Pneumolysin-macrophage interactions in *Streptococcus pneumoniae* infection

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By

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Abstract

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Streptococcus pneumoniae is a common human pathogen that accounts for more than 1 million deaths every year. Nasopharyngeal colonisation by *S. pneumoniae* is a necessary precursor to pneumonia and invasive disease and, thus, is a promising target for intervention. Pneumococcal virulence factors that maintain long-term carriage are of interest to the improvement of pneumococcal vaccines which currently fail to provide the required protection against >92 known serotypes of *S. pneumoniae*. The C-type lectin family member mannose receptor (MR) is expressed by macrophages and dendritic cells and has been identified as a non-opsonic receptor for *S. pneumoniae* in the lung. However, its functional role in pneumococcal infection has not been revealed, nor has its impact on nasopharyngeal carriage been assessed. I used MR-deficient mice and bone marrow derived macrophages to study the role of this receptor in the clearance of *S. pneumoniae*. Macrophages up regulate MR expression in response to pneumococcal infection both *in vitro* and *in vivo*, via a process dependent upon pneumococcal capsular polysaccharides and pneumolysin toxin. Furthermore, MR-expressing macrophages accumulate in the nasopharynx and draining cervical lymph nodes of mice during pneumococcal carriage. $MR^{-/-}$ macrophages are significantly attenuated in their ability to kill *S. pneumoniae* D39 *in vitro* and show reduced production of both inflammatory and immunomodulatory cytokines and chemokines, as compared to WT macrophages, in response to *S. pneumoniae*. MR is required for upregulation of expression of TLR-2 on macrophages in response to pneumococcal infection *in vitro* and also contributes to the activation of the NLRP3 inflammasome and the production of IL-1β. Domain four of the pneumococcal toxin pneumolysin binds MR, demonstrating that MR contributes directly to host-pathogen interactions. $MR^{-/-}$ mice have a defect in control of pneumococcal proliferation in the nasopharynx in the first 48 hours post-infection but accumulation of MR^{+} macrophages in the nasopharynx in wild-type mice takes place over weeks, suggesting a dual role of MR in control of both innate and adaptive immunity. The ability of MR^{+} macrophages to induce the differentiation of T regulatory cells *in vitro* suggests that they may contribute to the maintenance of prolonged carriage, in addition to their role in early clearance of colonising bacteria. Proteomic analysis reveals that pneumococcal infection induces a wide range of cellular processes in macrophages, with pro-inflammatory and apoptotic pathways particularly prominent. Macrophages infected with pneumolysin-deficient pneumococci produce high levels of proteins associated with healing and repair and are less geared towards inflammation, demonstrating the ability of pneumolysin to shape immune responses during infection. Taken together, my data add to our understanding of the interactions between *S. pneumoniae* and the host immune system. MR is a key contributor to macrophage responses against the pneumococcus and pneumolysin is both a crucial virulence factor and an inducer of host immune responses.

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Abbreviations

%	Percentage	ºC	Degrees centigrade
G	Gravity force	Amps	Amperes
BAB	Blood agar base	BHI	Brain heart infusion
CLN	Cervical lymph nodes	CFU	Colony forming units
CFU/ml	Colony forming units per milliliter	CNS	Central nervous system
CRP	C-reactive protein	dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay	Tris	Tris base
FACS	Fluorescence activated cells sorting	FCS	Fetal calf serum
GFP	Green fluorescent protein	M	Moles
HCl	Hydrochloric acid	IgG	Immunoglubulin G
I.n.	Intranasal	IgA	Immunoglobulin A
IgG	Immunoglubulin G	kDa	Kilodaltons
LPS	Lipopolyssacharide	LytA	Autolysin A
LytA	Autolysin A	ml	Millilitres
mg	milligrams	µl	Microlitre
MHC	Major histocompatibility complex	mg/ml	Milligrams per millilitres
mM	Millimoles	mins	Minutes
M	Moles	NaCl	Sodium chloride
NALT	Nasal associated lymphoid tissue	PBS	Phosphate buffered saline
PMN	Polymorphonuclear leucocytes	PLY	Pneumolysin
PspC	Pneumococcal surface protein C	MBL	Mannan binding lectin
PLY	Pneumolysin	O.D.	Optical density
RPM	Revolutions per minute	ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate	Tris	Buffered saline with tween
TEMED	Tetramethylethylenediamine	TMB	Tetramethylbenzidine

Tris	Tris base	WHO	World Health Organisation
HBSS	Hanks balanced salt solution	PspA	Pneumococcal surface protein A
IPD	Invasive pneumococcal disease	BMDMs	Bone marrow derived macrophags

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Chapter 1: Introduction

1.1 *Streptococcus pneumoniae*

1.1.1 Description

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive bacterium with distinctive diplococcoid morphology. It is encapsulated, alpha-hemolytic, and a facultative anaerobe that can be distinguished from other streptococcal species by optochin test, due to its sensitivity to this antibiotic. Individual cells are between 0.5 - 1.25 micrometers in diameter. Like other streptococcal species, they are catalase negative and able to ferment glucose to lactic acid (Struthers et al, 2003, Ryan et al, 2004, Frost et al, 2010).

The pneumococcus is a major human pathogen that was first recognized as a common cause of pneumonia in the late 19th century and has been the focus of much research since. The spectrum of pneumococcal disease includes pneumonia, bacteremia, sepsis, peritonitis, osteomyelitis, acute sinusitis, endocarditis, otitis media, cellulitis, pericarditis, and brain abscesses (Ryan et al, 2004). Recent work has also revealed a large burden of pneumococcal meningitis in developed countries and in large parts of Africa (Trivedi et al, 2010).

The pneumococcus is normally present in the nasopharynx of healthy adults (5-10%), and healthy children (20- 40%) in developed countries (Harboe et al, 2012). Faden et al. has shown that the nasopharyngeal flora in children usually established during the first weeks of their lives (Faden et al., 1997). The pathogen is believed to transmit more often in certain crowded environments (Brooks et al, 2010, Hamborsky and Kroger, 2015). Although mainly a harmless commensal in the nasopharynx, the pneumococcus can cause a range of diseases when it reaches other, normally sterile sites. Susceptibility to the pneumococcus is significantly age-related with the greatest disease burden in children under 5 years of age

and adults over 65 (Trivedi et al, 2010). Whereas the immaturity of the immune system is mainly responsible for poor protection in the former, the underlying causes of sensitivity in older adults is more complex including immune senescence and other cofactors (Gonçalves et al., 2016).

1.2 *S. pneumoniae* as a pathogen

1.2.1 Pneumococcal disease

Streptococcus pneumoniae has been recognized as a significant human pathogen for over 100 years and continues to be a common cause of diseases and mortality worldwide. The pathogen has been considered to be one of the most common bacterial respiratory pathogens, as it has been shown to be the main cause of meningitis and community-acquired pneumonia (CAP) in developed countries, which has greater than 20% mortality rates, with death usually the result of overwhelming septicemia (Rudan et al., 2008). Worldwide, mortality rates are unacceptably high; for example, pneumococcal septicemia is responsible for around 25% of preventable deaths in children under the age of 5 and more than 1.2 million infant deaths per year (Denny et al., 1986). Also, it has been reported that more than 800,000 children die annually in developing countries as a result of pneumococcal disease, particularly pneumonia (O'Brien et al., 2009). Furthermore, the pneumococcus has been shown to be the leading cause of acute otitis media (AOM) in developed countries among children (McEllistrem et al., 2005). Interestingly, in countries with high rates of HIV-1 infections, there are also increased rates of pneumococcal pneumonia and bacteremia cases, mostly in young adults (Berkley et al., 2005). The pneumococcus inhabits the mucosal surface of the upper respiratory tract, and can be cultured from the naso–oropharynx of humans and, infrequently, other large animals that live in close proximity to humans.

Although colonisation at this site seems to be asymptomatic, if the pathogen gains access to the lower airway, then a rapid inflammatory response develops which can lead to invasive disease (Kyaw et al., 2002, Bogaert et al., 2004).

1.2.2 Pneumococcal colonisation

S. *pneumoniae* has evolved over time to live in the human population, and to colonize the mucosal surfaces of the upper respiratory tract. The success of pneumococcal survival in the human population is verified by 1.9 - 5.8 billion individuals thought to be inhabited with pneumococcus at any given time (Bogaert et al., 2004, Rudan et al., 2008, O'Brien et al., 2009). Although S. *pneumoniae* has the ability to cause severe invasive disease, most pneumococcal-colonized people will not exhibit any clinical symptoms.

Colonisation is usually followed by the distribution of the pneumococcus to other individuals, leading to transmission within the population (Faden et al, 1990, Bogaert et al., 2004, Malley et al., 2007). Bacterial acquisition and carriage rates are influenced by many different factors such as: geographical area, age, genetic background, and socioeconomic conditions (Bogaert et al., 2001, Adrian et al., 2004). In addition, the rates of carriage are different among the 90 identified pneumococcal capsular serotypes (Park et al., 2007), which express antigenically and structurally different capsular polysaccharides (Calix et al., 2012). Protection against pneumocccal diseases is facilitated by opsonin-dependent phagocytosis. Antibody and complement-dependent opsonisation that initiates the classic complement pathway is believed to be the main immune mechanism by which the host protects itself against infection with the pneumococcus (Paton et al., 1993). Pneumococcal clearance depends on the interaction of type-specific antibodies (IgG, IgA, IgM), neutrophils and complement or phagocytic cells from liver and spleen (Bruyn et al., 1992).

The local host immune response has a significant regulatory role in the trafficking of microbes in the upper part of the respiratory system (Weinberger et al., 2009). A weak mucosal immune response may lead to persistent and periodic colonisation and subsequently infection, while a rapid immune response to the pathogen will lead to abolishment of colonisation and preclude re-colonisation (Harabuchi et al., 1994). As mucosal immunity develops earlier than systemic immunity, and is thought to be well developed by 6 months of age (Ghaffar et al., 1999, Bogaert et al., 2004), the early change in pneumococcal carriage rates is believed to be associated with the maturation of the immune system, since children at the age of 2 have been shown to generate weak antibody responses to thymus independent antigens for example; pneumococcal capsule polysaccharide (CPS) (Douglas et al., 1983, Abdullahi et al., 2008), and generate notably smaller amounts of anti-capsular IgG than their mother following colonisation with a particular serotype (Soininen et al., 2001). Some research has also revealed that IgG and secretory IgA antibodies against pneumococcal capsular polysaccharides and surface associated proteins have been detected in children's saliva in response to pneumococcal colonisation (Simell et al., 2001).

Furthermore, another study by Rodenburg et al. demonstrated salivary immune responses in children to the 7-valent pneumococcal conjugate vaccine in the first 2 years of life (Rodenburg et al., 2012). At 12 months, higher serum and saliva IgG-levels were observed against vaccine serotypes when compared to controls (Non-vaccinated kids), which continued for most serotypes until 24 months. Salivary IgG-levels were 10-20 fold lower compared to serum IgG, conversely, the IgG-levels in saliva and serum were greatly associated. Salivary and serum IgA-levels were greater at 12 months in children compared with controls. Greater salivary IgA levels stayed present for most serotypes till 24 months. Salivary IgA increased more than IgG, following carriage of serotypes 19F, 6B and 23F. In

conversion to IgG, salivary IgA-levels were similar to serum levels, suggesting local production of IgA. Interestingly, it has been suggested that competition for the space in the nasopharynx could affect the age associated prevalence of S. *pneumoniae*, since, while the rate of pneumococcal carriage decreases, colonisation of another nasopharyngeal resident such as *Staphylococcus aureus* increases, from about 10% to 50% at the age of 10 years (Bogaert et al., 2004). Moreover, a report by Faden et al. has proposed that interspecies competition develops and interfere with the natural composition of the nasopharyngeal flora during pneumococcal carriage (Faden at al., 1997). The balance between the microbial invaders and the local flora is significant. For example: α-haemolytic oral Streptococci (part of the resident flora) prevent colonisation by *S pneumoniae, S aureus* and *H influenzae* (Faden et al., 1997). The importance of this action was also shown by Ghaffar et al. (1999), when they demonstrated a competitive balance between α-haemolytic Streptococci and *S pneumoniae* and *H influenzae* that could be affected by the usage of antibiotics (Ghaffar et al., 1999). However, Madhi et al. has shown that the colonisation patterns for pneumococcus, *S. aureus* and *H. influenzae* did not change among HIV-infected and HIV-uninfected children (Madhi et al., 2005). Furthermore, a negative association has been reported between viridans streptococci and *S pneumoniae, H influenzae*, and *M catarrhalis*, since typically one pathogen (*H. influenza*) becomes predominant during upper respiratory tract infections (Faden et al., 1990, Ghaffar et al., 2002). Another study has showed that there were no constant variations in the carriage rates of *S. pneumoniae, S. aureus, M. catarrhalis, and H. influenzae* were observed over time (Spijkerman et al., 2012).

In addition to the huge shifts in pneumococcal serotypes, persistent high nasopharyngeal occurrence rates of *H. influenzae* and *S. aureus* were identified between young children and their parents following presentation of 7-valent pneumococcal conjugate vaccine (PCV-7). Pericone et al, have shown from in-vitro studies that a positive relationship exists between *S.*

pneumoniae and *N. meningitides* (Pericone et al., 2000). *S pneumoniae* growth is increased in the presence of *H. influenzae*, the growth rate was mediated by the catalase produced by *H. influenzae*. On the other hand, the growth of *H. influenzae* was reduced in the presence of the pneumococcal culture supernatant or pneumococci, the growth rate were affected by the presence of hydrogen peroxide, which known to be produced by pneumococcus as a mechanism for limiting competitive flora (Pericone et al., 2000).

1.2.3 Mechanisms of colonisation

Experimental colonisation studies in adults have been used to determine the host factors that could affect sensitivity to the acquisition of *S. pneumoniae* and its consequent clearance (McCool et al., 2002). More recently, Ferreira et al. validated that pneumococcal carriage in humans resulted in systemic and mucosal immunological responses that led to protection against re-colonisation and invasive pneumococcal infections (Ferreira et al., 2013). These findings show that carriage stimulates the production of both mucosal and systemic antibodies, which are generally strain and type specific. Although high levels of serotype-specific anti-capsular antibodies are generated following administration with the pneumococcal polysaccharide conjugate vaccine (PCV), it is not fully understood whether the relatively small amounts that are produced by colonisation are effective at improving clearance. In this respect, it has been observed that after childhood, carriage rates fall down among the different pneumococcal serotypes, which suggests that exposure to earlier colonisation events leads to immunity that is not strictly serotype-specific (Lipsitch et al., 2005). The pneumococcal external surfaces are protected and covered by a polysaccharide capsule. Capsular polysaccharides are varied, and about 100 different capsular serotypes have been defined so far (Malley and Anderson, 2012). The polysaccharide capsule is one of

the most significant virulence factors of pneumococci since it protects the microbe from phagocytosis by host immune cells (Kadioglu et al., 2008). Recent studies have determined the structure of capsular polysaccharide for various serotypes using advanced analytical technologies, providing explanation of genetic basis for the capsular types, and demonstrating the improvement of highly efficient pneumococcal conjugate vaccines (Habib et al., 2014, Geno et al., 2015, Park et al., 2015). Low expression levels of capsule will lead to increased complement and antibody deposition on the pneumococcal surface and consequently increased clearance by the immune system (Magee et al., 2001). Polysaccharide capsules are highly immunogenic since host antibodies deliver protection against homologous serotype infections by the stimulation of opsonophagocytosis. Capsule antigenicity is type-specific, although cross-reaction can occur due to common shared polysaccharides (Bruyn et al., 1992). Hyams et al. investigated the effects of capsular serotype on *S. pneumoniae* interactions with complement (Hyams et al., 2010). Significant differences in the deposition of C3b/iC3b were observed on opaque-phase variants of serotype 4 (TIGR4), 6A and 23F strains although the thicknesses of the capsule layers were similar. There was increased deposition of C3b/iC3b on TIGR4, 6A and +23F strains, and these variances remained even in serum depleted of immunoglobulin G, suggesting pneumoccocal resistance to complement-mediated immunity is dependent on the capsular serotype rather than capsule thickness. The layer under the capsule is the cell wall. It consists of a mixture of polysaccharides and teichoic acid that function as an anchor for cell wall associated surface proteins (Bruyn et al., 1992). The cell wall is the main cause of the strong inflammatory response that accompanies pneumococcal infections, as it activates the complement cascade, encourages the influx of inflammatory cells and induces cytokine production (Bruyn et al., 1991). To support this, a study by Moronal et al. has revealed that attachment of capsular polysaccharide to the cell wall of *Streptococcus pneumoniae* type 2 is required for invasive disease (Morona et al., 2006). Nasopharyngeal colonisation by *S.*

pneumoniae requires attachment to the epithelial lining of the respiratory tract. Successful adherence to nasopharynx epithelium has been considered to be the first important step to initiate the pathogenesis of pneumococcal asymptomatic carriage and diseases (Andersson et al., 1983). Asymptomatic colonisation initiates on non-inflamed resting epithelium by the binding of the pneumococcus to cell surface sugars (N-acetyl-glycosamine). Adherence to these carbohydrates is facilitated by some of the cell-wall-associated surface proteins such as pneumococcal surface adhesin A (PsaA) (Swiatlo et al., 2002). This surface protein is thought to be significant since it contributes to the hydrophobic and electrostatic surface features of the pneumococcus and facilitates partial adherence to host cells through physicochemical, non-specific interactions (Swiatlo et al., 2002). The identification of PsaA peptides has also showed to be important in the development of pneumococcal vaccine. Singh et al. has illustrated spleen and cervical lymph node (CLN) -derived T helper (Th) lymphocyte cytokine responses to PsaA peptides after challenging mice with S. pneumoniae strain EF3030 (Singh et al., 2014). Some of these peptides were responsible about the high responses and proliferation of interferon- γ , IL-2, IL-5 and IL-17, and the moderate responses of IL-10 and IL-4 by ex vivo re-stimulated splenic and CLN CD4+ T cells that isolated from challenged BALB/c mice with the pneumococcal strain EF3030.

