors were occupied following stimulation (n=6) showed a reduced ability of phagocytosis compared to control and no detectable differences between stimulated and non-stimulated groups were seen (Fcγ receptor occupied control 19.3% vs. Fcγ receptor occupied treated 19.8%, $p>0.05$).

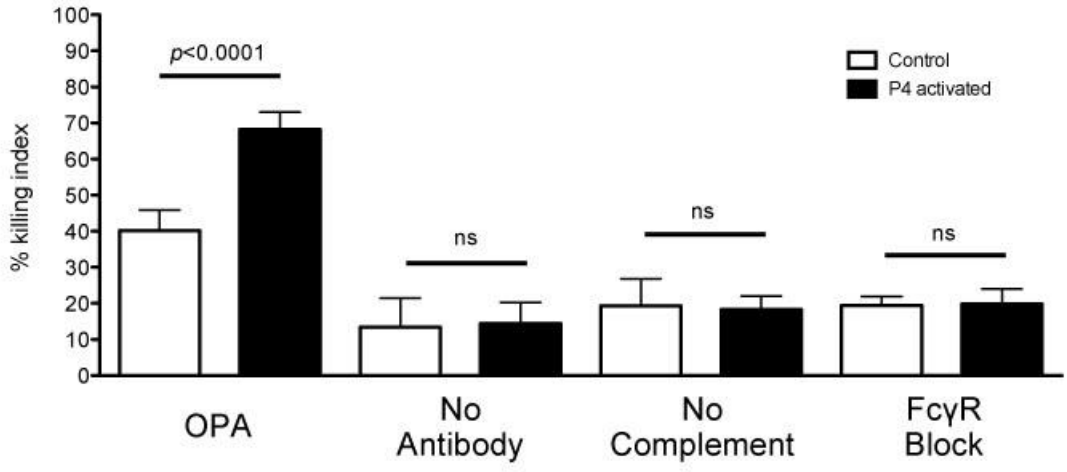


Figure 79 - Opsonophagocytosis of D39 pneumococci by human alveolar macrophages isolated from UK adult volunteers (n=6). "OPA" designates assays in which all components are included (macrophage, bacteria, antibody, complement), "No Antibody" designates assays without the presence of antibody, "No Complement" without the presence of complement and "FcγR Block" designates assays where Fcγ-receptors were occupied by IgG prior to the assay. (*) denotes statistical significance where $p<0.0001$ using a paired student's t-test.

6.14.4. HAM OPKA of ST23F *S. pneumoniae*

In addition, it was tested whether P4 mediated opsono-phagocytosis by alveolar macrophages was effective using a different capsular type of *S. pneumoniae* (Figure 80). Alveolar macrophages from the UK population were allowed to phagocytose opsonised ST23F pneumococci (n=6). Peptide stimulation led to significantly increased killing using the ST23F serotype (control 38.6 % vs. treated 75 %, p=0.02), similar to that seen using ST2 (D39) pneumococci.

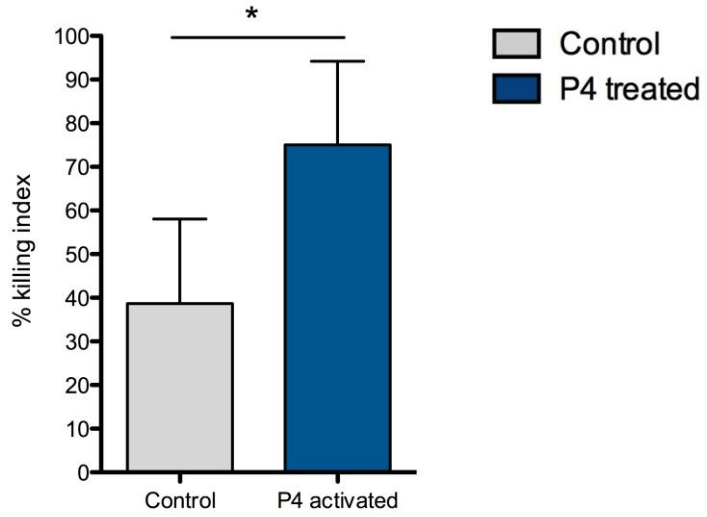


Figure 80 - Opsonophagocytosis of IVIG-opsonized ST23F pneumococci by human alveolar macrophages isolated from UK adult volunteers. (*) denotes statistical significance where p<0.05 using a paired student's t-test.

6.15 Intracellular killing following OPKA

P4 mediated opsonophagocytosis by alveolar macrophages leads to increased uptake of opsonized pneumococci. We wanted to test whether engulfed bacteria would persist intracellularly as a result of the higher amounts present due to P4 enhanced uptake.

Following opsonophagocytosis (n=3), peptide stimulated alveolar macrophages took up higher amounts of the bacterial dose than their unstimulated control (control 15.9% vs. treated 49.1%, p=0.005) (Figure 81) consistent with the increased uptake by P4 treated cells shown in Figure 5 . No CFU could be detected intracellularly in control cells following a two-hour OPKA, while P4 treated cells had small amounts of bacteria present intracellularly but this was not significant (control 0% vs. treated 1.5%, p=0.1).