The establishment of long-term pneumococcal colonisation and the transformation of asymptomatic colonisation to invasive infection require the help from several pneumococcal virulence factors at different structural sites (Orihuela *et al*., 2004). For example, the stimulation of local inflammatory factors during concurrent viral infections such as Tumour Necrosis Factor (TNF) and Interleukin1 (IL-1) are required for the transformation of asymptomatic colonisation to invasive diseases (Tuomanen et al., 1997). This inflammatory cascade affects the types and the number of receptors expressed on target epithelial and endothelial cells, facilitating greater pneumococcal adhesion and invasion.

1.3 Pneumococcal virulence factors

The pathogenicity of *S. pneumoniae* has been attributed to several virulence factors, the most significant being the capsule, and the toxin pneumolysin. The polysaccharide capsule helps the pneumococcus avoid phagocytosis and encourages its escape from host immune defenses (Chen et al., 2005). Pneumolysin is another significant virulence factor and a key driver of inflammation during pneumococcal infections. Both capsule and pneumolysin have been shown to have a wide range of important functions at different sites in the human or animal host. These include involvement in nasopharyngeal carriage and contributions to invasive diseases. Several studies in this field have been motivated by the understanding that the pneumococcal capsule and the multifunctional toxin PLY could be promising pneumococcal vaccine candidates. Inflammation is believed to be responsible for many of the symptoms of pneumococcal diseases (Johnston, 1991, Musher, 1992), and so the inflammatory properties of pneumolysin could be directly responsible for the high morbidity and mortality associated with pneumococcal infections. More details will be given in the next sections about these two factors, with a focus on their structures, functions and role in virulence.

1.3.1 Pneumococcal capsule

The pneumococcal polysaccharide capsule is 200-400 nm thick. It covers the outer layer of *S. pneumoniae*, and covalently attaches to the external part of the cell-wall peptidoglycan (Skov-Sorensen et al., 1988, Sorensen et al., 1990). There are 92 structurally and serologically described capsular serotypes of *S.pneumoniae* (Henrichsen, 1995, Kadioglu et al., 2008, Yother, 2011). Pneumococcal capsule structures vary between linear polymers composed of two monosaccharaides or more, to branched polysaccharides that have one to

six monosaccharaides in combination with extra side chains (Sorensen et al., 1990). The capsules of most serotypes of pneumococci are highly charged at physiological pH, which could directly influence the interaction with phagocytes (Lee et al., 1991). Another common feature among these different types of capsules is that none display a net positive charge (Hammerschmidt et al., 2005). The production of capsule is required for *S. pneumoniae* virulence, and is anti-phagocytic in non-immune hosts (Austrian, 1981, Hathaway et al., 2012). Although, non-encapsulated strains of *S. pneumoniae* have been associated with some superficial infections (Martin et al., 2003, Crum et al., 2004), clinical samples from different sterile sites are encapsulated, and unprompted non-encapsulated products of these pneumococcal strains are mainly a virulent.

Furthermore, some studies show that the virulence of pneumococci is correlated with the thickness of the pneumococcal capsule (MacLeod and Krauss, 1950, Kung et al., 2014) and different serotypes vary significantly in their capacity to cause infections (Austrian, 1981). Differences in the thickness of the capsule could lead to resistance to phagocytosis, alter stimulation of humoral immune responses, and confer adaptation to colonisation of different niches of the human body. Thicker capsules have shown reduction in entrapment within mucus (Nelson et al., 2007, Kung et al., 2014), and in opsonophagocytosis, leading to increased survival of the pathogen in the blood stream (Hyams et al., 2010). Whereas, thinner polysaccharide capsules offer an advantage to pneumococci to adhere to host tissues, due to the greater exposure of adhesion molecules (Magee & Yother, 2001, Kung et al., 2014). Exciting data have demonstrated that pneumococcal capsule can block the Fc region in IgG or complement component iC3b in order to prevent their interaction with the appropriate receptors on phagocytic cells (Musher, 1992, Hyams et al., 2010). Also, some data showed that pneumococcal capsule could form a protective shield to inhibit pneumococcal phagocytosis and killing by macrophages and neutrophils (Mitchell, 2003,

Wartha et al., 2007, Kadioglu et al., 2008, Levitz et al., 2012) and reduce the amount of complement (mainly C3b) deposited on the surface of the pathogen (Abeyta et al., 2003, Mitchell, 2003). This affords *S. pneumoniae* the advantage of colonizing and infecting different niches of the host (Ogunniyi et al., 2002, Hathaway et al., 2012). One study reported that the pneumococcal capsule could provide a noticeable level of resistance to antibiotic-induced autolysis, resulting in antibiotic tolerance in clinical isolates (Fernebro et al., 2004). Interestingly, the degree of toleration varied remarkably between the capsular serotypes. Although *S. pneumoniae* strains express different capsular types that show noticeable differences in virulence, non-capsular factors are also clearly significant (Kelly et al., 1994, Nesin et al., 1998, Hathaway et al., 2012). Recent molecular epidemiological analysis has determined that features that are mainly associated with a specific clonal type, as well as capsular serotype, influence the chance of pneumococci to cause serious infections in humans. A subsequent study demonstrated the contribution of host factors in which clinical isolates that had great human invasion potential presented significantly diverse virulence and infection kinetics in BALB/c mice when compared to C57BL/6 mice (Sandgren et al., 2005, Hathaway et al., 2014).

1.3.2 Pneumolysin

The pneumococcal toxin pneumolysin (PLY) was first described as a hemolysin produced by pneumococci in 1905 (Libman, 1905). PLY is a 53kDa intracellular soluble protein that belongs to the cholesterol-dependent cytolysin (CDC) family (Alouf and Geoffroy, 1991, Boulnois, 1992) It is expressed by virtually all pneumococcal isolates, and its amino acid sequence is well conserved (Lock et al., 1996, Kirkham et al., 2006). Several early studies described the biological effects of pneumolysin on polymorphonuclear leukocytes, and these were determined at the sub-lethal concentrations of the toxin in the absence of cell lysis

(Johnson et al., 1981). Some experiments demonstrated that PLY was present in the cytoplasm of the bacteria and was not secreted, a discovery which has essential consequences to understand the role of pneumolysin in the processes of disease (Johnson, 1977). PLY is released upon lysis of the pathogen, for instance, as a result of autolysis, impulsive bacterial cell death, due to phagolysosome degradation, or antibiotic treatment. One recent work suggested that PLY could be attached to the cell wall of the pneumococcus, however the mechanisms of active export have not been identified and the finding has not been replicated (Price & Camilli, 2009, Price et al, 2012). Moreover, an important study in mice showed that purified pneumolysin is a protective immunogen against pneumococcal infections in pneumonia models (Paton et al., 1983). This work led to many other investigations in order to develop pneumolysin-based vaccines, which may still play a significant role in reducing the frequency of pneumococcal infections in humans in the future.

1.3.2.1 The structure of Pneumolysin

PLY is a mono polypeptide chain that consists of 471 amino acids (Walker et al., 1987). Pneumolysin oligomerizes in the cellular membrane of target cells leading to formation of a ring-shaped pore. The transmembrane pore is composed of 40 monomer subunits that are 260 Å in diameter. The toxin undergoes sequences of remarkable changes in structure during its transformation from a soluble monomer to a membrane-inserted oligomer form (Tilley et al., 2005). It has been thought that the oligomers are responsible for both the cytolytic activity of pneumolysin and the cell modulatory events induced at sub-lytic concentrations. A pneumolysin homology model (Figure 1.2) shows that the toxin is long and rod shaped and composed of four domains (domains 1-3 form the N-terminal fragment and domain 4 forms the C-terminal part) that have particular functions in the formation of

membrane pores (Baba et al., 2001). Domain-4 has been identified to be necessary for cholesterol binding, and for the haemolytic activity of PLY (Gilbert et al., 1999, Tilley et al., 2005).

Figure 1.1. Structural model of the pneumococcal toxin pneumolysin. PLY has four domains; domain 1 (blue), domain 2 (green), domain 3 (red) with two groups of alpha helices (highlighted in orange) that play a significant role in the formation of pore in the host transmembrane, and domain 4 (yellow) associated with membrane binding (picture source; Tilley et al., 2005).

1.3.2.2 The role of pneumolysin in pathogenesis

The cytotoxic and hemolytic activities of PLY have been well defined over the last 70 years of research (Cohen et al., 1942, Halbert et al., 1946, Kreger & Bernheimer, 1969). The oligomeric pores formed at high concentration cause cell lysis (Boulnois, 1992). However, the toxin also has several important functions at lower concentrations, most of which have been demonstrated both in vitro and in vivo. PLY at sub-lytic concentrations has an ability to alter the function of immune cells (Mitchell et al., 1993, Alexander et al., 1998), encourage the production of proinflammatory cytokines such as interleukin-1β and tumor

necrosis factor alpha (TNF-α) by human monocytes (Houldsworth et al., 1994), prevent ciliary beating on human respiratory epithelial cells (Feldman et al., 1990, Feldman et al., 1991), damage alveolar epithelial barriers (Rubins et al., 1993) and the upper part of the respiratory tract (Feldman et al., 1990), and reduce neutrophil respiratory burst but stimulate their recruitment (Paton et al, 1983). PLY is also known to initiate the classical pathway of complement (Mitchell, et al., 1991, Paton et al., 1993), prevent the proliferation of lymphocytes and the synthesis of antibodies (Ferrante et al., 1984), and induce cytokine synthesis and CD4+ T-cell activation and chemotaxis (Hirst et al., 2004, Kadioglu et al., 2004). However, it is not clear if any of these features of the pneumococcal protein are necessary for virulence. The significance of pneumolysin as a key pneumococcal virulence factor was revealed clearly when PLY-negative pneumococcus was shown to be avirulent in mice when compared to the wild-type strain (Berry et al., 1989, Canvin et al., 1995). The PLY-deficient strain grew more slowly in the lungs, and showed reduced induction of cellular inflammatory responses (Canvin et al., 1995, Kadioglu et al., 2000). Although the levels and expression of pneumolysin may vary from strain to strain or serotype to serotype, several studies confirm that PLY is essential for pneumococcal virulence during pneumonia (Berry et al., 1995, Canvin et al., 1995, Rubin et al., 1996, Alexander et al., 1998, Jounblat et al., 2003), For instance, Alexander and colleagues (Alexander et al., 1994) discovered that mice immunized with sub-lethal doses of PLY were protected from nine strains of *S. pneumoniae*, although no protection was detected against a tenth strain. Another immunization study performed by Paton et al. exposed that inactivated PLY toxoid could provide sufficient protection against invasive pneumococcal infection in toxoid immunized mice (Paton et al., 1983). Moreover, the immunization of mice with PLY prolongs their survival after challenge with lethal doses of different serotypes of *S. pneumoniae* (Paton et al., 1983, Lock et al., 1988, Alexander et al., 1994). Pneumolysin is important for the spread of pneumococcus from the lungs to the bloodstream (Berry et al., 1989, Berry et al., 1999,

Kadioglu et al., 2002, Orihuela et al., 2004). In addition, some studies reveal that in bacteraemic infection the expression of pneumolysin is correlated with disease severity and high numbers of pneumococci in the blood (Berry et al., 1999, Orihuela et al., 2004). Conversely though, in the absence of the toxin, high numbers of the bacteria were tolerated in the bloodstream without any visible signs of infection (Kadioglu et al., 2000), suggesting pneumolysin is a major driver of pathology during tissue infection, but that its role is diminished once pneumococci reach the blood. It would appear therefore that Ply is a key virulence factor whose main function is restricted to the mucosal surfaces of the upper and lower airways.

1.4 Host defense systems

1.4.1 Innate immune response to *S. pneumoniae*

The term "innate immune system" covers a broad range of host defenses, involving mucociliary clearance, complement, neutrophils and macrophages. Innate immunity behaves as a non-specific defense that is able to identify and react rapidly against many pathogens. On the other hand, the adaptive immune system consists of pathogen antigen specific host defenses, coordinated by T and B cells. Pathogen recognition by the innate immune system is commonly achieved throughout a specific set of germline-encoded receptors without capacity for immunological memory. The arms of the immune system do not, however, work in isolation, since innate immunity plays important roles in the induction and development of the adaptive immune system. Therefore, in order to understand how these two immune systems recognize and respond to the pneumococcus, the next two sections will discuss these interactions in detail.

The responses of mucosal immunity in humans are a primary determinant of whether colonisation is cleared or long-term carriage arises. Immune response to *S. pneumoniae* is mediated mainly through the inflammatory response in the nasopharyngeal mucosa employing phagocytes such as neutrophils and macrophages, which identify and engulf and kill the pathogen, with the initiation of Th17 cells being necessary to maintain the recruitment of neutrophils and clear infection (Gonçalves et al., 2016).

The significance of neutrophil infiltration into lungs was revealed when a study by Kadioglu et al. showed that in mouse lungs infected with serotype 2 (D39) a significant infiltration of neutrophils was seen within 12 h of infection. Lung tissues were greatly infiltrated with neutrophils within 24 h, however their amounts were significantly decreased by 48 h post-infection. Interestingly, the number of neutrophils where higher than macrophages at equivalent time points, suggesting the importance of neutrophils in response to S. pneumoniae infections (Kadioglu et al., 2000). Furthermore, Van Rossum et al. has exposed noticeable inflammatory responses in mice during the first three days of pneumococcal colonisation. The inflammation in upper respiratory tract lavage was defined by the infiltration of neutrophils into the luminal spaces, however the influx of these cells did not aid bacterial clearance (Van Rossum et al., 2005). It has been observed that the accumulation of neutrophils was correlated with the increase of chemokine macrophage inflammatory protein-2 (MIP-2) production, which was dependent upon pneumolysin (PLY)-mediated pore formation, and the activation of mitogen-activated protein kinase p38 signaling pathways (Ratner et al., 2006). A recent study by Wilson et al. has shown that protection against serotype 19F S. pneumoniae lung infection after nasopharyngeal colonisation requires the response of neutrophils, since host protection was absent in neutrophil-deficient mice (Wilson et al., 2015).

Inflammation is mainly controlled by the recognition of bacteria through Toll-like receptors (TLR's), leading to activation of both inflammatory and modulatory signaling pathways (Van Rossum et al., 2005). These include the activation of TGF-β pathways to modulate epithelial barrier function and IL-6 signaling to recruit inflammatory mediators such as neutrophils into the luminal surface and thus help in the clearance of the bacteria (Beisswenger et al., 2009). Pneumococcal colonisation in mice has been shown to be more severe in the absence of TLR-2 (Van Rossum et al., 2005), TLR-4 and MyD88 (TLR signaling molecule) (Malley et al., 2003). Stimulation of isolated alveolar macrophages to generate TNF-α in response to heat-killed pneumococci is TLR-2 dependent (Knapp et al., 2004). Conversely, the immunohistochemical staining of infected lungs from TLR-$2^{-/-}$ mice has revealed that macrophages were generating high level of TNF-α comparable to that from wild-type mice. TLR-4 has also been suggested to be significant in pneumolysin-dependent activation of macrophages since TLR-4 mutant mice were susceptible to lethal infection after intranasal colonisation with pneumolysin-positive pneumococci than were control mice (Malley et al., 2003). However, in contrast to this earlier study, a more recent paper by McNeela and colleagues have showed that PLY has the ability to increase the production of cytokines independently of TLR4 (McNeela et al., 2010), as PLY and heat-killed S. *pneumoniae* stimulated splenocytes were able to generate both IFN-γ and IL-17 independently of TLR-4.

1.4.1.1 The role of macrophages in response to pneumococcal infection

In the respiratory tract, resident macrophages (alveolar macrophages) are the main effector cells in the early stages of host defense against respiratory infections (Janeway et al., 2006), due to their ability to phagocytose pathogens that may reach the terminal bronchioles and alveoli (Gordon et al., 2003). Macrophages have been considered as a significant innate

immune cells that have roles in the initiation and resolution of tissue inflammation (Gordon et al., 2003). Macrophages regulate the host inflammatory response to infection through production of antimicrobial molecules, the generation of cytokines and chemokines, and the presentation of microbial antigens on MHC, thus connecting innate to adaptive immunity (Martinez-Pomares and Gordon, 2012). However, the particular role for macrophages in host defence against pneumococcal infections is unclear.