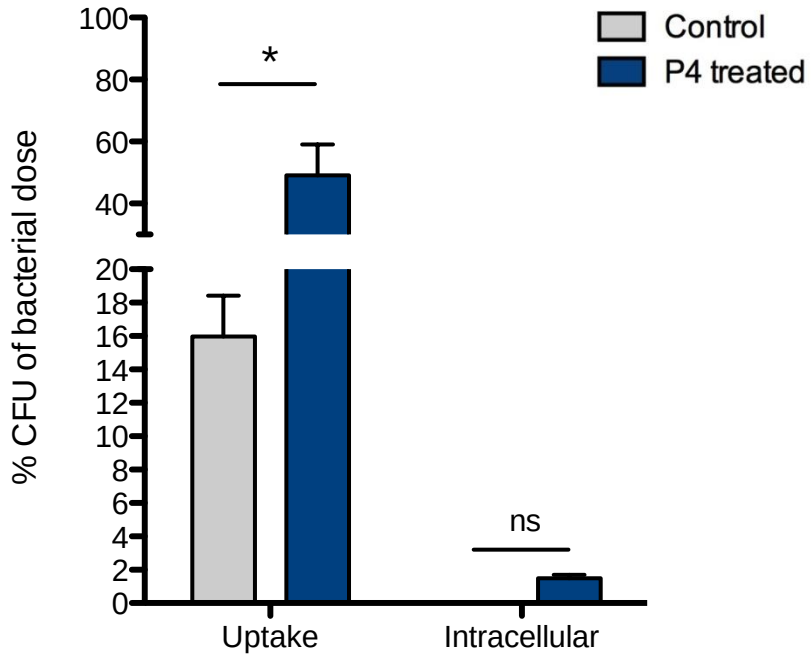


Figure 81 – Percentage of the bacterial dose taken up or found intracellular in human alveolar macrophages following an opsonophagocytosis killing assay (n=3). Statistical tests were done using a paired student's t-test.

6.16 Cytokine secretion following P4 mediated OPKA

Cytokine secretion of alveolar macrophages were measured following an opsonophagocytosis killing assay in the presence or absence of peptide stimulation. 2 hours following incubation with opsonized pneumococci and complement, alveolar macrophages (n=12) did not significantly alter their secretion of the cytokines MIP-1β, MCP-1, IL-10, IL12, IFN-γ, or RANTES (p>0.1). Higher levels, albeit non-significant, of IL-8 (control 6178 pg/ml vs. treated 8648 pg/ml, p=0.08), MIP-1α (control 144 pg/ml vs. treated 244 pg/ml, p=0.1), and IL-6 (control 71 pg/ml vs. treated 98 pg/ml, p=0.1) were detected following peptide stimulation (Figure 82).

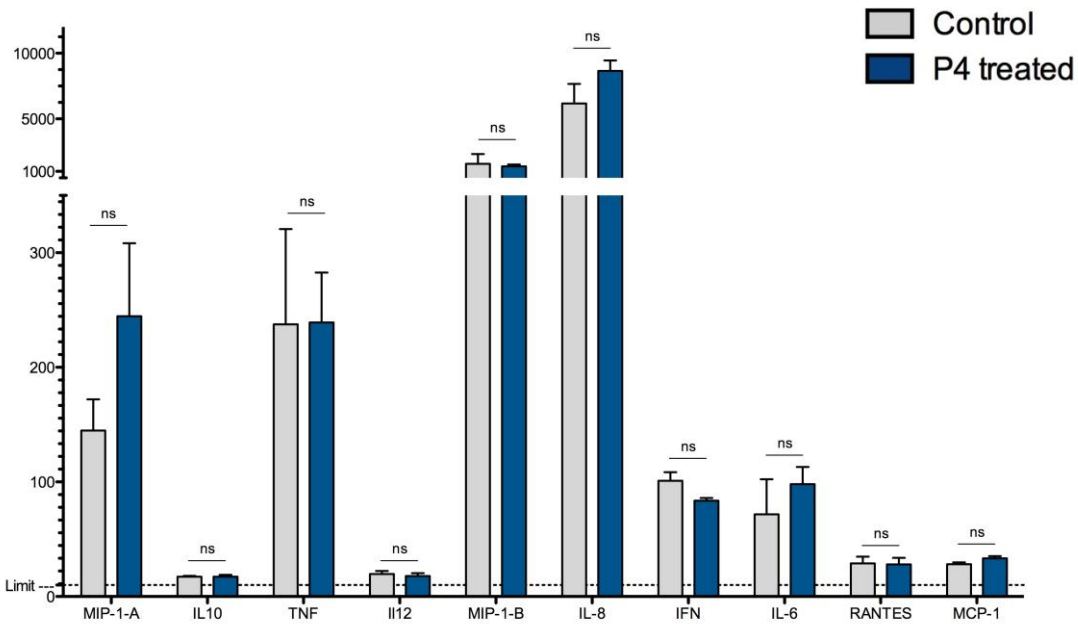


Figure 82 - Level of cytokine secretion in control or P4 activated alveolar macrophages (n=12) show no differences following a two-hour OPKA assay. Cytokine levels were detected in OPKA supernatant using a BD Flex Set kit following a two-hour OPKA. Statistical analysis was done using a Wilcoxon matched pairs t-test and defined not significant where p>0.05.

6.17 Receptor expression following OPKA

In order to compare differences in the expression of surface markers of activation, alveolar macrophages from the Malawian population were allowed to phagocytose opsonised pneumococci in the presence or absence of P4 peptide. Following OPKA, surface markers were assessed on alveolar macrophages using flow Cytometry. P4 treated AMs did not express higher levels (p>0.1) of HLA-DR, CD206 , CD11b, CCR7, CD163 or CD86 as compared to control cells (Figure 83).

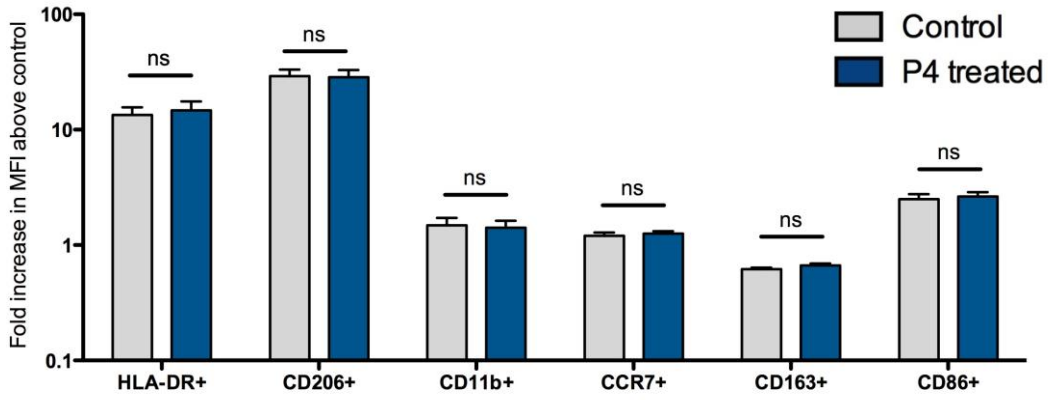


Figure 83 – Expression of protein markers of inflammation on alveolar macrophages is not modified as a result of peptide stimulation following phagocytosis. Alveolar macrophages were allowed to phagocytose opsonised pneumonia in the presence or absence of P4 peptide for two hours. Following the OPKA, alveolar macrophages were stained for surface markers and analyzed using flow cytometry.