Pneumococcal pneumonia cases have been examined and investigations have been made to study the mechanisms responsible for the resolution of inflammation and restoration of tissue homeostasis (Zychilnsky et al., 1997). These have revealed that long-term inflammation, which leads to organ failure and tissue damage, does not happen because of the persistent pro-inflammatory incidents alone, but could also as the result of inefficient resolution processes (Dockrell et al., 2012). Macrophages have been identified as key cells in this resolution process, in particular through phagocytosing apoptotic polymorphonuclear cells (PMNs) (Franke-Ullmann et al., 1996). During pneumococcal pneumonia, macrophages are the first line leukocytes to respond to pathogen encounter, since they have the ability to phagocytose and kill pneumococci (Kadioglu et al., 2004). Macrophages could also assist the killing process of phagocytosed pneumococci and keep the invasion of the pneumococcus into the bloodstream at the minimum (Marriott et al., 2006). The work of several laboratories has revealed that macrophages play significant roles in the elimination of pneumococci and the resolution of severe inflammation (Gratz et al., 2011). The killing and phagocytosis of pneumococci by macrophages in vitro is associated with the induction of apoptosis, which plays an essential role in host defence against pneumococcal infections (Cole et al., 2014). Also, phagocytosis of pneumococci has shown to be affected by the polysaccharide capsule and several pneumococcal proteins including pneumolysin and pneumococcal surface protein A or C (Preston and Dockrell, 2008). The degree of inhibition

to macrophage phagocytosis varies by capsule type and the strain that is able to colonize, as thick capsules (as compared to the thin ones), provide more protection for the pneumococcus against macrophage phagocytosis (Weinberger et al., 2009). However, when large numbers of *S. pneumoniae* attack the lower airways, neutrophils are recruited immediately and they become the leading phagocytic cells, particularly in the severely inflamed lung (Knapp et al., 2003). A study by Basran et al. has demonstrated that in human neutrophils are immediately recruited during inflammation, and could regulate the amount and outcome of the inflammatory response (Basran et al., 2013), throughout the release of significant cytokines and chemokines such as IL-1β and CXCL8 in patterns that reliant on the fundamental inflammatory stimulus. MΦs are subsequently relegated to the role of clearance of apoptotic neutrophils. Phagocytosis of neutrophils by macrophages will lead to reduction in the secretion of pro-inflammatory cytokines and induction of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (McGrath et al., 2011). The recruitment of neutrophils into infected sites is very important to clear invading pneumococci (McNamee et al., 2006). This process is initiated by the production of chemokines, which could be released by macrophages (Standiford et al., 1996). During pneumococcal pneumonia, the induction of specific chemokines and the recruitment of neutrophils require upstream signaling from macrophages and, in particular, production of the early response cytokines such as TNF-α, IL-6 and IL-1β (Jones et al., 2005). The generation of these cytokines is mediated by several transcription factors, such as nuclear factor-kappa B (NF-kB). Of the NF-kB proteins, p50 is the only detectable protein in the nuclear fractions of lung during acute respiratory infection (Mizgerd et al., 2008). Saccani et al. demonstrated that pneumococcal-infected WT mice show an ability to control and limit the production of pro-inflammatory cytokines and inhibit inflammatory lung damage that is absent in p50 knockout mice (Saccani et al., 2006).

Several studies by Knapp et al. have shown the importance of macrophages against pneumococcal infections and investigated the effect of macrophage depletion on the host response to pulmonary infections; these studies have shown that macrophages are required for bacterial clearance and to reduce inflammation in lungs (Knapp et al., 2003) (Knapp et al., 2006). Furthermore, Harvey et al. demonstrated the impact of the pneumococcal toxin pneumolysin on the macrophage response to *Streptococcus pneumoniae* (Harvey et al., 2014). Strains that expressed cytolytic Ply were found to produce an important increase in IL-1β release from macrophage-like cells compared to the non-cytolytic PLY, confirming that pore formation is required for the activation of the NLRP3 inflammasome. The cytolytic activity of PLY in the D39 background was also found to induce the expression of GM-CSF, IFNβ (IFNB1) and p19 subunit of IL-23 (IL23A) when compared to the non-hemolytic and Ply-deficient D39 mutants.

The importance of macrophages in the recognition of the pneumococcal polysaccharide capsule has been revealed by Kang et al. who demonstrated the role of the capsular polysaccharide of S. pneumoniae (CPS) in the interaction of SIGN-R1 with pneumococci (Kang et al., 2004). The study has shown that macrophages expressing the C-type lectin SIGN-R1 in mouse spleen have the ability to bind pneumococci and clear the pneumococcal capsule from four different serotypes by marginal zone macrophages in vivo (Kang et al., 2004). Another study by Zamze et al. has shown that macrophage mannose receptor is able to bind the capsular polysaccharide from S. pneumoniae (Zamze et al., 2002), suggesting a potential role for the mannose receptor in the recognition of *S. pneumoniae* by innate and adaptive immune systems. Another macrophage receptor that has been suggested to play a key role in pneumococcal infection is the scavenger receptor MARCO (Arredouani et al., 2004). The genetic deletion of this receptor in mice made them more susceptible to pneumococcal pneumonia, lack the ability to clear the bacteria

from lungs, and increased their morbidity (Arredouani et al., 2004). *In vitro* isolated alveolar macrophages from the knockout mice had lost their capability to bind and engulf pneumococci. Another interesting study by Dorrington et al. has revealed that during pneumococcal colonisation MARCO is required to response and clear S. pneumoniae from nasopharynx, as MARCO -/- mice significantly lost the ability to clear S. pneumoniae from the nasopharynx (Dorrington et al., 2013).

Macrophages have also been described as important mediators for the activation of neutrophils through the release of MIP-1/2, granulocyte macrophage colony-stimulating factor (GM-CSF), and Keratinocyte chemo-attractant (KC), which are known to be involved in the activation and recruitment of neutrophils (Cailhier et al., 2006). Interestingly, it has been found that the response of innate immunity to pneumococcal infection is weak in the nasopharynx when compared to its response to the pneumococcus in either the blood or lungs (Mahdi et al., 2008). Bogaert et al. have also evaluated the immune responses of neonatal and infant mice to *S. pneumoniae* during colonisation (Bogaert et al., 2009). Like human infants, infant mice show impaired clearance of nasopharyngeal colonisation with S. pneumoniae. Macrophages from neonatal and infant mice treated with killed pneumococci in vitro revealed notably reduced cytokine production, involving granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage chemo-attractant protein 1, IL-6, IL-1α, TNF- α, and INF-γ, while IL-10 expression was considerably increased compared to that in macrophages from adult mice. The production of IL-17A from adult immune CD4+ T cells was delayed when neonatal macrophages instead of adult macrophages were used as antigen-presenting cells. Another study has shown that during nasal infection, pneumococcal colonisation was correlated with the release of high levels of significant pro-inflammatory cytokines by macrophages such as tumor necrosis factor-alpha (TNF-α),

Interleukin 6 (IL-6), monocyte chemo-attractant protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-2), which have been shown previously to be correlated with the pathogenesis of *S. pneumoniae* infections (Mahdi et al., 2008). It is known that both TNF-α (a powerful stimulator of inflammation), and IL-6 (a potent activator of neutrophils) are required for early responses of macrophages to pneumococcal infections (Borish et al., 1996, Dallaire et al., 2001). Mahdi et al has shown that both TNF-α and IL-6 were released in high amount by macrophages stimulated in the nasopharynx with virulent serotype 2 D39, but were released in smaller amounts when cells stimulated with PLY-deficient PLN-A (Mahdi et al., 2008). Furthermore, IL-12 has been shown to be significant since it encourages the production of Th-1 cytokines that boost neutrophil recruitment and enhance the humoral immune response (Arulanandam et al., 1999) (Sabirov and Metzger, 2008). Moreover, Das et al. demonstrated that the clearance of pneumococcal colonisation is promoted by Macrophage migration inhibitory factor (MIF) (Das et al., 2014). Primary human monocyte-derived macrophages and THP-1 macrophages have shown also that PLY is required for the production of MIF during pneumococcal infection, as MIF production required its pore-forming activity and phosphorylation of p38-MAPK in macrophages, with constant p38-MAPK phosphorylation abolished in the setting of MIF deficiency. Challenge with pneumolysin-deficient pneumococci showed weak MIF up-regulation, reduced number of macrophages in the nasopharynx, and poor clearance (Das et al., 2014).

1.4.2 Adaptive immunity to *S. pneumoniae*

CD4+ T cells in addition to their independent role in adaptive antigen-specific responses have an ability to play a significant role in the early response to pneumococcal infections, whereby numbers of CD4+ T cells were decreased in MHC-II knockout mice 72hrs after intranasal infection with pneumococcus as compared to wild type controls, which revealed a significant role for the T cells in response to pneumococcal pneumonia at early stages of infections (Kadioglu et al., 2004). The susceptibility of these knockout mice to pneumococcal infection was enhanced, since the number of pneumococci in blood and lungs was significantly elevated when compared to their wild type counterparts. Furthermore, the percentage of mortality in knockout mice after 3 days post-infection were 100%, while wild type mice survived and resisted the challenge (Kadioglu et al., 2000, Jounblat et al., 2003). The pneumococcal toxin pneumolysin has been shown to play an important role in the migration of T cells, since pneumolysin-negative pneumococci stimulate less T cell migration compared to WT pneumococci, but the mechanism that PLY used to stimulate these cells is still unclear and requires more investigation. However, one study has reveled that the expression of TLR-4 by T cells could be of relevance (Komai- Koma et al., 2004). A study by Malley and colleagues verified an essential function for T cells in antibody independent acquired immunity to *S. pneumoniae* colonisation (Malley et al., 2005). However, the migration and activation of CD4+ T cells in response to pneumococcal infection during nasopharyngeal colonisation is still not clear, despite having been described during pneumonia. It has been shown that CD4+ T-cells play an important role in providing acquired protection against pneumococcal disease, since mice treated with anti-CD4+ antibodies or MHC-II knockouts mice were not protected against pneumococcal infection after vaccination

with whole-cells vaccine (WCV) compared to wild-type mice (Malley et al., 2005). The significance of CD4+ T cells in response to pneumococcal infections is also evident in the increased sensitivity of HIV infected patients to pneumococcal nasopharyngeal colonisation (Low et al., 2008) and invasive infection (Dworkin et al., 2001). Furthermore, T cells have also shown their capability to deliver the required co-stimulation to B-cells that in turn encourages class switching to IgG, and the initiation of long-term memory responses (McHeyzer-Williams et al., 2009).

T-helper-1 (Th-1) cells are host immunity effectors that function against extracellular bacteria. They produce Interleukin 12 (IL-12) and interferon gamma (IFN-γ), which have been shown to provide protection against pneumococcal infections (Kemp et al., 2002), and carriage (van Rossum et al., 2005). Mice that were lacking IL-12, which is known to be a powerful stimulator for Th-1 response, lack the ability to clear colonisation inversely to Th-2 deficient mice (van Rossum et al., 2005). Furthermore, IL-18 is another common stimulator for Th-1 biased immunity via IFN-γ and an interesting study has revealed that mice lacking IL-18 were not able to clear pneumococcal colonisation (Paterson et al., 2005).

IL-17A is another mechanism the immune system uses to provide protection against the carriage of pneumococci. IL-17A is produced predominantly by a subset of T-cells, called Th-17 cells. These cells have been shown to provide effective protection against a wide range of extracellular bacteria such as *Klebsiella pneumoniae* and *S. pneumoniae* (Happel et al., 2005, Higgins et al., 2006). It has been observed that during intranasal vaccination with pneumococcal cell-wall polysaccharides the production of IL-17A cytokine by Th-17 was enhanced, while the deletion of this cytokine at the time of immunization led to a reduced protective

response of vaccination (Malley et al., 2006). Interestingly, the production of IL-17A by splenocytes has been described *in vivo* to be encouraged by the pneumococcal toxin pneumolysin (McNeela et al., 2010). Also, another study by Li et al. has suggested that IL-17 is significant to limit pneumococcal carriage, and can provide novel insight into the design of pneumococcus vaccine (Li et al., 2012). The study has showed that the stimulation of type I interferon (IFN) during a first non-lethal influenza virus infection is necessary to encourage a lethal S. pneumoniae secondary infection. Furthermore, mice lack type I interferon receptor (IFNAR knockout mice) successfully cleared the secondary pneumococcal infection from their lungs, improved the recruitment of neutrophils, and established an enhanced the expression of interleukin-17 (IL-17) relative to wild-type (WT) mice. Interestingly, the data has also revealed that lung γδ T cells were responsible for the production of IL-17, and their role was compromised during secondary pneumococcal infection of WT but not IFNAR KO mice. Also, the adoptive transfer of γδ T cells from IFNAR KO mice reduced the sensitivity to the secondary infection with S. pneumoniae in the lungs of WT mice. Clearance of *S. pneumoniae* from the nasopharynx during naïve carriage is mediated by the influx of monocytes or macrophages to the upper respiratory tract, in particular to the luminal areas, whereas a subsequent encounter of carriage will lead to infiltrate of neutrophils only to the nasal spaces (Zhang et al., 2009). Excitingly, it has been shown that the deletion of IL-17A in both instances leads to abolishment of infiltration of both neutrophils and macrophages, and consequently reduced clearance of pneumococci from the nasopharynx (Zhang et al., 2009). In summary, Th-17 cells are required to link the gap between innate and adaptive immunity, encouraging the production of antimicrobial products, and to recruit effector immune cells in response to the site of pneumococcal infections.

Regulatory T (T-reg) cells are another subpopulation of T cells with well-described roles in regulation of immune responses, maintenance of tolerance, inhibition of autoimmune disease and prevention of chronic inflammatory diseases. Recent evidence proposes that T regulatory cells play a significant role in nasopharyngeal colonisation, since children with pneumococcus positive nasopharyngeal swabs had larger number of T regulatory cells in their adenoidal tissues when compared to children with pneumococcus negative cultures (Zhang et al., 2011). A more recent study has revealed that S. *pneumoniae* encourages an immuno-regulatory response in the naso-oropharynx, defined by significant infiltration of T regulatory cells and production of transforming growth factor beta (TGF-β), leading to long-term asymptomatic carriage. The study has shown that mice intranasally infected with PLY-negative mutant of *S. pneumoniae* D39 (PLN-A), at the same low dose that establishes persistent carriage when wild-type (WT) D39 are used, fails to encourage carriage but leads to short-term colonisation (Richard et al., 2010). The study suggested that the failure to carry high concentrations of D39 or a lower concentration of PLN-A could be as a result of an altered balance of pro-inflammatory and anti-inflammatory cytokine production in the upper airways as compared with low-density WT carriage, which stimulates conditions that favor stable carriage. Furthermore, Neill et al. demonstrated that pneumococcal colonisation in both mice and humans are associated with induction of the immunoregulatory cytokine TGFb (Neill et al, 2014). Low-dose nasopharyngeal colonisation in mice induced TGFb production and an associated accumulation of T regulatory cells in the nasopharynx. By contrast, high-dose infection or infection with pneumolysin-deficient PLN-A induced a response dominated by inflammatory cytokines and which lacked T regulatory cell accumulation. Consequently, carriage was cleared over 7-14 days. Inhibition of

TGFb during low-dose colonisation similarly resulted in an inflammatory driven response that cleared colonisation. Significantly however, this clearance was accompanied by dissemination of bacteria to the lungs, suggesting that TGFb-driven responses act to limit bacterial dissemination (Neill et al., 2014).

TGF-β is an immunosuppressive cytokine that has a key role in innate and adaptive immunity (Li et al., 2006). The activity of TGF-β limits pro-inflammatory responses and stimulates tissues healing after damage (Li Mo et al., 2006). The importance of TGF-β in invasive pneumococcal infections has been verified, as the inhibition of TGF-β significantly increased the sensitivity of normally disease-resistant mice to invasive pneumonia (Neill et al., 2012). The correlation of high levels of TGF-β and high regulatory T cell numbers with reduced lung apoptosis indicates that the regulation of immune system plays a significant role in inhibiting the dissemination of pneumococci by preventing the inflammatory damage to the lung epithelial barrier that can be induced by excessive inflammation (Neill et al., 2012).

1.5 Mannose Receptor (MR)

1.5.1 Description

MR is a 175-kDa-endocytic protein that belong to the C-type lectin family, as it has multiple C-Type Lectin-Like Domains (CTLDs) with a single polypeptide backbone. It is a type I membrane protein, which has single transmembrane and cytoplasmic domains that play important roles in facilitating MR internalization and recycling. MR is expressed mainly by both macrophages, and dendritic cells (DCs) (Janeway et al., 2006, Martinez-Pomares, 2012). This receptor has numerous important roles in

host immunity including; clearance of endogenous molecules, modulation of cellular activation, trafficking and promotion of antigen presentation, promoting T cell differentiation and cellular stimulation (Pontow et al., 1993, Stahl and Ezekowitz, 1998). Mannose receptor consists of two independent carbohydrate-binding domains, binding mannosylated and sulfated sugars, respectively (Linehan et al., 1999). Carbohydrate-binding function can be abolished through proteolyticleavage and alterations in glycosylation and conformation. Although predominantly expressed on macrophages and DCs, studies have reported that MR could be detected on the surfaces of tracheal cells, hepatic cells, kidney cells, retinal pigment epithelium, and lymphatic endothelia (Sallusto et al., 1995, Engering et al., 1997).

1.5.2 MR Structure

In figure 1.1 MR structure has been showed. MR consists of three extracellular domains; an N-terminal cysteine-rich (CR) domain, which is required for Ca2+ independent binding to sulphated sugars terminated in SO4-3-galactose (Gal) or SO4-3/4-N-acetylgalactosamine (GalNAc) (Taylor et al., 2005), a Fibronectin type II (FNII) domain which able to detect and bind collagen type I, II, III, and IV (Martinez-Pomares et al., 2006, Janeway et al., 2006), and the third domain is the C-Type Lectin-Like Domains (CTLDs), consisting of eight organized CTLD motifs that are responsible for Ca2+ dependent binding to sugars ended with D-mannose and L-fucose or N-acetylglucosamine (GlcNAc) (East and Isacke, 2002, Taylor et al., 2005, Janeway et al., 2006). Mannose receptor is the only protein of the MR family that has a functional N-terminal CR domain and the interaction with galactose or GalNAc occurs through a neutral-binding pocket in a calcium-independent manner (Leteux et

al., 2000, Taylor et al., 2005). The responsible region for sugar binding does not exist in the N-terminal CR domain of the other members of the MR family (Liu et al., 2000).

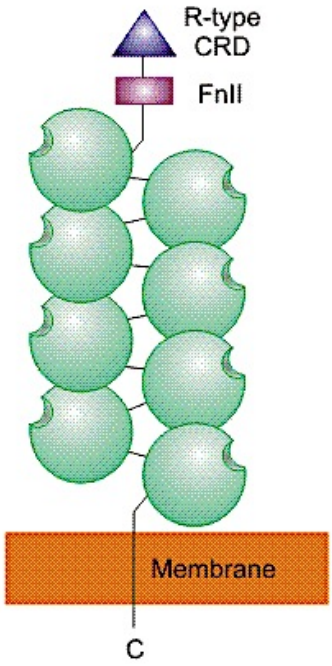


Figure 1.2. MR is a type I membrane molecule that has three extracellular domains: cysteine-rich domain (CRD) (Navy), which binds sulfated glycan in lymphoid tissues. Fibronectin type II (FNII) domain (Purple), which binds collagen. The Cytoplasmic tail is another domain that consists of eight CTLD motifs (green), which bind endogenous and exogenous molecules, involving allergens and pathogens products such as LPS and capsule (Source https://www.imperial.ac.uk/animallectins/ctld/mammals/Groups/GroupVI.html).