6.18 Intracellular oxidation

Intracellular oxidation was measured during an opsonophagocytosis assay with and without peptide stimulation (10) and in the presence of P4 peptide, LPS or PBS alone (11).

6.18.1 Intracellular oxidation during OPKA

Peptide treatment of alveolar macrophages exposed to opsonised bacteria and complement (n=6) led to a significantly greater increase in intracellular oxidation after 140 minutes of measurement (control 81% vs. treated 135% at 140-200 minutes, $p<0.05$) when compared to the non-stimulated control.

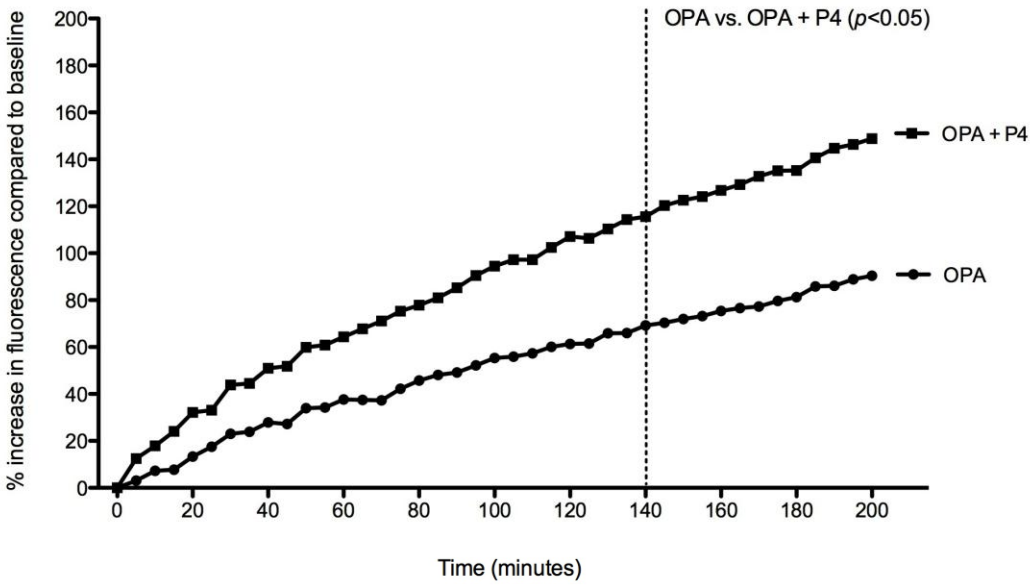


Figure 84 – Measurement of intracellular oxidation in human alveolar macrophages (n=6) during the opsonophagocytosis of IVIG-opsonized ST23F pneumococci. The dashed line at 140 minutes indicates the time-points from when statistical differences were detected using an ANOVA test (where $p<0.05$ from 140-200 mins).

6.18.1 Intracellular oxidation in the absence of infection

The effect of direct stimulation of alveolar macrophages in the absence of an immune complex was also tested (Figure 85). Here we tested the effect of P4 and compared it to a positive control (LPS) and a negative control (PBS). In P4 peptide treated macrophages (n=6) greater fluorescence compared to non-stimulated control cells was detected over time but did not reach statistical significance (control 84% vs. treated 102% at 160 minutes, p=0.30). In macrophages stimulated with LPS, however, fluorescence at 160 minutes was significantly higher than control macrophages (control 84% vs. LPS treated 139% at 160-200 minutes, p<0.05).

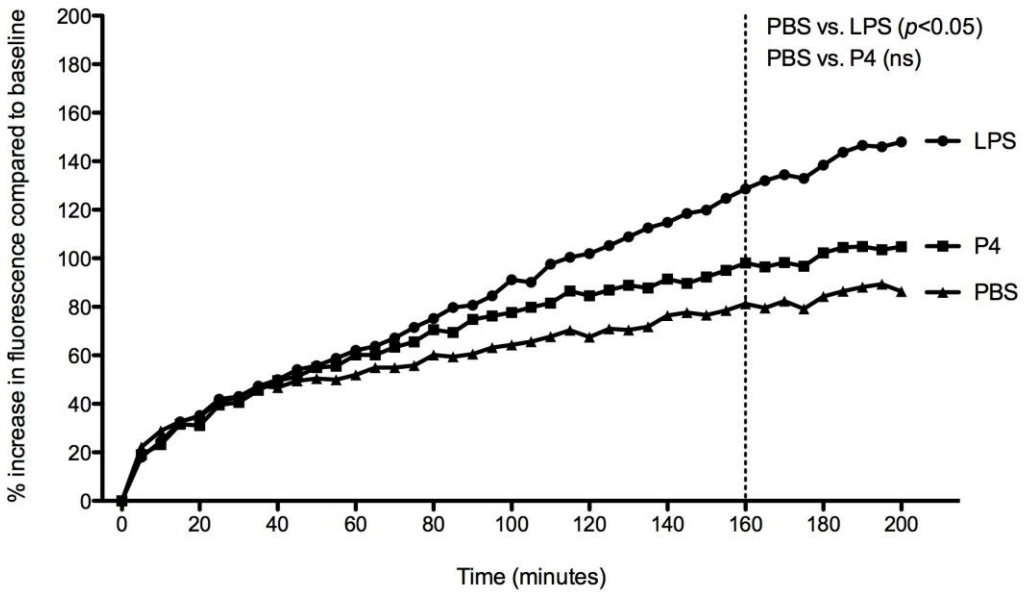


Figure 85 – Measurement of intracellular oxidation in human alveolar macrophages (n=6) following stimulation with PBS, LPS (100 μg/ml) or P4 (1mg/ml). Dashed line at 160 minutes indicates the time-points from when statistical differences were detected using an ANOVA test (where p>0.05 from 160-200 mins).