1.5.3 Initiation of immune responses

The *MR* gene has a promoter region with binding sites for two unique transcription factors: PU.1 and SP.1. Both these factors are involved in initiation of immune responses and are required for proper development of myeloid progenitors (Egan et al., 1999). A range of cytokines have been shown to up regulate the expression of MR including IL-10, IL-4 and IL-13, and have been linked to the alternative activation of macrophages (Doyle et al., 1994, Martinez-Pomares et al., 2003). Also, the anti-

inflammatory molecules prostaglandin E (PGE) and dexamethasone stimulate MR expression on MΦ, while IFN-γ encourages MΦ classical activation and thus down regulates the expression of MR (Schreiber et al., 1990). These observations suggest MR may play a role in alternative activation, determination of inflammation and prevention of self-damage.

1.5.4 Role in immunity

MR has a wide range of functions in response to infections. For example, MR could work as a Pattern Recognition Receptor (PRR) through its CTLD motifs, as fucose, mannose, and N-acetylglucosamine are not usually present in mammalian glycoproteins as terminal residues. Also, MR has been shown to identify a wide range of pathogens including *S. pneumoniae* and *C. albicans* (Zamze et al., 2002, Martinez-Pomares, 2012). However, despite its identified function in the recognition of pathogens, the importance of this unique receptor in immunity is not fully understood. In the following sections, the role of MR in phagocytosis, antigen processing and presentation, and intracellular signaling response to pathogens will be discussed.

1.5.5. Phagocytosis

The role of MR in phagocytosis is not fully understood. Although mannose receptor has been shown to be involved in the phagocytosis of several microbes, including *M. tuberculosis* (Kang et al., 2005), and *C. albicans* (Marodi et al., 1991), some studies have shown that despite the occurrence of mannosylated glycoprotein endocytosis, MR was not able to phagocytose *M. kansasii* or any mannosylated latex beads (Le Cabec et al., 2005). Furthermore, several *in vivo* models have shown that uptake of *C.*

albicans is unaffected in MR -/- mice (Akilov et al., 2007), and MR expression was not observed in the early stages of phagosome formation (Heinsbroek et al., 2008). In fact, MR can offer pathogens a safe route for entry, as MR engagement leads to the inhibition of phagosome maturation and phagosome-lysosome fusion following phagocytosis of *Mycobacterium avium* (Shimada et al., 2010) and mycobacteria (Astarie-Dequeker et al., 1999). Many of these studies have confirmed the role of MR in phagocytosis by using Mannan, since the sugar acts as a specific inhibitor for MR, although the existence of other Mannan binding receptors (dectin-2 and DC-SIGN) may influence the efficiency of inhibition. The use of MR-specific antibodies with J774 macrophages has been shown to be more reliable and effective, and WT cells display greater efficiency of *Francisella tularansis* ingestion as compared to MR-blocked cells (Schulert and Allen, 2006).

1.5.6 Antigen processing and presentation

MR is expressed mainly by macrophages on early Rab5a+ endosomes, which play an important role in the recruitment of Rab7 and in the process of maturation to late endosomes. The ability of IL-4 and PGE to induce the expression of MR may contribute to the recycling of endocytic proteins Rab11+ and Rab7+ from endosomes (Wainszelbaum et al., 2006). Thus, MR plays a crucial role in the transportation of bound molecules into late endosomes, and is essential for the presentation of pathogen antigens. However, the role of MR as an endocytic receptor in the presentation of microbial antigens is controversial (Napper and Taylor, 2004, Taylor et al., 2005). Several in vitro studies show that blocking MR with either mAb or mannosylated ligands enhance both MHC class I, and MHC class II pathways for antigen

presentation and induction of T-cell responses (Keler et al., 2004). In addition, an *in vivo* model using knockout mice revealed that MR functions as a catabolic receptor for serum glycoproteins (Lee et al., 2003) without showing an important role in generating protective immune response against a model infection (Lee et al., 2003).

Several co-localisation studies have hypothesized an important role for MR in antigen presentation as it has been found in association with CD11b and MHC class II (Sallusto et al., 1995, Engering et al, 1997, Martinez-Pomares, 2012). Another co-localization study suggested that MR is vital in the presentation of antigen in the context of CD11b and lipoarabinomannan (LAM) with MHC II molecules (Prigozy et al, 1997, Engering et al, 1997). The seemingly dichotomous findings of some of these studies could be explained by differences in the type of MR ligand used, and the possible effect of other PRRs and endocytic receptors that share ligands with MR, such as DC-sign and dectin-2. Additionally, a study by Burgdorf *et al*. has revealed that MR in context with MHC class II molecules is able to stimulate release of exogenous proteins, and to support the cross-presentation of internalized microbial antigens (Burgdorf et al., 2008).

1.5.7 Intracellular signaling

Several studies have suggested that MR plays important roles in intracellular signaling that results in the regulation of gene expression (Yamamoto et al., 1997, Chieppa et al., 2003). However, Mannose receptor requires the assistance of other receptors in order to initiate cytokine signaling cascades, since the MR cytoplasmic domain does not contain any signaling motifs (Zhang et al., 2005). TLR2 is thought to be the candidate receptor for this cross talk with MR. A recent study revealed that

both TLR2 and MR are required for the secretion of IL-8 in response to *Pneumocystis carinii* (Tachado et al., 2007). The two receptors seem to form a functional complex on the surfaces of cells during the microbial recognition process. Another study using a different model revealed that MR has a direct role in inducing T cell production of T helper 2 and T regulatory cell cytokines but not T helper 1 (Chieppa et al., 2003). Furthermore, a study by Pathak et al. confirmed that MR could deliver negative signals to inhibit the induction of pro-inflammatory cytokines. The study has shown that the engagement of MR leads to up-regulation and induction of expression of IRAK-M, which is a negative regulator for TLRs signaling, preventing the dissociation of IRAK-1, and IRAK-4 from MyD88 and the formation of IRAK-TRAF6 complexes (Pathak et al., 2005).

1.5.8 Mannose Receptor in response to *S. pneumoniae*

The role of the MR in response to *S. pneumoniae* is not well defined and more studies are needed to investigate how the pneumococcus and MR interact with each other, in particular during nasopharyngeal colonisation. Little is known about the role of MR in activation and recruitment of other immune cells such as neutrophils during pneumococcal infection. With regard to *S. pneumoniae* and MR interaction, Zamze et al. (2002) has shown that MR was able to recognize and bind purified capsular polysaccharides from *Streptococcus pneumoniae* but not from *Klebsiella pneumoniae*. Surprisingly, the recognition was inhibited with D-mannose and also was Ca^{2+}-dependent. Furthermore, the carbohydrate recognition of Mannose receptor domains 4–7 was shown to provide sufficient binding toward pneumococcal polysaccharides when compared to other domains suggesting that these particular domains are

required for the recognition of these capsular polysaccharides. However, no direct correlation was found between the structure of pneumococcal polysaccharides and the binding to the mannose receptor, proposing that the conformation of the polysaccharides may has a significant role in recognition (Zamze et al., 2002). However, this study did not cover the interaction of MR with other significant pneumococcal virulence factors including pneumolysin. Therefore, this PhD project has explored how pneumolysin and MR interact with each other. This has revealed the importance of MR in response to the pneumococcal toxin pneumolysin during infection and inflammation.

1.6 Pneumolysin and host immunity

The inflammatory responses to PLY are well defined, and the enhancement of TNF-α, IL-1β, IL-6 and IL-8 production has been validated in a range of host immune cells including macrophages, dendritic cells, endothelial cells, and epithelial cells (McNeela et al., 2010, Luttge et al., 2012, Neill et al., 2014). This activity is believed to contribute to the generation of anti-pneumococcal immune responses and to the enhancement of pathology. For example, pneumolysin stimulates the production of the potent antimicrobial compound nitric oxide (NO), however this is also a strong contributor to tissue pathology. NO production by macrophages develops through an IFN-γ dependent pathway, which includes the up-regulation of cox-2 gene expression (Braun et al., 1999). Several groups have documented that PLY induces production of IL-6 and CXCL8 in upper airways cells, and stimulates MAPK, NFκB, and TLR-4 signaling pathways (Dogan et al., 2011). Interestingly, the degree of PLY-induced cytokine production seems to be limited in the presence of the polysaccharide capsule

(Kung et al., 2014). Suppression of CXCL8 and MIP-2 production by the pneumococcal capsule was detected in a murine model of nasopharyngeal carriage, however this phenomenon was only detectable in the absence of pneumolysin. Therefore, the polysaccharide capsule may have a role in regulating the pro-inflammatory response of the toxin in vivo. The mechanisms of *S. pneumoniae* recognition by the innate immune system remain to be fully elucidated although some pattern recognition receptors have been implicated, including Toll-like receptors 2 and 4 (Malley et al., 2003, McNeela et al., 2010).

1.6.1 Recognition by Toll-Like receptors (TLRs)

Although TLR-4 has long been known to be a key part of the innate immune response to Gram-negative infection by its recognition of bacterial LPS, work by Malley et al. has also demonstrated an unexpected potential role for this receptor in facilitating inflammatory responses to the pneumococcal toxin pneumolysin. The study revealed that the expression of TLR-4 and MyD88 protein are essential for the production of TNF-a and IL-6 by macrophages, since TLR-4 deficient mice were susceptible to acute infection after pneumococcal colonisation (Malley et al., 2003, Malley et al., 2005). The possible explanation for this unique observation was that PLY binding to TLR-4 induced host-mediated apoptosis and thus led to clearance of the bacteria. The authors subsequently also showed that PLY interacted directly with TLR-4 during a solid phase binding assay, and thus mediated signaling in epithelial cells and macrophages (Srivastava et al., 2005). Also, it has been postulated that the interaction between pneumolysin and TLR-4 could stimulate the activation of caspase-1, and the production of significant cytokines, such as IL-1α, IL-1β and IL-18 (Shoma et al.,

2008). The activation of caspase-1 has been revealed recently to be reliant on NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasomes (Fang et al., 2011). These inflammatory responses are not dependent on the hemolytic and complement-activating activities of PLY, as the mutant type of pneumolysin, which lacks these virulent properties, showed the same effects as wild type PLY. The significance of this detected interaction was examined during pneumococcal colonisation of wild type and TLR4 knock out mice in a nasopharyngeal carriage model (Malley et al., 2003). The study showed that TLR4 knock out mice were colonized at a greater density and were significantly more likely to develop invasive disease than were wild type controls. Consequently, through its ability to recognize pneumococcal toxin, TLR4 was able to limit the proliferation of the pneumococcus in nasopharynx (Srivastava et al., 2005). Moreover, the importance of TLR4 in the recognition of pneumococcal infections seems to be limited since an earlier study found that the absence of TLR4 did not affect survival rates of mice infected with the pneumococcus, or bacterial counts in blood after intravenous challenge (Benton et al., 1997). However, the role of TLR4 in pneumococcal infection remains controversial and several studies with LPS-free purified PLY showed no significant role for TLR4 in pneumolysin-induced maturation of dendritic cells or in the stimulation of cytokine production (McNeela et al., 2010). Endotoxin-free PLY was able to induce the expression of co-stimulatory molecules on dendritic cells, and to boost the secretion of cytokines, including IL-12, IL-6, IL-23, TNF-α, IL-1α, and IL-1β, but only in the *presence of TLR agonists*. The synergistic influence of PLY on the responses to TLR agonists was completely independent of TLR4. These latest data would suggest that the stimulation of PLY-dependent cytokines could occur through a mechanism independent from the stimulation of TLR4.

1.6.2 Inflammasome activation

PLY induces the production of IL-1b, which is required for NLRP3 activation, demonstrating the role of pneumolysin in the activation of the inflammasome. Inflammasome activation is an important step in the maturation process of anti-pneumococcal immunity, since NLRP3 knockout mice lack the ability to control bacterial numbers during pneumococcal pneumonia. The signaling of the inflammasome appears to be necessary for the IL-17 responses that have been implicated in resistance to pneumococcal pneumonia and carriage (Malley et al., 2006, Lu et al., 2008), as PLY-deficient *S. pneumoniae* induce lower production of IL-17A and INF-g in mice as compared to wild-type pneumococci. Further supportive evidence for the role of NLRP3 in anti-pneumococcal immunity comes from the observation that several pneumococcal serotypes, such as serotype 1, 7F and 8 (Brueggemann et al., 2003, Song et al., 2013), that are associated with enhanced bacterial invasiveness, produce PLY with low hemolytic activity that fails to activate the inflammasome (Kirkham et al., 2006). The activation of the inflammasome is clearly correlated with vigorous immune responses, and with the control of pneumococcal numbers, however a potential outcome of stimulation of every inflammatory pathway is bystander tissue injury. In a murine model of pneumococcal pneumonia, the induction of the inflammasome adaptor molecule ASC and the sensor NLRP3 is correlated with increases in pathology and infection score (Hoegen et al., 2011). ASC knockout mice showed high level of lethality and pneumococcal dissemination when compared to NLRP3 knockout mice, despite notably reduced cytokine production in both mouse strains (Van Lieshout et al., 2014). This observation may be explained by the theory that the attenuation of IL-17, integrin-αM, and GM-CSF responses combined to reduce the signaling of the adaptive

immune response in the ASC knockout mice (Van Lieshout et al., 2014). In contrast to the effect of PLY on inflammasome activation (as mentioned above), a recent study has demonstrated that pneumolysin could also prevent the generation of inflammatory responses in human antigen presenting cells (APCs) such as dendritic cells (Littmann et al., 2009). Wild type and PLY knockout serotype 4 (TIGR4) pneumococci were used in this study, which revealed that PLY has the ability to enhance the uptake of pneumococci by human DCs by 50%. On the other hand, this uptake was correlated with increased DC caspase-dependent apoptosis, and this activity was dependent on PLY. Moreover, the expression of the pneumococcal toxin was associated with some activities including the reduction of cytokine responses by DCs, down-regulation of inflammasome activation, and reduction of the expression of co-stimulatory molecules on DCs. These experimental results are in contrast to the current evidence of the role of the pneumococcal toxin PLY in the activation of the inflammasome, and it has been argued that the outcomes suggest dissimilarities in the signaling pathways between human and murine cells. Conversely though, several pneumococcal studies have described similarities in the inflammatory responses to PLY in human and murine cells (McNeela at al., 2010, Witzenrath et al., 2011, Hoegen et al., 2011). Hence, the reasons behind these conflicting observations of the special effects of PLY on the activity of inflammasome may be due to serotype specific differences although more detailed investigation is needed to clarify the issue.

1.6.3 T-cell differentiation

T cells are crucial for anti-pneumococcal immune responses, as the depletion of T cells reduces the clearance of pneumococci in colonisation and pneumonia models

(Kadioglu et al., 2000, Kadioglu et al., 2004, Van Rossum et al., 2005). The relationship between PLY and T cell immunity is not fully understood, however the current data suggests that the toxin is required for the activation of T cells and for their production of cytokines (Houldsworth et al., 1994, Kadioglu et al., 2004, Kadioglu and Andrew, 2004). These data also shown that during pneumococcal pneumonia, T cells respond rapidly to infection and accumulate in the lungs. Interestingly, the accumulation of these cells was slower and notably reduced when mice were infected with pneumolysin-deficient pneumococci (Kadioglu et al., 2000). Furthermore, a recent study has revealed new roles for the pneumococcal toxin PLY in the initiation of memory T cell responses and in the proliferation of CD4+ T cells isolated from either human nasal-associated lymphoid tissue or human blood in response to stimulation with pneumolysin domain 4 (Gray et al., 2014). Moreover, memory Th17 responses were prompted in response to PLY, and this is more noticeable in cells isolated from carriage-negative children when compared to carriage-positive children, where a more regulatory response dominates. These data suggest that PLY has a role in the induction of Th17 immunity and thus may provide children with protection against future pneumococcal colonisation. In contrast though, earlier work had reported that PLY at high concentrations inhibited the proliferation of lymphocytes and reduced the production of pro-inflammatory cytokines (Ferrante et al., 1984). In general, it seems clear that pneumolysin produced during pneumococcal infection has strong influences on the responses of T cells and other inflammatory responses that could be activating or inhibitory in nature, depending on toxin concentration.

1.6.4 Role in pneumococcal carriage

Nasopharyngeal colonisation with *S. pneumoniae* is a prerequisite for invasive disease; therefore it is essential that we understand the role of host defenses and bacterial virulence factors in carriage in order to develop appropriate strategies to control and eliminate infection. The first demonstration of the role of Ply in pneumococcal infection of the nasopharynx was revealed by Kadioglu et al. (Kadioglu et al., 2002). Recent studies in mice have reported that pneumolysin is required for prolonged nasopharyngeal colonisation (Richards et al., 2010, Neill et al., 2014). Several studies have shown that wild-type serotype 2 (D39) pneumococci were able to colonize the nasopharynx for more than four weeks without showing any sign of clearance or development of severe infection, whereas an isogenic pneumolysin-negative pneumococcus lacked the ability to establish prolonged colonisation, and was cleared completely within one to two weeks post-infection (Kadioglu et al., 2002, Richards et al., 2010, Neill et al., 2014). Conversely, an earlier paper had reported that a pneumolysin-negative pneumococcus showed low level of adherence to murine epithelia cells, however no defect was determined in pneumococcal colonisation (Rubins et al., 1998). A recent study by Neill et al. has revealed that D39 pneumococci and purified PLY induce the production of TGFβ1 from nasopharynx and lung epithelium, while PLN-A induces significantly lower levels of this immune modulatory cytokine (Neill et al., 2014). This observed activity requires the signaling of Toll-like receptors, and is dependent on the efflux of potassium, and the rupture of phagosomes, implicating the NLRP3 inflammasome. Significantly, the host TGFβ1 response to PLY revealed a unique dose response, since high concentrations of PLY or pneumococci led to production of INF-γ and reduced the response of TGFβ1, whereas intermediate or low doses of PLY or pneumococci encouraged TGFβ1

production in a dose- dependent manner. This dose response was evident in the outcomes of pneumococcal colonisation with low or high numbers of bacteria. Colonisation with high number of pneumococci or PLY-negative bacteria leads to transient carriage, while colonisation with low number of pneumococci leads to long-term carriage. These differences are explained by the altered balance in the responses of pro-inflammatory and anti-inflammatory cytokines following colonisation with high density of pneumococci or pneumolysin-negative bacteria. Long-term carriage was associated with induction of TGFβ1 and T regulatory cell responses that act to reduce the production of pro-inflammatory cytokine, and limit the infiltration of neutrophils, and consequently allow the maintenance of stable numbers of pneumococci in the nasopharynx. However, pneumolysin-negative pneumococci fail to induce the production of TGFβ1 and the response of T regulatory cells, and so are eliminated from the upper airways by the resulting inflammatory responses. Relatedly, colonisation with high-density of bacteria led to induce the response of pro-inflammatory cytokines in the airway of epithelial cells, interrupting the induction of TGFβ1 production and leading to clearance of the bacteria. When TGFB1 production was inhibited following colonisation with low numbers of pneumococci in mice, carriage was cleared totally within 7-14 days. Clearance was associated with dissemination of bacteria from nasopharynx to lungs, suggesting the presence of a fine line between inflammatory responses that eliminate colonisation, and those that cause damage and poor prognosis. This reported phenomenon could explain the observed differences in the virulence of pneumolysin-negative bacteria in different experimental models, and would also help to understand why most pneumococcal serotypes that express PLY with different hemolytic activity are correlated with severe infections, and found rarely in carriage (Douce et al., 2010). In support of this

hypothesis, in experimental human pneumococcal carriage (Gritzfeld et al., 2013, Ferreira et al., 2013), TGFβ1 levels in nasal wash at 48 h post-pneumococcal challenge were associated with the establishment of successful pneumococcal carriage (Neill et al., 2014). Individuals in whom carriage was established had significantly higher TGFβ1 levels than those in whom carriage failed to establish. In addition, the number of T regulatory cells in nasal associated lymphoid tissue and the inhibitory capacity of those cells has been reported to be elevated in pneumococcal carriage in children (Pido-Lopez et al., 2011, Zhang et al., 2011).

The emerging theory from all these data can be described as follows; invasive pneumococcal infections could arise from the failure to maintain or stimulate the proper response of T regulatory cells during nasopharyngeal colonisation. The cell-driven responses of TGFβ1 and T regulatory cells have been shown to have a role in the induction of immune tolerance to pneumococcal colonisation in the upper airways. The significance of this immune tolerance is to preserve tissue integrity and thus prevent the dissemination of pneumococci within the host, however this immune phenomenon will also inhibit the clearance of pneumococci during colonisation and allow maintenance of carriage. Colonisation with high density of bacteria or with pneumolysin-negative pneumococci leads to a failure to induce tolerance and thus activates the inflammatory pathways, and pneumococci are either cleared or else disseminate within the host through the damaged tissue causing pneumococcal pneumonia and invasive infections. Moreover, a recent work has revealed a comparable role for T regulatory cells during pneumonia, where they limit inflammation and prevent the spread of pneumococci (Neill et al., 2012).

1.7 Research aim and vision

The central aim of this project was to understand how pneumolysin activates and potentially drives different mechanisms of host immunity in the nasopharynx during colonisation. We have recently demonstrated that the pneumococcal toxin pneumolysin is a potent activator of both mucosal and systemic immune responses, directly stimulating macrophages and dendritic cells and dramatically amplifying their production of pro-inflammatory cytokines independently of TLR-4. We have also shown for the first time that pneumolysin activates the NLRP3 inflammasome complex leading to release IL-1β, which is required for protection against invasive pneumococcal pneumoniae (McNeela et al., 2010). Our recent data reveal an interesting dichotomy of PLY; that it is crucial to the pneumococcus for successful asymptomatic colonisation of the nasopharynx (Kadioglu et al., 2002, Richards et al., 2010), without generating pro-inflammatory immune responses, unlike its powerful ability to drive inflammation in the lungs during pneumonia. Based on new preliminary data, I propose that during nasopharyngeal colonisation, nasal mucosa-associated macrophages become activated by PLY and subsequently drain to cervical lymph nodes to initiate immune responses that act to prevent damage to host tissues and systemic pneumococcal dissemination. In order to determine the mechanistic detail of these interactions, I will address the following research questions:

(1) How does pneumolysin induce immune tolerance during nasopharyngeal colonisation?

(2) What is the role of nasal mucosa-associated macrophages in the generation of immune tolerance during nasopharyngeal colonisation?

(3) What are the host-cell signalling pathways activated by pneumolysin during colonisation?

(4) What are the changes in host and bacterial conditions that allow an asymptomatic carrier state to progress to invasive disease?

My hypothesis is that during colonisation of the nasophrynx a state of immune tolerance is generated involving pneumolysin and pneumococcal pathogen associated molecular patterns (PAMPs). Nasal mucosal-associated macrophages are key to initiating this tolergenic state; T-regulatory cells, TGF-β and IL-10 are key to its long-term regulation. During pneumonia however, a different mechanism operates where pneumolysin drives pro-inflammatory responses in alveolar macrophages and dendritic cells. Interestingly, mice that clear their lung infection are still colonised by the same pneumococcal strain in their nasopharynx (Richards et al., 2010), demonstrating that immune responses in the lung have no protective effect in nasopharyngeal colonisation. This provides evidence of a different immunological mechanism operating in the nasopharynx. Our confocal studies of nasal mucosa and cervical lymph nodes during a 14-day pneumococcal nasopharyngeal colonisation study revealed a narked increase in CD11c/CD68/CD169/MHC-II positive macrophages in the subscapular sinus regions of cervical lymph nodes over time. We also showed that these macrophages are present in the nasal mucosa of mice, and increase in numbers during early stages of colonisation accompanied by increased expression of mannose receptor, which have been shown to bind to pneumococci (Zamze et al., 2002).

My hypothesis is that this subset of mannose receptor-expressing, CD68/CD169-positive macrophages are critical gatekeepers at the nasal mucosal tissue/cervical lymph node interface and are the first cells to sample bacteria and present antigen to

B- and T-cells in CLN. There is no increase in CLN size during colonisation and no change in CLN structure, i.e. B- and T-cells area remain intact and the proportion of each area is maintained, indicative of absence of inflammation. Furthermore, CD11c/CD68/CD169/MHC-II-positive macrophages are the only cells that increase over time, there being no increase in dendritic cells (CD11c/MHC-II-positive but CD68/CD169 negative), monocytes, B- or T-cells, or neutrophils in either CLN or nasal mucosa. This is in keeping with our previously published FACS analysis of leucocyte populations in nasal mucosa and CLN during a 28-day pneumococcal nasopharyngeal colonisation study, where we showed that macrophages were the only cell type to show significant increases in numbers over time with no significant increases in neutrophils, B- or T-cells (Richards et al., 2010).

It is clear that pneumolysin has pro-inflammatory effects in the lungs based on different modes of action; it can stimulate cells directly by its pore-forming activity (via NLRP3 activation) but also through non-pore forming NLRP3-independent mechanisms. It is likely that NFkB activation is responsible for this mechanism. This research will elucidate the different cell signalling pathways pneumolysin activates and the pro-inflammatory/protective role of the cells and cytokines induced by activation of these pathways during colonisation. The knowledge gained from this research will provide the basis for potential treatments focused on preventing the transition of colonisation into invasive disease. Furthermore, the knowledge gained of pneumolysin-host immune cells interaction in the nasopharynx will inform us of correlates of immunity to aid future vaccine development.

Chapter 2: Materials and methods

2.1 Growth conditions and media:

S. pneumoniae was grown on Blood agar base (Oxoid) plates supplemented with 5% v/v horse blood (Oxoid), with the selective antibiotics required for the different mutants (Table 2.1). Under sterile conditions, a full loop of colonies was streaked out from the bead stocks or from existing good condition plates, and the new fresh plates were incubated inverted at 37 °C overnight in a sealed jar. A sweep of overnight grown pneumococci was transferred from the plate to 10 ml of sterile Brain Heart Infusion (BHI) broth (Oxoid). Cultures were grown overnight for 16-18 hours at 37 °C, in a strictly closed universal tube (Sterilin). Following overnight cultures, stock aliquots were grown for five to eight hours; in 80% (v/v) BHI supplied with 20% (v/v) of sterilized Foetal Bovine Serum (FBS, Sigma). These cultures were incubated statically at 37°C in a candle jar. Media used in this PhD study were prepared according to Table 2.1.

Medium name	Recipe	Supplier
Blood agar base (BAB)	16g in 400ml dH2O Horse Blood	Oxoid, UK
Brain heart infusion (BHI)	14.8g in 400ml dH2O	Oxoid, UK
Luria Broth (LB)	4g NaCl, 2g Yeast extract, 4g Tryptone in 400ml dH2O	NaCl – Fisher Scientific Yeast Extract – Oxoid, UK Tryptone – Oxoid, UK
Dulbecco's Modified Eagle's Medium (DMEM)	10% foetal bovine serum (FBS) 100 mg/ml Streptomycin 100 mg/ml Penicillin 20ng/ml macrophage colony-stimulating factor (M-CSF)	FBS – Sigma Streptomycin - Sigma Penicillin – Sigma M-CSF – R&D system

Table 2.1. Media used in this PhD study

2.2 Bacterial strains

Pneumococcus isolates were distinguished from other bacterial species by Gram stain, hemolytic test on blood agar plates, optochin (Ethylhydrocupreine hydrochloride) sensitivity and finally by catalase test. Pneumococcal isolates were stored at -80 °C for long-term storage in brain-heart infusion media (BHI, Oxoid) supplemented with 15% glycerol (Sigma Aldrich). Wild type (WT) serotype 2 *S. pneumoniae*, its mutants and purified proteins were used in this PhD project listed in Table 2.2 and Table 2.3. The pneumolysin-deficient mutant was used previously by McNeela et al. (McNeela et al., 2010). The capsule-deficient mutant was provided kindly by Doctor Kathrin Mühlemann (University of Bern) (Battig and Muhlemann, 2007). The pneumolysin mutants were a kind gift from Professor Tim Mitchell (University of Birmingham, UK) (Yuste et al., 2005). The pneumolysin and capsule mutant DKO D39 was provided kindly by Doctor Lucy Hathaway (University of Bern) (Engel et al., 2013).

Strain	Serotype	Strain background	Strain designation	Source	Antibiotic resistance
D39 (WT)	2	WT	7466	NCTC	-
ΔPLY (Pneumolysin deletion mutant)	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-
D39-J (Capsule negative mutant)	2	D39	-	Dr Kathrin Mühlemann (University of Bern)	Penicillin 0.1 μl/ml
DKO D39 (Capsule & pneumolysin negative mutant)	2	D39	-	Dr Lucy Hathaway (University of Bern)	Spectinomycin 0.1 mg/ml

Table 2.2. Pneumococcal strains used in this PhD study

Strain	Serotype	Strain background	Strain designation	Source	Antibiotic resistance
Pneumolysin domain 1-3	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-
Pneumolysin domain 4	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-
eGFP whole pneumolysin	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-
eGFP pneumolysin domain 1-3	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-
eGFP pneumolysin domain 4	2	D39	-	Prof. Tim Mitchell (University of Birmingham)	-

Table 2.3. Pneumococcal proteins used in this PhD study

2.3 Pneumococcal viable counting:

Sterile round-bottomed 96 well plates (Thomas Scientific) were using to determine the number of bacteria in liquid culture or in homogenised mouse tissue. Serial dilutions from 10^1-10^6 were performed by adding 20 μl of the liquid culture or homogenised tissues to 180 μl of sterile phosphate-buffered saline (PBS, Sigma), in triplicate, according to standard Miles and Misra methodology. 60 μl of each dilution was spotted on blood agar plates and incubated inverted overnight in a

closed jar at 37 °C. The next day, grown pneumococcal colonies were examined and counted from the dilution where between 30 and 100 colonies were visible. The number of bacteria was determined as colony forming units (CFUs) per ml, using the following equation:

$$\text{CFU/ml} = \frac{\text{(Total number of colonies counted in sector)}}{\text{60 (total volume)}} \times 100$$

2.4 Mice

WT C57BL/6 and MF-1 outbred mice were used in this PhD study (Purchased from Charles River, UK). MR $^{-/-}$ mice (genetically deficient in mannose receptor) were generously provided by the laboratory of Dr. Luisa Martinez-Pomares (University of Nottingham, Nottingham, U.K.). MR $^{-/-}$ mice were bred at the Biomedical Services Unit of the University of Nottingham. Experimental animals were housed in individually ventilated cages (IVCs) of up to 5 mice, with ready access to food and water, in a specific pathogen free facility at the University of Liverpool. Animals were handled according to institutional and UK Home Office guidelines and were kept under specific pathogen-free conditions. Females mice were used at 7–10 weeks of age. Approval before all experimentation was obtained from the University of Liverpool ethics committee and the UK Home Office; project licence 40/3602 and personal licence number: (PIL 40/10224).

2.5 Preparation of infectious dose:

Aliquots of frozen pneumococci of known CFU/ml were thawed quickly in the palm of the hand and centrifuged at 13,000 rpm using a micro-centrifuge (Sigma). The

supernatant was discarded and the bacteria were re-suspended in 400 μl of PBS. As required, dilution of this stock was performed in PBS to give a final concentration of 1 x 10^7 CFU / ml, which provides an infectious dose of 1 x 10^5 CFU per 10 µl. All mice in the same experiment were infected using the same aliquot to ensure consistency.

2.6 Carriage study

Female outbred MF1 (Charles River, UK) mice (7–10 weeks old, 30–35 g) were used for infection studies. Mice were lightly anaesthetised with 2.5% (v/v) Isofluorane USP (Isocare) over oxygen (1.4–1.6 litres/min), in an anaesthetic box. 10µl of PBS containing 1x10^5 CFUs of *S. pneumoniae* were administered into the nostrils (equally between both nostrils) using a Gilson pipette. The mice were left on their backs to recover from the effect of the anesthesia, and to prevent the inoculum from discharging from the nose. The dose was confirmed by viable count following infection. At pre-chosen time intervals following infection, mice were sacrificed and nasopharynx, nasal mucosa and lymph nodes were collected, manually pushed through a 30µm cell strainer or else homogenized with an Ultra-Turrax T8 homogeniser (IKA, Germany). CFU counts from tissue were determined by viable count on blood agar plates following Miles and Misra methodology.

2.7 Pneumolysin expression and purification

Pneumolysin (Ply) was expressed in *E. coli* and purified as previously described

(Mitchell et al., 1989). Briefly, recombinant Ply was expressed in Escherichia coli (*E. coli*) strain MC1061 harboring plasmid pJW208 (which has an IPTG-inducible promoter and carries resistance to kanamycin and ampicillin). MC1061 was grown overnight in 8 L of Luria-Bertani (Sigma) broth supplemented with 100μg/ml ampicillin (Sigma). Bacterial cells were collected by centrifugation (14000xg for 20 minutes at 4 °C) and washed once in Equilibration buffer (Equilibration buffer (PH7); 10 mM $NaPO_3$, 250 mM NaCI). Cells were re-suspended in 30 ml of Equilibration buffer and disrupted by sonication (Cole-Parmer). The extract supernatants were clarified by centrifugation and stored at -20 °C until further use. Pneumolysin was purified from cell extracts supernatants using the high- performance Affinity chromatography system (GE healthcare). 50 ml of the supernatants were loaded onto the purification column and the column was washed with 5 column volumes of 10 mM equilibration buffer, 10 mM Imidazole, 20 mM Imidazole and 100mM Imidazole. Proteins were detected by Bradford assay using the kit supplied (Bio-Rad). Pneumolysin concentrations were measured using Nanodrop at 280 nm, then the toxin was passed 3 times through an EndoTrap endotoxin removal column (Profos AG, Germany) after which LPS was undetectable using the PyroGene Recombinant Factor C assay (Lonza; detection limit 0.01 EU/ml) and PLY purity was > 97% as determined by SDS-PAGE gel stained with Coomassie brilliant blue R250 (Sigma). Purified pneumolysin was filter sterilized and stored at -80°C.

2.8 Pneumolysin quantification

PLY was measured by Nanodrop spectrophotometer (Thermo Scientific), measuring at a wavelength of A280. Briefly, 2 μl of nano-pure water was dropped on the sensor

and the system was calibrated. Then the sensor was wiped clean. Following this, 2 µl of the buffer that the proteins were being stored in, was added to the sensor as a blank control. The protein elutions were then added to the sensor, separately to determine the concentrations, and in between each elution measurement the machine was blanked and wiped. Pneumolysin concentrations were determined in mg/ml.

2.9 SDS-PAGE and Western blot

To confirm the molecular weight (M.W) of the purified pneumolysin, and to verify that the pneumococcal protein had no other contaminants in the collected elutes, a 12% v/v SDS-PAGE gels was used. Individual SDS-PAGE gel was prepared using the chemical reagents listed in Table 2.3. Cell extracts and the different elutions collected from the columns were analyzed for protein content. PLY samples were prepared with NuPAGE LDS sample buffer 4X (Invitrogen). The samples were then incubated for 4 minutes at 100°C. Then, 16µl of each sample was loaded into separate wells in the SDS gel. A 4µl of precision plus protein standard (Bio-rad) was also added to each SDS gel, as a molecular weight marker. TGS buffer ((10X)- 250mM Tris, 10% SDS, 2M glycine, 1L dH2O) was added to the tank, and the gel was run at 0.4 amps for1 hour.