D. Discussion

6.19 Discussion

In this chapter, both human phagocytes involved in defending the host against pneumococcal infection during health and disease were shown to respond to P4 treatment. These data are the first for human alveolar macrophages and confirm previous work on peripheral blood neutrophils [279]. It was also shown that P4 upregulates alveolar macrophage intracellular oxidation only in the presence of immunecomplexes and that no changes in inflammation could be detected by measuring cell surface marker expression and cytokine secretion following OPK assays.

6.19.1 Alveolar macrophage and neutrophil phagocytosis

Two different human populations with different experience of pneumococcal disease were deliberately compared. UK adults of the age recruited have a low incidence of pneumonia (<1/1000/yr) and pneumococcal carriage (<10%) but in Malawi both disease and carriage are much higher, related to exposure and risk factors including malnutrition [359], smoke exposure [360] and concurrent infections [361]. In this chapter, both the UK and Malawian alveolar macrophages showed a significant increase in opsono-phagocytosis following P4 peptide treatment. Whilst both population groups showed enhanced activity, this was more pronounced in the UK population. The lower phagocytic enhancement seen in the Malawian population was possibly due to the heavy carbon load of Malawian alveolar macrophages [362], which has been shown to reduce phagocytosis [363]. Nevertheless, it is promising to see P4 stimulation effective in populations most susceptible to pneumococcal disease. These results translate observations from chapter III, where murine alveolar macrophage augmented their phagocytic killing of pneumococci following peptide stimulation. In addition, peripheral blood neutrophils form

healthy adults augmented their phagocytic killing of IVIG opsonised pneumococci as has previously shown [280]. As neutrophils are heavily recruited to lungs following infection [306], these results further support the use of API to augment lung defence during pneumonic infections.

Phagocytosis is triggered when the Fc region of a pathogen-bound antibody comes into contact with Fcγ receptors on immune cells, an event which can be mediated by complement [71]. In this chapter, phagocytosis of bacteria in the absence of antibodies or complement was greatly decreased and P4 did not up-regulate function. This suggests that the P4 enhanced phagocytosis by alveolar macrophages is specific, relying on the presence of the components necessary for phagocytosis to occur. In previous chapters, Fcγ receptors have been associated with P4 administration and in this chapter their involvement in P4-mediated opsono-phagocytosis by alveolar macrophages was confirmed. Immunoglobulin G is used as a standard method in immunofluorescent staining to reduce non-specific binding by occupying Fcγ receptors. This technique was adapted to occupy Fcγ receptors following peptide stimulation. Similar to HL-60 Fc fragment occupation studies, occupation of Fcγ receptors on alveolar macrophages reduced phagocytic killing in both control and P4 stimulated cells. Possible mechanisms for the reduced killing by P4 treated cells in these assays were suggested to be due to (a) augmented FcγR expression being annulled through receptor occupation, (b) lack of TLR stimulation following internalization of free IgG and (c) ligation of inhibitory FcγR by IgG.

Invasion assays in this chapter were performed to determine whether internalized pneumococci following OPK assays were also killed intracellularly. Following OPKA, cells were washed and incubated with a bactericidal antibiotic then lysed using a detergent to culture intracellular pneumococci. It was hypothesized that P4 not only enhanced phagocytic uptake but also phagocytic killing. No pneumococci could be cultured

intracellularly in control cells while small amounts (>2% of bacterial dose) were cultured in P4 stimulated cells. As >50% of the pneumococcal dose was internalized in P4 stimulated cells (compared to 16% in control cells), the amount of internal pneumococci cultured is relatively small and would have most likely been killed intracellularly if given more time. This could have been confirmed by allowing alveolar macrophages additional time to kill the larger proportion of internalized phagocytes. These results confirm that the OPK assay measures killing of opsonised pneumococci and that augmented phagocytosis of P4 stimulated alveolar macrophages also leads to intracellular killing. It would be interesting to assess P4 mediated killing of intracellular pathogens such as *Legionella pneumophila*, *Listeria monocytogenes* and *Mycobacterium tuberculosis* which allow themselves to be phagocytosed in order to escape the phagosome and reside intracellular [364].

6.19.2 Intracellular oxidation by alveolar macrophages

Phagocytic cells use intracellular oxidation and proteases to break down and kill ingested bacteria [356]. This chapter investigated whether alveolar macrophages would alter their intracellular oxidation as a result of peptide treatment. In the presence of immune complexes and complement, oxidation levels were higher in P4 stimulated alveolar macrophages as compared to the non-stimulated control. In the absence of immune complexes however, P4 stimulation alone leads to little increase in intracellular oxidation as compared to LPS where levels rise significantly. The DCFH assay does not differentiate between oxidation in the cell and oxidation in the phagosome so it is unclear where the enhanced oxidation is occurring. As peptide treatment alone does little to alter oxidation levels however, the increased levels seen during phagocytosis are most likely due to larger amounts of pneumococci present intracellularly as a result of P4 stimulation.

In addition to the generation of reactive oxygen species, a recent study by Nunes *et al* has shown that high Ca^{2+} levels within and in proximity of the phagosome can augment killing of internalized pathogens through the interaction of endoplasmic reticulum and phagosomes [365]. As calcium signaling is important for the maturation of phagosomes and therefore killing of pathogens [366], it would be of interest to assess the flow of intracellular calcium during P4 mediated opsonophagocytosis.

6.19.3 Cell surface markers and cytokine secretions following OPKA

During severe infections, an exaggerated immune response is often the main cause of tissue damage and reduced recovery [367]. In this chapter, it was investigated whether P4 treatment would exacerbate inflammation by measuring inflammatory cytokine and cell surface markers following opsonophagocytosis killing assays. P4 treatment did not significantly alter either cytokine secretion or surface marker expression immediately after stimulation, suggesting that P4 therapy could be used clinically in severe inflammatory conditions such as those seen during sepsis. These results are in line with results from chapter V, where P4 stimulation in the absence of infection did not alter expression of FcγRI, a marker of cell activation.

Additional work is necessary, however, to ascertain the benign effect of long-term peptide stimulation as the time point sampled in these assays may be to short to detect phenotypical changes on cells and cytokine secretions. mRNA studies could help detect rapid changes following peptide stimulation while extended timepoints, in the presence and absence of infection, . In addition, the use of IVIG which may contribute to reduced inflammation during these studies due to sialylated pooled immunoglobulin G acting on inhibitory Fc receptors [368]. This needs to be taken into consideration when assessing activation of cells following API.

CHAPTER VII.

Discussion

Since the validation of the "germ theory of disease" by Louis Pasteur in the 19th century, modern medicine has made great leaps in reducing deaths from infectious diseases. Imaginative and sometimes counter-productive treatments were replaced by two strategies: (1) targeting the disease through the use of antimicrobial compounds and (2) preventing disease through vaccination of the host. These strategies work in separate ways: one targets the pathogen to assist the immune system while the other assists the immune system to target the pathogen. There is no doubt that both strategies (as well as improvements in hygiene) have contributed to immense reductions in death. While vaccination continues to prevent >3million deaths every year, the efficacy of antibiotic treatment is fading. The development of new antimicrobial compounds is important and needs to be further pursued. It is, however, equally important to appreciate the achievements of vaccination and remember the successes of passive immunotherapy when developing new treatments.

In this thesis, the efficacy of augmented passive immunotherapy (API) to treat pneumococcal disease was investigated. API is a synergistic treatment of pooled antibody in order to assist the host in recognizing pathogens and the immunomodulating peptide P4 to encourage the clearance of pathogen. In order to investigate its effectiveness, *in vivo* models were used to assess API in treating invasive pneumonia and *ex vivo* / *in vitro* assays were used to assess the effect of peptide stimulation on phagocytic cells.

In summary, API significantly improved the survival of mice in all models of invasive pneumococcal disease tested. Three strains of mice were chosen with varying genetic backgrounds and ages as well as varying severity of infection. Improved survival was associated with reduced pneumococcal burden in lungs and blood and the recruitment and activation of phagocytic leukocytes following treatment. Activation of phagocytes was associated with increased expression of the opsonic Fcγ receptors. During acute infections of

pneumonia, peptide treatment of the lung alongside antibody administration prevented the onset of septicemia and subsequent death of mice. Peptide or antibody treatment alone did not significantly improve survival in all models tested. In the absence of infection and IVIG, *in vivo* peptide administration led to the recruitment and activation of phagocytes in a manner similar to the infection studies.

Peptide stimulation of phagocytes derived from various tissues or through cell culture led to significantly enhanced killing of opsonized *S. pneumoniae*. Phagocytes tested included murine alveolar and peritoneal macrophages as well as a murine macrophage cell line. Human alveolar macrophages from two donor populations, peripheral blood neutrophils and HL-60 cell lines were also tested. Peptide treatment of a monocyte-like cell line did not lead to enhanced phagocytosis. Increased killing of *S. pneumoniae* by phagocytes was dependent on the presence of antibody, functional complement and the availability of opsonic Fcγ receptors.

The data in this thesis support previous data that have shown the beneficial effect of API in treating bacterial diseases. Since it's conception in 2008, API has been able to rescue moribund mice infected with invasive pneumonia [280], influenza-pneumonia co-infections [284] and MRSA [282]. API has also been administered alongside low dose antibiotics leading to enhanced survival rates [281]. Repeat therapy in mice whom had previously survived pneumococcal infection due to API was also successful [281].

Murine models of disease

Mouse models of pneumococcal disease have been used to assess the efficacy of vaccination, therapeutic drugs, and host-pathogen interactions. The choice of mouse strain is important, as differences in susceptibility to infection have been described in various strains of mice [310, 369, 370]. Differences in

susceptibility with respect to the sex of mice have also been described [367]. Inbred mice allow for reproducible and uniform immune responses and have therefore frequently been used to assess vaccine and therapeutic drug efficacy [371]. Outbred strains are used to observe immune responses in a heterozygote population. The phenotypic differences in outbred mice, despite being lower than in humans, can better reflect the natural variations in response to infection [372]. Assessing the efficacy of therapeutic agents in aged mice is important as ageing has been shown to increase susceptibility to pneumococcal infection due to the diminished capabilities of a range of effector functions [314, 373, 374]. The infecting dose and strain is also important. Laboratory strains of *S. pneumoniae* are frequently used for infection as they assure reproducibility and allow for comparison between different research groups. The problem is, however, that frequent passaging of bacteria and long-term storage changes the phenotype and virulence of the bacterium [375]. To overcome this, laboratory strains of D39 *S. pneumoniae* were passaged once *in vivo* prior to infection to increase and standardize their virulence. For all models of invasive disease, a high dose (10^6 CFU) of *S. pneumoniae* is instilled directly into the lungs of mice. Depending on the host, pneumonia is established within 12-48 hours after which pneumococci seed into blood and cause septic infections.