Reagents	**12% Separating Gel (15 ml)**	**5% Stacking Gel (5 ml)**

Nanopure Water	4.9 ml	3.4 ml
30% acrylamide (protogel, geneflow)	6 ml	0.83 ml
1.5M Tris HCl pH 8.8 (Sigma)	3.8 ml	-
1M Tris HCl pH 6.8	-	0.63 ml
10% SDS (Sigma)	0.15 ml	0.05 ml
10% Ammonium persulphate (Fisher Scientific)	0.15 ml	0.05 ml
TEMED (Sigma)	0.006 ml	0.005 ml

Table 2.3. Chemical reagents used for SDS PAGE gel preparation

Following the preparation of SDS PAGE gel, western blot was performed for each protein sample. To start the assay, each filter paper and sponge was equilibrating with transfer buffer (0.02M Tris, 0.03M glycine, 20% methanol, 0.03% SDS, 1L dH2O).

Western blot apparatus was assembled as shown in Figure 2.4, and the proteins were transferred onto a nitrocellulose membrane (G E Healthcare), for 1 hour at 0.25 Amps. The membrane was then blocked overnight with 5% v/v of milk in TBST (10mM tris, 0.15M NaCl2, 0.05% tween 20, 400ml dH2O pH- 8) buffer, at 4°C. Next day the membrane was washed 3 times, with TBST buffer. The nitrocellulose membrane was then incubated for 2 hours with primary anti-pneumolysin monoclonal antibody (Statens Serum Institute, Denmark), at a 1:1000 dilution. Subsequently, the membrane was washed three times with TBST, then the membrane was incubated for two hours, with the secondary antibody, anti-rabbit IgG, (raised in goat) which was conjugated to alkaline phosphatase (Invitrogen). Following this step, the membrane was washed again three more times with TBST and 5 ml of BCIP/NBT one-step solution (Sigma) were added for 10 minutes to develop the bands. Following this step, 5 ml of water were added to the membrane to stop the reaction of BCIP/NBT solution.

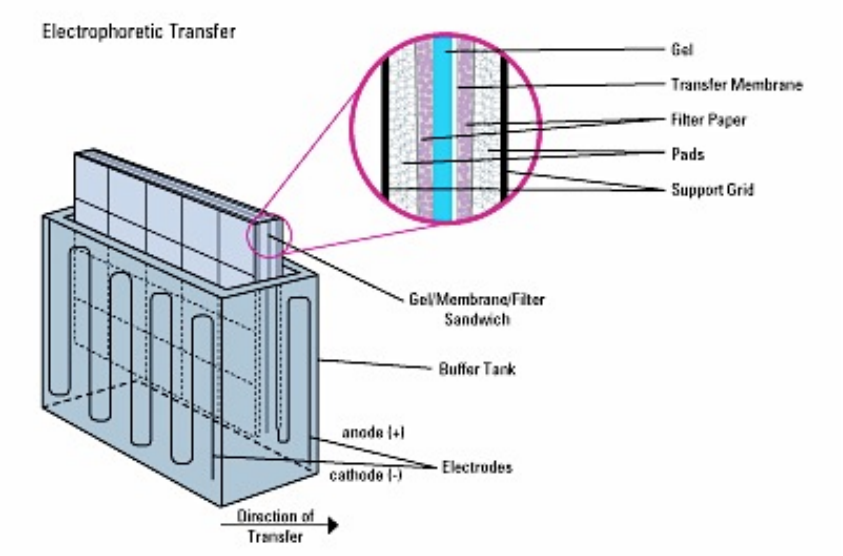


Figure 2.4. The picture showing the assembly of the Western blot apparatus.
(Picture from https://www.thermofisher.com/sa/en/home/brands/thermo-scientific/pierce-protein-biology.html

2.10 Hemolytic assays

The pneumolysin activity in fractions collected from chromatography columns was determined semi-quantitatively. 50 µl of each sample was diluted serially two-fold in phosphate-buffered saline (PBS, Sigma) along one row of 12 wells in a 96-well microtiter plate (Thermo scientific). 50 µl of 1% (vol/vol) fresh sheep erythrocyte (Oxoid) suspension was added to each well, and the plates were incubated at 37°C for 30 min and then centrifuged at 3,000 xg for 5 min to remove unlysed RBCs and the absorbance of the supernatant at 540 nm was measured. The percentage of erythrocytes lysed was plotted against dilution for the protein (Figure 2.5), and the pneumolysin activity of the sample was defined as the reciprocal of the estimated dilution at which 50% of the erythrocytes would have lysed. The highest dilution of each sample resulting in at least 50% hemolysis was then estimated visually. This activity was expressed as hemolytic units (HU) per milliliter.

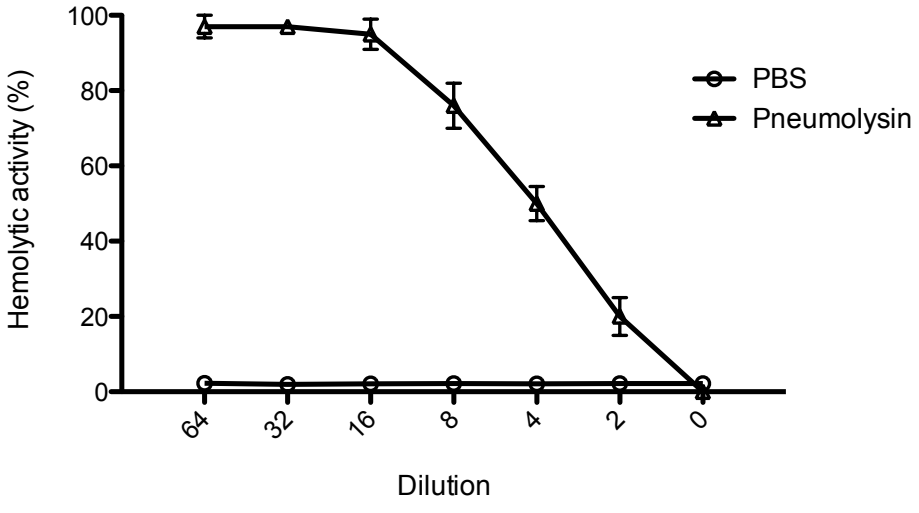


Figure 2.5. The hemolytic activity of wild type Ply against 1% (vol/vol) sheep RBCs.

2.11 Culturing of Bone marrow derived macrophages (BMDMs)

BMDMs were prepared by culturing murine bone marrow cells using protocols adapted from Lutz et al. (1999) and Davies et al. (2005). Briefly, bone marrow cells from wild type C57Bl6 or knockout mice (Table 2.6) (TLR knockout bone marrows were provided by the laboratory of Dr. Ed Lavelle, Trinity college Dublin, Dublin, Ireland) were flushed aseptically from the femurs and tibia of mice. Macrophages were grown in Dulbecco's Modified Eagle's Medium (Sigma) 10% v/v fetal calf serum (FCS; Sigma), 100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mM L-glutamine (Sigma) and supplemented with macrophage colony-stimulating factor (M-CSF; R&D system) (final concentration of 20ng/ml). Cultures were maintained in a humidified atmosphere (5% CO2) at 37°C, and medium was replaced on days 3 and 6. On day 6 of the culture, mature macrophages were plated ($6.25x10^5$ cells per well) onto 48 well plates (Sigma), and incubated over-night to adhere before adding the bacteria. Next day, cells were cultured with media or D39, ΔPly, D39-J, DKO D39 (1 MΦ: 10 bacteria) and PLY alone (4μg/ml). Following 24 h incubation at 37°C, supernatants were collected and stored at -80°C. Macrophages were removed using warmed trypsin (5 mg/ml) with 0.02% EDTA and used directly for flow cytometry staining and marker detection.

Cell Type	Mouse background	Source

Mannose receptor knockouts BMDM (MR $^{-/-}$)	C57BL/6	Dr. Luisa Martinez-Pomares (University of Nottingham)
Toll-like receptor 2 knockouts BMDM (TLR-2 $^{-/-}$)	C57BL/6	Dr. Ed Lavelle (Trinity college Dublin)
Toll-like receptor 4 knockouts BMDM (TLR-4 $^{-/-}$)	C57BL/6	Dr. Ed Lavelle (Trinity college Dublin)
Toll-like receptor 2 and 4 knockouts BMDM (DKO)	C57BL/6	Dr. Ed Lavelle (Trinity college Dublin)

Table 2.6. The type of knockouts cells used in this PhD research

2.12 MARCO blocking assay

The macrophage receptor with collagenous structure (MARCO) is a class A scavenger receptor. MARCO is inhibited by the Class A scavenger receptor (SR) blocker dextran sulfate (DxSO4, Sigma), but not chondroitin sulfate (Control) (ChSO4, Sigma), which does not block the SR or MARCO (Bowdish et al., 2009).

Briefly, mature macrophages were incubated with 100 μg/ml of dextran sulfate or chondroitin sulfate for 30 minutes at 37°C in DMEM media prior to stimulations or infections. Macrophages were washed gently with 300 μl of dPBS (Sigma), and then cells were stimulated with pneumococcus for 24 h.

2.13 Macrophage images

Wild type and $MR^{-/-}$ BMDMs were stimulated with D39 and ΔPLY at an MOI of 1:10. Macrophages were photographed by phase-contrast microscopy (20X) at 0, 24 and 72 h post-infections.

2.14 Stimulation of macrophages with pneumococcus and co-culture with T cells

Naïve CD4+ T cells were purified from spleens of C57BL/6 mice (Charles river) using magnetic separation with a CD4+ T Cell isolation kit (Miltenyi Biotec). Spleen were washed three times with dPBS (Gibco), manually pushed through a 40μm Cell strainer (Falcon), and centrifuged for 10 minutes at 300xg. Cell pellets were re-suspend in 0.5% BSA buffer (Sigma) and counted using a hemocytometer (Thomas Scientific). Cell suspensions were labeled with a cocktail of Biotin-conjugated antibodies (biotin-conjugated monoclonal antibodies against CD8a, CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC Class II, Ter-119, and TCRγ/δ), and then passed through a magnetic LS MACS column containing anti-Biotin MicroBeads. Negative selection of $CD4^{+}$ T cells were obtained using LS

MACS magnetic columns (Miltenyi Biotec) according to the manufacture's instructions. The CD4+ T-cells purity was >90% as assessed by flow cytometry. For in vitro stimulation, 24 hours pneumococcal stimulated macrophages were washed gently with 200 μl of PBS. Purified T-cells were re-suspended in DMEM and added gently to the stimulated macrophages at a ratio of 1:15, and incubated for 5 days at 37°C. Supernatants were collected and stored at -80°C for cytokines detection. Cells were removed using warmed trypsin (5 mg/ml) with 0.02% EDTA and used directly for surface marker determination by flow cytometry.

2.15 Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants from stimulated cells were collected (approximately 500 μl) and stored at -80°C until needed. The levels of IL-6, IL-10, IL-12, IL-1β, INF-γ, IL-17, IL-4 and MIP-2 cytokines in the supernatants were determined by ELISA (Table 2.7). 96-well plates (NUNC Maxisorp) were coated overnight with 10 μg/ml of IL-6, IL-10, IL-12, IL-1β, INF-γ, IL-17, IL-4 and MIP-2 capture antibodies in PBS, at 4 °C. The following day, plates were washed 3 times with PBS + 0.05% v/v Tween 20 (Sigma Aldrich), allowing the washing buffer to stand for 20 seconds in the wells before continuing with following washes. All wells then were blocked for 2 h at room temperature with assay diluent, diluted (4:1) in PBS to prevent non-specific binding. Wells were then washed again 3 times as previously described. Standards and samples were added into the wells in duplicate. Supernatants were diluted (1:5) in assay diluent for IL-6, IL-12 and IL-1β, diluted (1:10) for MIP-2, and used neat for IL-10, INF-γ, IL-17 and IL-4 and left for 2 hours at room temperature. Wells were washed before the addition of 10 μg/ml of the detection antibodies and plates

were incubated at room temperature for 1 hour. Wells were washed again 3 times with the washing buffer and diluted Avidin-HRP (1:1) in assay diluent was added to all wells and left at room temperature. After 30 minutes incubation, wells were wash 5 times as described previously. TMB substrate solution were added to all wells and incubated for 15 minutes in dark at room temperature. Colour development action was stopped by 0.16M of sulphuric acid (life technologies). Plates were read by micro-plate reader at 450nm in order to create the standard curve and determine the level of IL-6, IL-10, IL-12, IL-1β, INF-γ, IL-17, IL-4 and MIP-2 in cells supernatants.

Cytokine Name	**Type**	**Kit Brand**
Interleukin 6 (IL-6)	Mouse	eBioscience (Ready-SET-Go)
Interleukin 10 (IL-10)	Mouse	eBioscience (Ready-SET-Go)
Interleukin 12 (IL-12)	Mouse	eBioscience (Ready-SET-Go)
Interleukin 1 beta (IL-1β)	Mouse	eBioscience (Ready-SET-Go)
Interferon gamma (INF-γ)	Mouse	eBioscience (Ready-SET-Go)
Interleukin 17 (IL-17)	Mouse	eBioscience (Ready-SET-Go)
Interleukin 4	Mouse	eBioscience (Ready-SET-Go)

(IL-4)		
Macrophage inflammatory protein 2 (MIP-2)	Mouse	R&D systems

Table 2.7. ELISA kits used in this PhD research

2.16 Cell Survival Analysis

For analysis of cell survival, macrophages membrane integrity was determined using two assays; lactate dehydrogenase (LDH) and PrestoBlue assays. All experiments were performed in triplicate cultures. LDH assay was used to measure the released LDH from damaged macrophages, as a biomarker for cellular cytotoxicity and cytolysis. LDH released into the supernatant was measured using Cytotoxicity detection kit (Roche). 96 well tissue culture plates (Sigma) were filled with 100 ul of the assay medium. Cells were washed with 300 ul of the assay medium before adding them to the 96 wells plate. Cells suspensions were adjusted to a concentration of 2 X 10^6 cells/ml, and cells were titrated by two-fold serial dilutions across the plate. 200 ul of the standards were diluted in assay medium (1:2) and added to the plate. Cells were incubated at 37 °C, 5% CO_2, 90% humidity for 30 minutes. Microplates were centrifuged at 250xg for 10 minutes and supernatants removed carefully and transferred into a new clear 96- well flat bottom microplate (Sigma). LDH activity in these supernatants was determined by adding 100 ul of freshly prepared reaction mixture to each well, and plate were incubated in dark for 30 minutes at 25 °C. The absorbance of the samples was measured at 490 nm and 600 nm using a microplate reader.

PrestoBlue Cell Viability Assay (Life technologies) was performed following the manufacturer's protocol. PrestoBlue is a ready to use cell permeable resazurin-based solution that works as an indicator for cell viability. When added to macrophages, the reagent is changed by the reducing environment of the viable cell and turns red in color, becoming highly fluorescent. This changed color can be determined using absorbance measurements. Briefly, pneumococcal treated macrophages were washed 2 times with 400 μl of Dulbecco's phosphate-buffered saline (dPBS, Life technologies), then 250 μl of PrestoBlue reagent were added to the washed cells and incubated for 10 minutes at 37ºC. samples absorbance were measured at 570 nm using a micro-plate reader.

2.17 Antibodies and flow cytometry

Flow cytometry was performed on fresh cells suspensions. BMDMΦ or CD4+ T cells or Nasal mucosa tissue were incubated for 15 minutes with Fc-block (anti-CD16/32) and then stained for 20 minutes with the following antibodies in PBS 2% fetal bovine serum (FBS): Anti-CD206-APC (Mannose receptor), anti-CD69-PE/CY7 (T lymphocyte), anti-GATA3-APC (T helper 2), isotype Ctrl IgG2b-APC, isotype Ctrl IgG2b-PE (BioLegend), anti-CD11-b-PE (Macrophages), anti-FOXP3-PE (Regulatory T cells), anti-RORγt-APC (T helper 17), anti-CD45-FITC (T lymphocytes), anti-T-bet-PE (T helper 1) and anti-CD4-PE/CY7 (T helper cells) (eBioscience), anti- MARCO-FITC (AbD Serotec), Anti-Gr-1 (eBioscience), Anti-F4/80 (eBioscience), Anti-CD19 (eBioscience), Anti-CD8 (eBioscience), Anti-CD3

(eBioscience) . After staining, cells were washed in PBS 2% FBS and re-suspended in 300 μl of PBS and used immediately for data collection using a FACSCalibur flow cytometer (BD). Results were analyzed by FlowJo software (version 8.8.3, Tree Star). Reagents for cell fixation and permeabilization for detecting intracellular cytokines and Foxp3 were obtained from eBioscience, and staining was performed according to the manufacturer's instructions.

2.18 Immunohistochemistry

This work was performed at the university of York, York, U.K. by Dr. Alun Kirby. For immunofluorescence, Day 1 and day 7 tissue samples of cervical lymph nodes (CLN) and nasal mucosal were used and 7 μm sections were cut. Cells were stained using the following primary antibodies: BM8 (anti-F4/80), N418 (anti-CD11c), M5/114 (anti-MHCII) RM4-5 (anti-CD4), 53-67 (anti-CD8), H57-597 (anti-TCRb) (eBioscience), ED3 (anti-CD169) (AbD Serotec), FA11 (anti-CD68) (Acris Antibodies, Germany), ERTR9 (anti-SIGNR1) (Bachem), (anti-CD31) biotin, (anti-CD19), (anti-Meca32) biotin 546, (anti-PNAg) biotin 647, (anti-CD206) (biotin) (BD biosciences). Most of the antibodies were directly conjugated to fluorochromes but where indicated biotin antibodies were conjugated to secondary antibody Alexa-Fluor conjugates (Invitrogen). Sections were mounted in ProLong Gold (Invitrogen) and images were taken using a Zeiss Axioplan LSM 510 confocal microscope as single optical slices of between 0.8 and 1.0 μm. The images were analyzed using Zeiss LSM image browser software v4.