API against invasive disease

In this thesis, outbred strains were used to model human invasive pneumonia. These were MF1 (48 hours mean survival [369]), Swiss Webster (72 hours mean survival) and BALB/c (168 hours mean survival [369]). In addition, Swiss Webster and BALB/c mice were allowed to age until >50% of the initial colony succumbed of natural causes, thereby establishing an "aged population". Survival of API treated mice was significantly increased in both young and aged populations of BALB/c and Swiss Webster mice. The MF1 host was chosen to model acute infections that progress rapidly. In a clinical

setting, acute infections are responsible for the majority of deaths due to the time taken for antimicrobial compounds to take effect [318]. To challenge the treatment model, API was administered when bacteraemia had already set in. The sole administration of either P4 or IVIG did not lead to significant increases suggesting a synergistic effect of APIs

IVIG as an opsonin

Immunoglobulin present in pooled preparations such as IVIG is derived from 1,000-3,000 blood donors and includes antibodies generated naturally through exposure to pathogens as well as antibodies generated following vaccination. Naturally derived antibodies have evolved in parallel to the evolution of pathogens and directed against several antigens. Vaccine generated antibodies against encapsulated bacteria are usually strong opsonisers [376, 377] and aimed at inactivating virulence factors essential for causing disease. This combination reduces the ability of pathogens to avoid detection and evolve, making antibody administration feasible as a long-term treatment strategy. Several studies have shown that IVIG preparations contain antibodies against a wide range of common bacterial and viral pathogens [378, 379] and in this thesis IVIG was able to opsonize eight serotypes (including un-encapsulated strains) of *S. pneumoniae* as determined by opsonophagocytic killing assays in the presence and absence of IVIG opsonisation. This broad specificity allows for pre-emptive administration against a range of clinically important pathogens prior to diagnostic results.

A problem with the use of IVIG to treat infectious disease is the lot-to-lot variation in the neutralizing titres of pathogen specific immunoglobulin [380-382]. This is because currently commercial IVIG preparations are not tailored for the use against infectious diseases. This could be overcome, however, by establishing international standards of neutralizing antibody titres in IVIG preparation using existing methods [383]. In addition, IVIG preparations

could be further potentiated for the use against infectious diseases by supplementing collected serum with monoclonal antibodies against the most important human pathogens. There are many FDA approved monoclonal antibodies against a wide range of infectious pathogens that could be used for IVIG potentiation [274, 384].

IVIG as an anti-inflammatory

The anti-inflammatory properties of IVIG have long been recognized [368, 385]. It is believed that sialylation of the Fc portion of a small percentage of immunoglobulin present in IVIG leads to significant anti-inflammatory effects through it's interaction with inhibitory Fc receptors on leukocytes [386, 387]. Specifically, IVIG induces the expression of inhibitory FcγRIIb, which is then ligated by exogenous antibody leading to dampening of immune responses [388]. As such, IVIG preparations are frequently used to treat inflammatory diseases such as arthritis [389]. In the context of infectious diseases, this is beneficial as an exaggerated immune response, often seen during bacterial infections, can be minimized or prevented leading to faster resolution of infection [390]. During API of pneumonia-influenza co-infected mice, lung tissue damage was significantly lower in API mice as judged by histopathological analysis [284]. This was suggested to be due to a decrease of bacterial burden, but reduced inflammation due to IVIG administration may have also contributed.

In the search for treatments against sepsis, anti-inflammatory compounds have frequently undergone clinical trials with mixed results [391]. There have so far only been a few clinical trials of IVIG in sepsis [392] where most successful results are reported when IVIG is used as an adjunct to conventional treatments [273, 393]. Reduced inflammatory stimuli, however, also decreases the effectiveness of phagocytic clearance of pathogens. As such, immunomodulation to enhance phagocytosis is of benefit. In this thesis,

the sole administration of IVIG in the control groups increased survival of mice, but in combination with the immunomodulating peptide P4 this was significantly improved. In order to elucidate the role of IVIG in containing inflammation during API, studies comparing "classical" serum from immunized mice (i.e. no sialylation of IgG molecules, titrated to have the same opsonic efficacy as IVIG) to IVIG could be performed. Similarly, treatments using anti-pneumococcal monoclonal antibodies could be tested against IVIG.

Intravenous administration

IV administration of P4 was effective in all models of invasive pneumococcal disease. It has the advantage that administered peptide will circulate systemically thereby affecting a wide range of cells. A rapid effect is important, as survival in patients presenting with acute pneumonia or sepsis is greatly increased if treatments are administered during the "golden hours", i.e. immediately after presenting at the clinic [394]. Antimicrobial compounds have not been shown to reduce deaths that occur in the initial 48-hour period following admission [395-397]. A rapidly effective "bridging treatment" could therefore contain infections before antimicrobial compounds take effect. In this thesis, acutely infected mice treated with API survived significantly longer than untreated mice. API halted the progression of pneumonia and bacteraemia within 12 hours of P4 & IVIG administration in the MF1 host. In a clinical setting this could allow for further antibiotic therapy to take effect as well as time for a full diagnosis and further targeted treatment.

Intranasal administration

Intranasal administration of P4 results in the exposure of the peptide to all sites of pulmonary immunity, including resident alveolar macrophages. It is assumed that peptide solution administered IN would also diffuse through

tissue and enter the blood circulation although at lower concentrations than when administered IV [398-400]. IN treatment was given at 12 hours following acute infection, which in the MF1 mouse model is prior to the onset of bacteraemia. This resulted in significant reductions of pneumococcal loads in lungs and therefore decreased neutrophil infiltration and the potential for "collateral damage" caused by them [151]. The administration of P4 at 12 hours as compared to 24 hours in the IV model meant that treatment was given while infection was still at a crucial stage (in lungs rather than blood). Hence, IN administration of P4 alongside IVIG as a pre-emptive treatment of acute invasive pneumonia was able to prevent exacerbation of the disease by prevention of pneumococcal seeding into blood and thereby no sepsis, leading to complete host survival.