2.19 Binding Assay

According to Martinez-Pomares et al. (2006) and Chavele et al. (2010), Nunc Maxisorp 96 well flat-bottomed plates (Sigma) were coated overnight in the range of 1.25-10 μg/ml (100 μl per well) of: mannose receptor (MR) construct antibody (CTLD4-7-Fc), negative control construct (CR-FNII-CTLD1-Fc) (Both constructs provided generously by Dr. Luisa Martinez-Pomares, University of Nottingham), Mannan-BSA, Galactose (Sigma), Mannose (Sigma) in coating buffer (15 mM Na2CO3, 35mM NaHCO3, pH 9.6). Wells were blocked with 200 μl of 20% (v/v) FBS in PBS buffer, and then washed three times with 250 μl of PBS with 0.05% (v/v) Tween 20 (Sigma). 10 μg/ml of PLY or PdB, or domain 1-3 or domain 4 were added, and incubated on shaker at 37ºC for 1 h. Wells were washed again with PBS and bound proteins were detected using PLY polyclonal antibody (abcam) in blocking buffer. Plates were covered with anti-rabbit IgG alkaline phosphatase (abcam) in blocking buffer. Bound antibodies were detected in dark using the chromogenic substrate pnitrophenylphosphate (pNPP) for 30 mins. 1M of NaOH were added to all wells and the developed color was measured at 405nm.

2.20 Uptake Assay

This assay was performed at the University of Nottingham, Nottingham, U.K. by Dr. Luisa Luisa Martinez-Pomares. Both Chinese Hamster Ovary cells; wild type CHO and MR $^{-/-}$ CHO were harvest by trypsin/EDTA treatment. The cells suspensions were collected and washed twice with DMEM/F12 complete medium to remove trypsin. The pellets were re-suspended in 8 mL of medium culture and counted without any

further dilution. On a 24 well plate, 250000 cells/well were plated in DMEM/F12 complete medium (added of 0.6 mg/mL of geneticin for CHO-MR) and culture overnight (500 µl). The cells were washed twice with PBS (500 µlx2). 500 µl of opti-MEM were added to each well and the cells were incubated for 30 minutes at 37°C. The medium culture was discharged and 500 µl of each endocytic tracer (PLY, D4 and D1-3) were added at 5 µg/mL to the wells and incubated for 1 h at 37°C in the dark (covered with aluminum foil). The plates were then washed with PBS (3x 1mL). The cells were harvested by trypsin/EDTA treatment adding 200 µl of trypsin / EDTA diluted 1:1 with PBS. After 2 minutes at room temperature the cells suspensions were transferred to a FACS tube containing 200 µl of 4% p/v paraformaldehyde solution (final concentration in the tube 2% p/v). The FACS samples were stored in the fridge in the dark until FACS analysis on FC 500 MPL Flow Cytometry System, Beckman Coulter.

2.21 The preparation of macrophages for label free quantitative proteomics

Bone marrow cells from wild type C57B16 mice were prepared and cultured as mentioned previously in section 2.11 but in the absence of FBS. Proteomic work and data analysis were performed by Dr. Stuart Armstrong, at the University of Liverpool, Liverpool - U.K. Briefly, macrophages (in triplicate for each condition) were lysed in 50mM ammonium bicarbonate, 0.1% (w/v) RapiGest (Waters) then heated at 80ºC for 10 minutes before centrifugation at 12,000 xg for 15 minutes. The soluble fraction was transferred to low adhesion micro-centrifuge (Eppendorf) tubes. Total protein concentration was measured by Pierce™ Coomassie Plus (Bradford) Assay Kit assay

(Thermo scientific). Sample protein content and volume was normalised with 50mM ammonium bicarbonate. Samples were then heated at 80ºC for 10 minutes, reduced with 3 mM dithiothreitol (Sigma) at 60ºC for 10 minutes then alkylated with 9 mM iodoacetimde (Sigma) at room temperature for 30 minutes in the dark. Proteomic grade trypsin (Sigma) was added at a protein:trypsin ratio of 50:1 and samples incubated at 37ºC for 16 hrs. Rapigest was removed by adding TFA to a final concentration of 1% (v/v) and incubating at 37ºC for 2 hrs. Peptide samples were centrifuged at 12,000xg for 60 min (at 4ºC) to remove the precipitated Rapigest.

Samples were analysed by on-line nanoflow LC using the Thermo EASY-nLC 1000 LC system (Thermo Fisher Scientific) coupled with Q-Exactive mass spectrometer (Thermo Fisher Scientific). Samples were loaded on a 50cm Easy-Spray column with an internal diameter of 75μm, packed with 2μm C18 particles, fused to a silica nano-electrospray emitter (Thermo Fisher Scientific). The column was operated at a constant temperature of 35°C. Chromatography was performed with a buffer system consisting of 0.1% formic acid (buffer A) and 80% acetonitrile in 0.1% formic acid (buffer B). The peptides were separated by a linear gradient of 3.8 – 50% buffer B over 90 minutes at a flow rate of 300nl/min. The Q-Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 70,000. Up to the top 10 most abundant isotope patterns with charge states +2, +3 and/or +4 from the survey scan were selected with an isolation window of 2.0Th and fragmented by higher energy collisional dissociation with normalized collision energies of 30. MS/MS scans were acquired at a resolution of 17,500. The maximum ion injection times for the survey scan and the MS/MS scans were 250 and 50 ms, respectively, and the ion target value was set to 1E6 for survey scans and 1E5 for the MS/MS scans. Repetitive sequencing of peptides was minimized through dynamic exclusion of the

sequenced peptides for 20s. Thermo RAW files were imported into Progenesis LC–MS (version 4.1, Nonlinear Dynamics). Runs were time aligned using default settings and using an auto selected run as reference. Peaks were picked by the software and filtered to include only peaks with a charge state of between +2 and +6. Peptide intensities were normalised against the reference run by Progenesis LC-MS and these intensities are used to highlight differences in protein expression between control and treated samples with supporting statistical analysis (ANOVA adjusted p-values) calculated by the Progenesis LC-MS software. Spectral data were transformed to .mgf files with Progenesis LC–MS and exported for peptide identification using the Mascot (version 2.3.02, Matrix Science) search engine. Tandem MS data were searched against the mouse (16,868 sequences; 9,451,355 residues) and bovine (6,159 sequences; 2,427,109 residues) predicted proteomes (Uniprot release 2015_02). Mascot search parameters were as follows; precursor mass tolerance set to 10ppm and fragment mass tolerance set to 0.02 Da. Two missed tryptic cleavages were permitted. Carbamidomethylation (cysteine) was set as a fixed modification and oxidation (methionine) set as a variable modification. Mascot search results were further processed using the machine-learning algorithm Percolator. The false discovery rate was < 1%. Individual ion scores > 13 indicated identity or extensive homology ($p < 0.05$). Protein identification results were imported into Progenesis LC–MS as .xml files.

2.22 Statistical analyses

Unless otherwise stated, data were analyzed in GraphPad Prism and compared by one-way and two-way ANOVA. The Tukey-Kramer multiple-comparison test was

used to identify significant differences between individual groups. Results with P values < 0.05 were considered significant.

Chapter 3: Macrophage-pneumolysin interactions *in vitro* and *in vivo*

3.1. Introduction:

The aim of this chapter was to understand how pneumolysin activates and potentially drives different mechanisms of host immunity in the nasopharynx during colonization since the pneumococcal toxin pneumolysin have shown to be a potent activator of both mucosal and systemic immune responses, directly stimulating macrophages and dendritic cells and dramatically amplifying their production of pro-inflammatory cytokines independently of TLR-4. Also, we have shown for the first time that pneumolysin activates the NLRP3 inflammasome complex leading to release IL-1β, which is required for protection against invasive pneumococcal pneumoniae (McNeela et al., 2010). Our recent data expose an interesting dichotomy of PLY; that it is important to the pneumococcus for successful asymptomatic colonisation of the nasopharynx (Kadioglu et al., 2002, Richards et al., 2010), without generating pro-inflammatory immune responses, unlike its powerful ability to drive inflammation in the lungs during pneumonia. Based on new preliminary data, I propose that during nasopharyngeal colonisation, nasal mucosa-associated macrophages become activated by PLY and subsequently drain to cervical lymph nodes to initiate immune responses that act to prevent damage to host tissues and systemic pneumococcal dissemination

3.2 Macrophages accumulate in the draining lymph nodes of the nasopharynx during carriage.

Confocal imaging of cervical lymph nodes (CLN) during asymptomatic nasopharyngeal pneumococcal carriage in mice, identified an accumulation of a population of CD68, CD11c, and CD169 expressing macrophages (Figure 3.1). The aim of my PhD was to explore the role of these macrophages in anti-pneumococcal immunity and to understand how they behave during pneumococcal carriage.

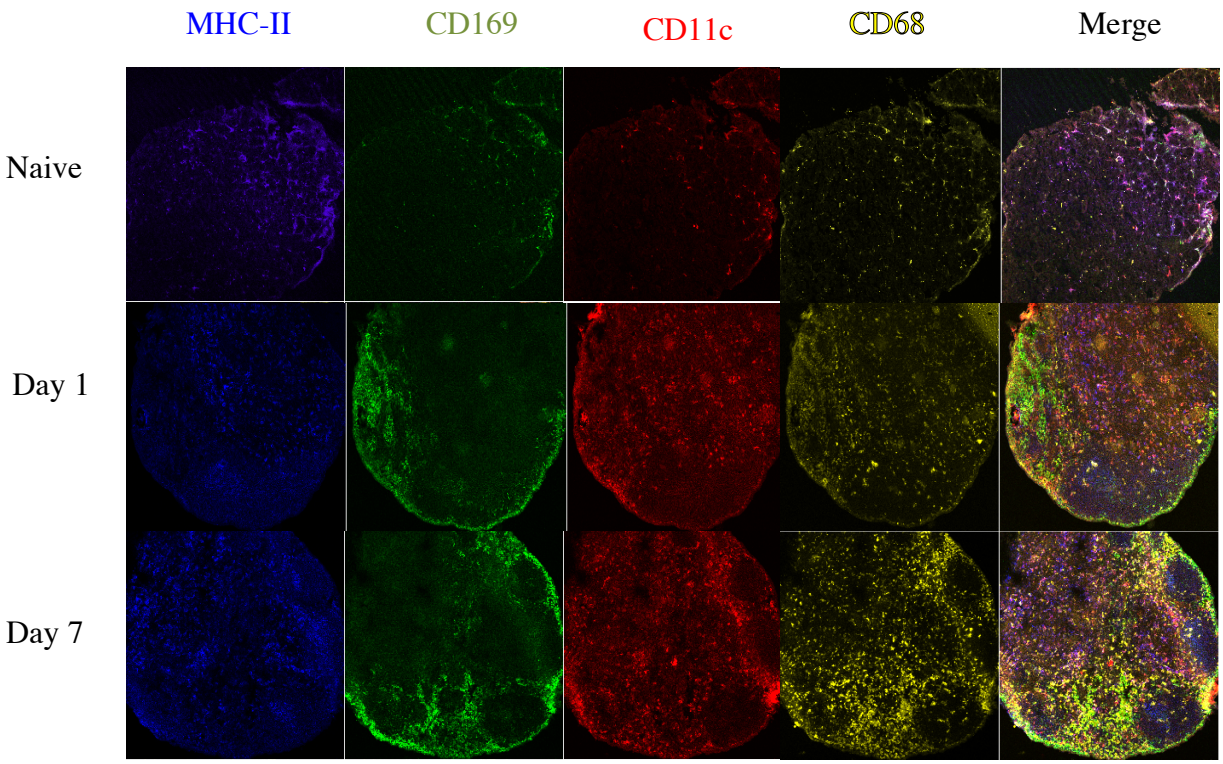


Figure 3.1. Confocal microscopy of macrophages in cervical lymph nodes during pneumococcal carriage. Cervical lymph node samples from MF1 mice on day 0, 1 and 7 of carriage with Wild type (WT) D39 were sectioned and stained with the following surface markers; CD68 (yellow), CD169 (green) and CD11c (red) and MHCII (blue), and a composite image. CD11c+ CD68+ CD169+ Pictures were taken with objective lens x 10.

3.3 Pneumococcal pneumolysin induces mannose-receptor expression in vitro and in vivo

We have previously identified a population of macrophages in the nasopharynx of mice undergoing prolonged pneumococcal carriage that express the mannose receptor (MR) marker of alternative activation (Neill et al., 2014). To determine whether pneumococci induce MR expression, wild type and $MR^{-/-}$ bone marrow derived macrophages (BMDM) were incubated with serotype 2 (D39) *S. pneumoniae* at a 1:10 ratio. MR expression was evident on a small proportion (5%) of un-stimulated wild type BMDM but not on $MR^{-/-}$ BMDM (Fig 3.2A and B). Furthermore, D39 induced a significant up-regulation in MR expression on wild type but not $MR^{-/-}$ BMDM (Figure 3.2A). Pneumococcal-induced changes in MR expression were partially dependent upon the pneumococcal toxin pneumolysin as a D39 mutant lacking toxin expression (ΔPLY) induced only moderately increased MR expression (Fig 3.2B). The contribution of polysaccharide capsule to pneumococcal-induced MR up-regulation appeared minimal as a capsule-deficient D39 strain (D39-J) induced MR up-regulation to a comparable extent to wild type D39 and a mutant strain deficient in both capsule and pneumolysin production (DKO) behaved similarly to ΔPLY (Fig 3.2B).

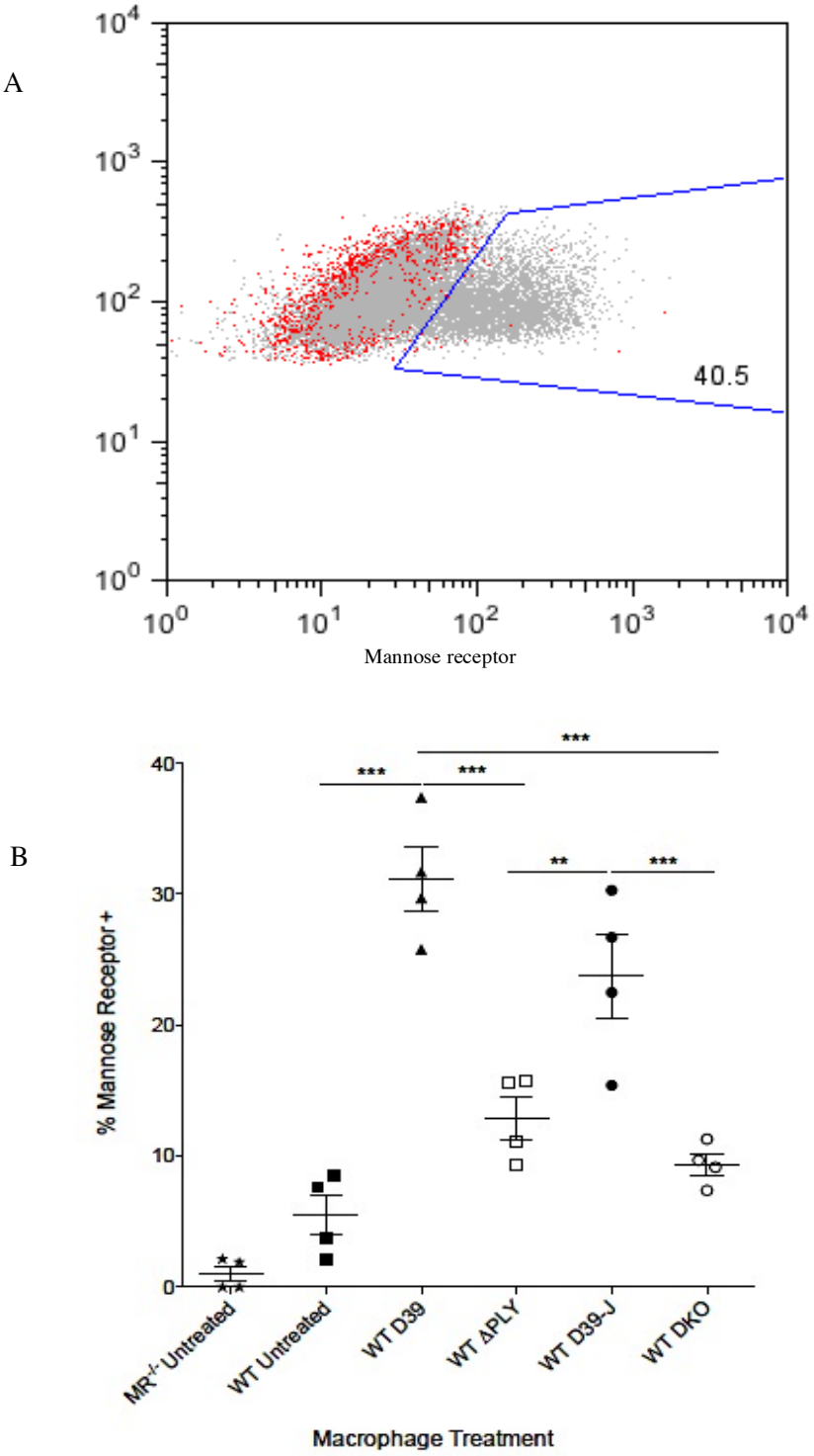
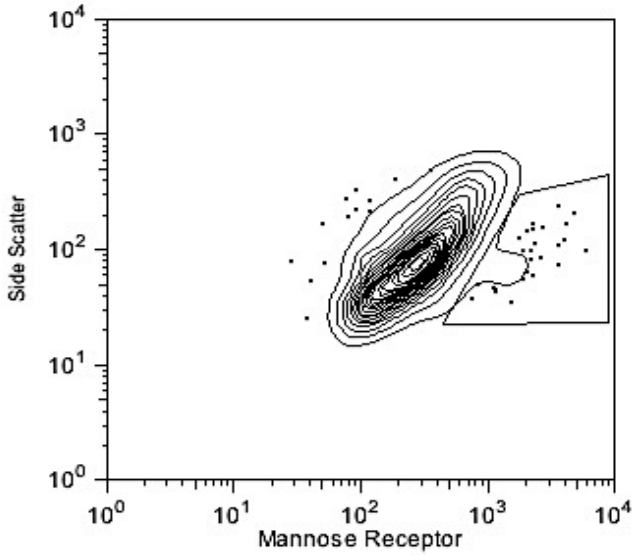


Figure 3.2. Pneumolysin induces macrophage mannose receptor up-regulation. Wild type (WT) or mannose-receptor deficient ($MR^{-/-}$), BMDM were incubated with *S. pneumoniae* D39, pneumolysin-deficient D39 (ΔPLY), capsule-deficient D39 (D39-J) and D39 lacking both pneumolysin and capsule (DKO) for 24 hours at a 1:10 ratio. **(A)** Expression of MR on BMDM at 24 hours post-infection with D39 (Red: $MR^{-/-}$ BMDM + D39) (Gray: WT BMDM + D39). 40.5 are the percentage of macrophages expressing mannose receptor. **(B)** Quantification of MR+ BMDM, gated relative to isotype control staining of F4/80+CD11b+ WT BMDM. Result in figure A a representative of one experiment of at least 3 wells per condition; therefore no statistical conclusion can be made. Results in figure B are representative of three independent experiments of at least 3 wells per condition. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

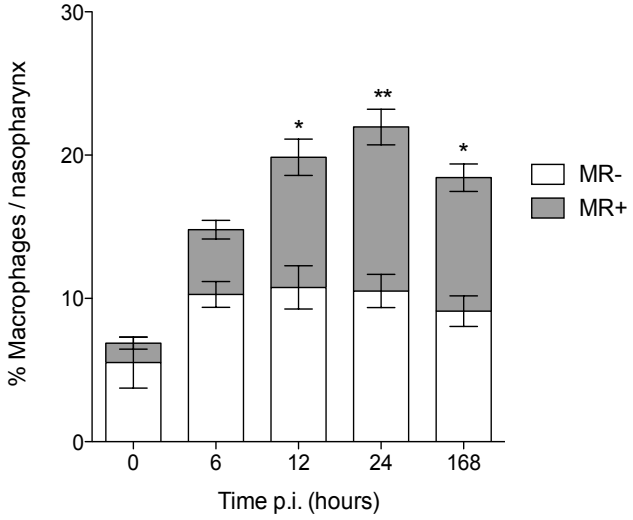
3.4 Mannose receptor expressing macrophages rapidly accumulate in the nasopharynx following pneumococcal colonisation.