To the best of my knowledge, this is the first time intranasal therapy of immunomodulating compounds has led to host protection after the onset of acute pneumonia and offers the potential of targeted treatments against bacterial respiratory infection and septicemia secondary to such infections. IN treatment has the advantage of directly stimulating alveolar macrophages. AMs make up a large phagocytic population in lungs but remain mostly anti-inflammatory, a natural mechanism evolved to avoid unnecessary damage to lungs during mild infections. It has been shown that alveolar macrophages from patients with severe pneumonia have a reduced ability respond to LPS stimuli compared with healthy controls [401]. As *ex vivo* peptide treatment of alveolar macrophages led to increased bacterial killing, IN administration has the potential to harvest the phagocytic potential of macrophages in addition to recruited neutrophils and monocytes. Intranasal administration of IVIG has also been shown to reduce bacterial loads during pneumococcal infection (but to a lesser extent than intravenous administration) suggesting that both P4 and IVIG could be administered intranasally during API to treat pneumonia [402].

Nasopharyngeal carriage of the pneumococcus precedes invasive disease. It was tested whether API would be able to affect pneumococcal carriage following intranasal administration of P4. 48 hours following the first administration of peptide to the nasopharynx, levels of pneumococci in the nasopharynx significantly increased without causing invasive disease. This is perhaps not surprising, as it has previously been shown that P4 affects the cytology of nasopharyngeal epithelial cells, including the remodeling of actin, thereby facilitating invasion by the pathogen [279]. This is in line with studies on PsaA, the protein from which P4 is derived, which showed that PsaA facilitates adhesion and subsequent invasion of epithelial cells by pneumococci [277]. In addition, the administration of IVIG during carriage would not be able to effectively opsonise pneumococci as IVIG is predominantly made up of IgG immunoglobulin, which is less efficient at entering mucosal areas compared to IgA.

These findings suggest that intranasal administration has the potential of harvesting the phagocytic potential of alveolar macrophages and neutrophils recruited through the up-regulation of Fcγ receptors and subsequent clearance of pathogens. Care needs to be taken, however, to avoid exposure of P4 to the nasopharynx as it may facilitate the invasion by pneumococci.

Modulating phagocyte recruitment

Infection of the lung leads to the secretion of cytokines to recruit phagocytes to the site of infection. Following pneumococcal infection in this thesis, control MF1 mice recruited both neutrophils and monocytes to the lungs as has been seen in previous studies [306]. Alveolar macrophages did not change their cytokine secretions following peptide mediated phagocytosis *ex vivo* but a slight increase in IL-8 and MIP-1α secretion (responsible for neutrophil recruitment) as well as IL-6 (functions include neutrophil production) was detected. Intravenous treatment with peptide, however, had no effect on pulmonary neutrophil recruitment during *in vivo* infection studies. Intranasal

administration of peptide actually led to a reduction of neutrophils in the lungs. The significantly increased neutrophil recruitment in peripheral blood after intravenous administration of peptide in the absence of infection, however, would suggest that P4 peptide does influence neutrophil migration.

Neutrophils become the major cell phagocytosing bacteria in the alveolar compartment in established pneumonia [403] but can also cause "collateral damage" by releasing reactive oxygen species and proteolytic enzymes [404-407]. In both intranasal and intravenous peptide administrations, significant reductions in bacteria were detected in lungs. This could suggest that neutrophils were recruited and began phagocytosing bacteria (hence the reduction in bacterial burden) after which they induced apoptosis. Studies looking for apoptotic neutrophils in lungs or the presence of cytokines responsible for neutrophil recruitment or release from the bone marrow (i.e. IL-8, G-CSF) would help better understand the role of neutrophil recruitment in API. An augmented, but not exaggerated recruitment of neutrophils from API would be beneficial as neutrophils from septic patients, particularly non-survivors, exhibit significantly less chemotactic activity *in vitro* than neutrophils obtained from healthy volunteers [408].

Most strikingly, however, was the detection of a rapid increase in blood monocytes following intravenous administration of P4 peptide in the presence and absence of infection. As previously described there are two monocyte sub-populations that have distinct functions. Resident tissue monocytes initially phagocytose bacteria and recruit leukocytes but then switch into anti-inflammatory immune cells [298]. Inflammatory monocytes are recruited to inflamed tissue to phagocytose bacteria and further recruit phagocytes but then differentiate into anti-inflammatory macrophages [409]. This suggests that the increased monocyte population observed following peptide administration could be inflammatory monocytes sub-population that may function to either help in clearing opsonized pathogens or act as

anti-inflammatory cells. In order to verify this, isolated F4/80^{+} monocytes following peptide administration could be further categorized by staining for Ly-6C^{high} (resident monocytes) and Ly-6C^{low} (inflammatory monocytes) expression. Inflammatory monocytes have previously been shown to be essential in containing septic infections and as such and augmented monocytic response from API could be of benefit [319].

While the *in vivo*, peptide stimulation led to large effects on monocyte populations, this was not true *in vitro*. Using a monocytic THP-1 cell line, peptide stimulation did not lead to enhanced opsonophagocytosis in this thesis. It could be that THP-1 monocytes require further differentiation into macrophages for enhanced phagocytosis or that P4 peptide indirectly contributes to monocyte activity through IL-2 secretion by other effector cells [410]. P4 mediated phagocytosis assays using THP-1 or freshly derived monocytes alongside co-stimulation, such as IL-2, could help inform on the effect of P4 on monocyte phagocytosis.

Similar to PsaA, the virulence factor NadA from *Neisseria meningitidis* is an invasin that promotes bacterial adherence and invasion into epithelial cells [324]. Purified soluble NadA was shown to efficiently stimulate monocytes/macrophage secretion of IL-8, IL-6, MCP-1, and MIP-1α as well as low levels of TNF-α and IL-1. In addition, NadA also inhibited monocyte apoptosis and induced the differentiation into a macrophage-like cells [411]. It is possible that P4 acts on monocytes in a similar way as NadA by enhancing recruitment of neutrophils and differentiating into macrophages in order to assist in the bacterial clearance. Studies looking at cytokine secretion and monocyte differentiation would help better understand the role of monocytes in API.

Modulation of Fcγ expression

A reoccurring observation of API is the increased expression of opsonic Fcγ receptors on phagocytic cells. This was true in the presence and absence of infection, *in vitro* and *in vivo*. Using, flow cytometry, Fcγ blocking studies and immunogold staining it was determined that P4 up-regulates Fcγ expression within one hour of peptide stimulation and that this is necessary for enhanced phagocytosis to occur. In vivo, the IV administration of P4 solution in the absence of infection led to significant up regulation of Fcγ expression on circulating blood monocytes and neutrophils at 1 hour, peaking at 24 hours. Infection of these mice at 24 hours led to significantly increased survival and reduced bacterial load in the absence of IVIG. At 24 hours in this model, control mice had pneumococci seeding into blood but this was not true for P4 pre-treated mice suggesting that host immune cells were initially primed and able to effectively clear invading pathogens, but the overwhelming infection and lack of opsonic molecules may have led to host mortality.

In order to visualize increased Fcγ receptors expression, peripheral blood monocytes were collected 24 hours following peptide administration in the absence of infection and stained for FcγR using immunogold antibodies. This confirmed the enhanced expression of FcγR observed using flow Cytometry studies but also showed an augmented ability of P4 treated monocytes to internalize immunogold complexes. This suggests that enhanced internalization is independent of the presence of inflammatory mediators triggered when pathogens are internalized. This could have potential therapeutic benefits as some pathogens, such as the parasitic leishmania species, do not trigger phagocytosis of monocytes and macrophages in the absence of further stimuli such as LPS binding to TLR [150].

Fcγ expression is naturally increased through inflammation and P4 was able to augment this during infection *in vivo* and *in vitro*. It is unclear whether P4

enhances the natural signaling pathway of Fcγ expression or modulates expression via another signaling pathway. As increased expression is seen *in vitro*, it can be assumed that up-regulation is independent of stimuli secreted by other immune cells. A better understanding of the pathways of Fcγ regulation is necessary to further elucidate the mechanism of P4 augmented expression.

Modulating phagocytosis

Previously it was shown that the phagocytosis of opsonized pneumococci by HL-60 neutrophil like cells as well as RAW macrophages would be up-regulated following peptide treatment [279, 280]. A standardized killing assay was chosen which measures the ability of phagocytes to take up and kill pneumococci in the presence of immunoglobulin and complement, serum proteins important for the opsonisation of pathogens. Titrated IVIG was used as a source of pathogen specific antibody and titrated baby rabbit complement as a source of complement. The previous observations were extended to alveolar as well as peritoneal macrophages in mice and peripheral blood neutrophils and alveolar macrophages in humans as well as monocyte and neutrophil like human cells lines.

For all immune cells tested, the presence of antibody was necessary for P4 to up-regulate phagocytic function. Antibody bound to pathogen initiates phagocytosis via FcγR and activates complement through classical activation of the complement cascade. As such, the absence of antibody greatly diminishes the ability for FcγR phagocytosis, complement activation and subsequent phagocytosis to occur.

Similarly, the presence of complement was necessary for P4 augmented phagocytosis. This could be due to the regulatory function of FcγR by complement. Increased expression of activating Fcγ receptors is dependent on the presence of C5a, an anaphylactic protein activated in the final stages of the

complement cascade [80]. Similarly, the production of C5a protein is dependent on the presence of Fcγ receptors [81] suggesting that complement acts in a way that regulates FcγR expression and vice versa [412]. As such, it may be that enhanced Fcγ expression is dependent on the presence and activation of complement and the P4 stimulation does not override complement regulatory mechanisms. Further work looking at the role of complement during API is needed to confirm this. The necessity of the presence of serum components in theses assays, however, suggests that P4 augments a natural process of phagocytosis and does not cause unspecific bacterial killing through other mechanism.

Many patients suffering from chronic obstructive pulmonary disease (COPD) are colonized by bacteria in their lower respiratory tract and >50% of COPD exacerbations are associated with bacteria, which was suggested to be due to a reduced ability of phagocytes to clear pathogens [413]. Similarly, neutrophils from septic patients show reduced phagocytosis [414, 415] as do immature neutrophils released from the bone marrow during sepsis [416]. Septic patients who have neutrophils with reduced phagocytic activity have lower chances of survival [417]. Phagocytosis is thus an important component of host responses in critically ill patients but relies on the presence of opsonins for effective clearance of pathogens. The ability of API to upregulate phagocyte function and clear IVIG opsonised pathogens therefore offers a potential new treatment strategy against fulminant bacterial infections.

Nature has provided … in the phagocytes … a natural means of devouring and destroying all disease germs. There is at bottom only one genuinely scientific treatment for all diseases, and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion.

The Doctor's Dilemma, *George Bernard Shaw, 1911*

Publications arising from work in this thesis

Mathieu Bangert, Adam K Wright, Jamie Rylance, Matthew J Kelly, Gowrisankar Rajam, Edwin W Ades, Aras Kadioglu and Stephen B Gordon. "P4 peptide augments human lung defense by modulating alveolar macrophage and neutrophil phagocytosis" (manuscript prepared).

Mathieu Bangert, Laura M. Bricio, Suzanna Gore, Gowrisankar Rajam, Edwin W. Ades, Stephen B. Gordon, Aras Kadioglu. "P4 mediated antibody therapy in an acute model of pneumococcal disease." *The Journal of Infectious Diseases* (2012):205(9):1399-1407

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Daniel R. Neill, Sarah Smeaton, **Mathieu Bangert** and Aras Kadioglu. "Nasopharyngeal carriage with *Streptococcus pneumoniae* augments the immunising effect of pneumolysin derivative PdB". *Journal of Allergy and Clinical Immunology* (2012, *In press*)

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