The induction of MR up-regulation observed *in vitro* occurred within 24 hours of infection. We previously highlighted an accumulation of MR^+ macrophages in the nasopharynx *in vivo* at day 7 post-induction of pneumococcal carriage in mice (Neill et al, 2014). However, in that study we had not examined MR expression in the nasopharynx during the earliest stages of pneumococcal colonisation and carriage in mice. When we compared the proportion of macrophages amongst the leukocytes in the nasopharynx over the first 24 hours post pneumococcal colonisation we observed a significantly rapid increase that was almost entirely attributable to an increase in the MR^+ fraction of macrophages and that was maintained up to day 7 post-infection (Fig 3.3A and B). Furthermore, wild type mice infected with D39 had significantly lower densities of pneumococcus in the nasopharynx over the first 24 hours of pneumococcal carriage when compared to $MR^{-/-}$ mice (Figure 3.3C). Thus, the accumulation of MR^+ macrophages coincided with the period in which bacterial numbers in the nasopharynx stabilized following initial infection in WT mice. Interestingly, the absence of MR was correlated with significantly reduced polymorphonuclear cell (PMN) recruitment to the nasopharynx over the first 12 hours of infection (Figure 3.3D), suggesting an important role for MR in early control of bacterial numbers and recruitment of neutrophils during *S. pneumoniae* carriage. Confocal imaging of nasopharynx at day 7 post-infection confirmed the accumulation of MR-expression cells (Figure 3.3E).

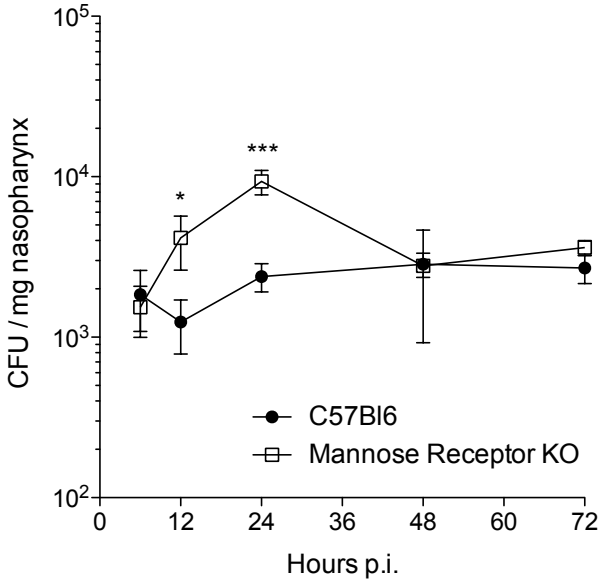
A


Side Scatter
Mannose Receptor

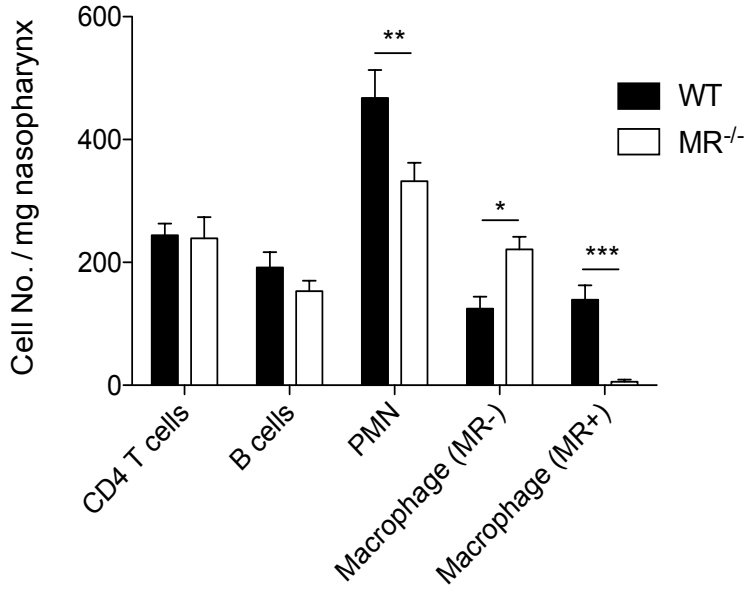
B


% Macrophages / nasopharynx
Time p.i. (hours)
MR-
MR+

C


CFU / mg nasopharynx
Hours p.i.
C57Bl6
Mannose Receptor KO

D



E

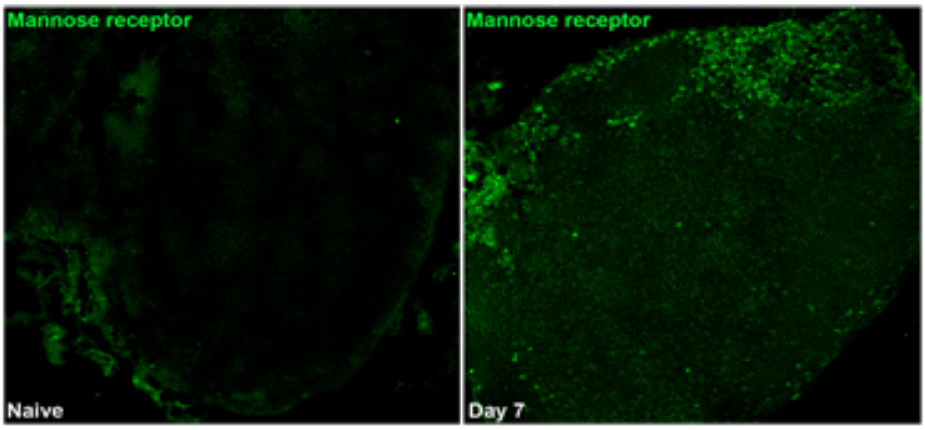


Figure 3.3. Mannose receptor expressing macrophages rapidly accumulate in the nasopharynx following pneumococcal colonisation. (A) MR expression on nasopharyngeal F4/80+CD11b+ cells from a naïve mouse. Gated area show the numbers of MR expressing macrophages. **(B)** Wild type mice were intranasally infected with $1x10^5$ CFU D39 and nasopharyngeal homogenates were stained for flow cytometry. Data shown are $F4/80^+CD11b^+$ cells as a proportion of total CD45+ cells. Shaded bars represent $MR^+F4/80^+CD11b^+$ cells. (**C**) Colony-forming units (CFU) per mg nasopharynx. (**D**) Cell number per mg nasopharynx 12 h post infection. **(E)** MR-stained nasopharyngeal tissue sections from naïve (left panel) and *S. pneumoniae* D39-colonised mice (right panel). Results A and E are representative of one experiment of at least 3 wells per condition; therefore no statistical conclusion can be made. B, C and D results are representative of three independent experiments of at least 3 wells per condition. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.5 Mannose receptor is important for the regulation of pro-inflammatory and anti-inflammatory cytokines during pneumococcal carriage in vivo

In line with a defect in neutrophil recruitment, nasopharyngeal homogenates from D39-infected $MR^{-/-}$ mice contained markedly less IL-6 than their WT counterparts (Figure 3.4). Furthermore, levels of the immune regulatory cytokine TGF-β were also significantly reduced in nasopharyngeal homogenates of $MR^{-/-}$ mice as compared to wild type controls (Figure 3.5), suggesting disruption of the normal pathways that lead to establishment of a tolerogenic nasopharyngeal environment conducive to carriage (Neill et al., 2014).

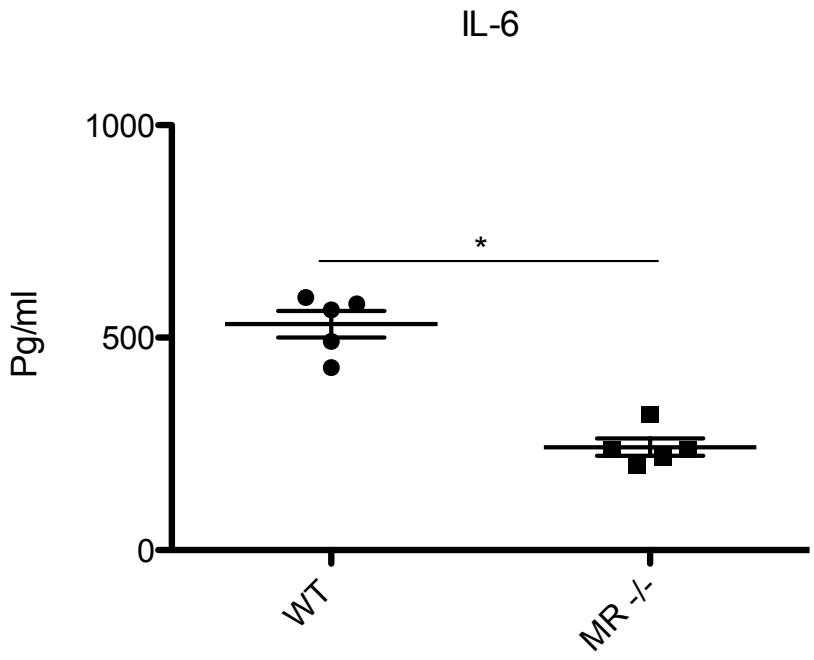


Figure 3.4. MR is important for the production of IL-6 during pneumococcal carriage. WT or $MR^{-/-}$ mice were infected with *S. pneumoniae* serotype 2 D39 for 24 h. ELISA measured cytokine levels in nasopharynx supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

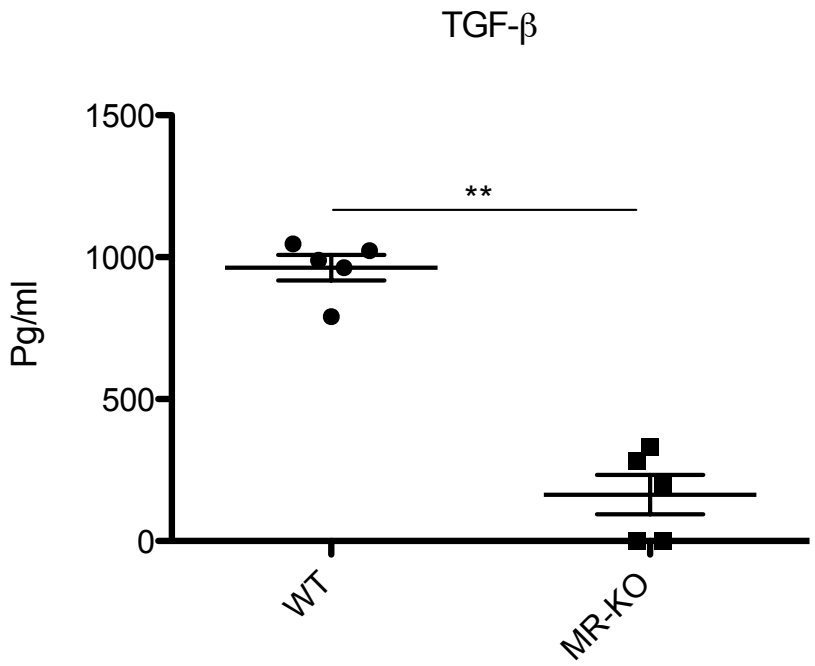
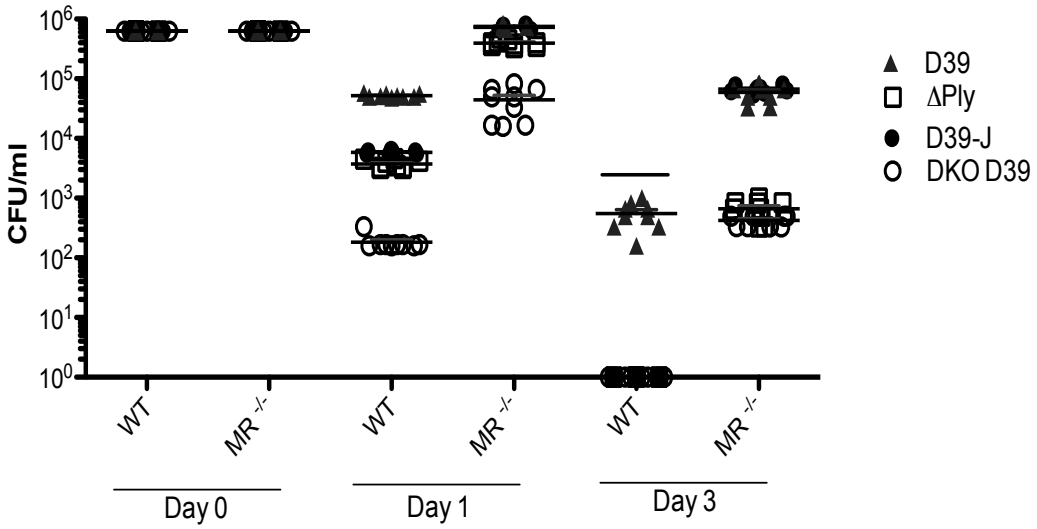


Figure 3.5. WT mice have higher levels of TGF-β during pneumococcal carriage. WT or MR $^{-/-}$ mice were infected with *S. pneumoniae* serotype 2 D39 for 24 h. ELISA measured cytokine levels in nasopharynx supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.6 Mannose receptor contributes to macrophage phagocytosis

I next sought to determine the role of mannose receptor expression on macrophages in defense against pneumococcal infection. *In vitro* phagocytosis assays performed with wild type and $MR^{-/-}$ BMDM revealed a significantly reduced ability to clear pneumococcal infection in the absence of MR expression (Figure 3.6A). When pneumococci were incubated with wild type BMDM, bacterial numbers were reduced by 99.95% (for D39) or were completely cleared (for pneumolysin-deficient, capsule-deficient and pneumolysin and capsule-deficient strains) within the first 72 hours post-infection as compared to starting dose. By contrast, when $MR^{-/-}$ BMDMs were used, none of the infecting bacterial strains were cleared. $MR^{-/-}$ BMDM had an attenuated ability to clear all pneumococcal strains tested but defense against D39 and the capsule-deficient strain was particularly compromised, with a 2-log deficit in D39 clearance (versus wild type BMDM) and a 5-log deficit in D39-J clearance by 72 hours post-infection. To determine whether this effect was due to impaired $MR^{-/-}$ macrophage survival and viability during *S. pneumoniae* infection, BMDM were seeded into 48-well tissue culture plates and cultured for 72hrs with D39, ΔPLY, D39-J, DKO D39 and purified LPS-free PLY. During, and at the end of, the culture period, supernatants were removed and PrestoBlue cell viability reagent was added to all wells (including the untreated wells) to determine cell death at day 0, day 1, and day 3 (Figure 3.6 B). The PrestoBlue analysis showed few differences in macrophage death following exposure to pneumococcal infections. Approximately 50% of both WT and $MR^{-/-}$ macrophages had died by day 3 post-infection but the rate of death did not differ between WT and $MR^{-/-}$ cells. The exception to this was for ΔPLY treated macrophages, where $MR^{-/-}$ cells showed a moderately increased susceptibility to cell death as compared to wild type controls (Figure 3.6B).

A



D39 VS. ΔPLY	D39 VS. D39-J	D39 VS. DKO D39
WT VS. MR -/- (Day 0) = ns	WT VS. MR -/- (Day 0) = ns	WT VS. MR -/- (Day 0) = ns
WT VS. MR -/- (Day 1) = ***	WT VS. MR -/- (Day 1) = ***	WT VS. MR -/- (Day 1) = ***
WT VS. MR -/- (Day 3) = ***	WT VS. MR -/- (Day 3) = ***	WT VS. MR -/- (Day 3) = ***

B

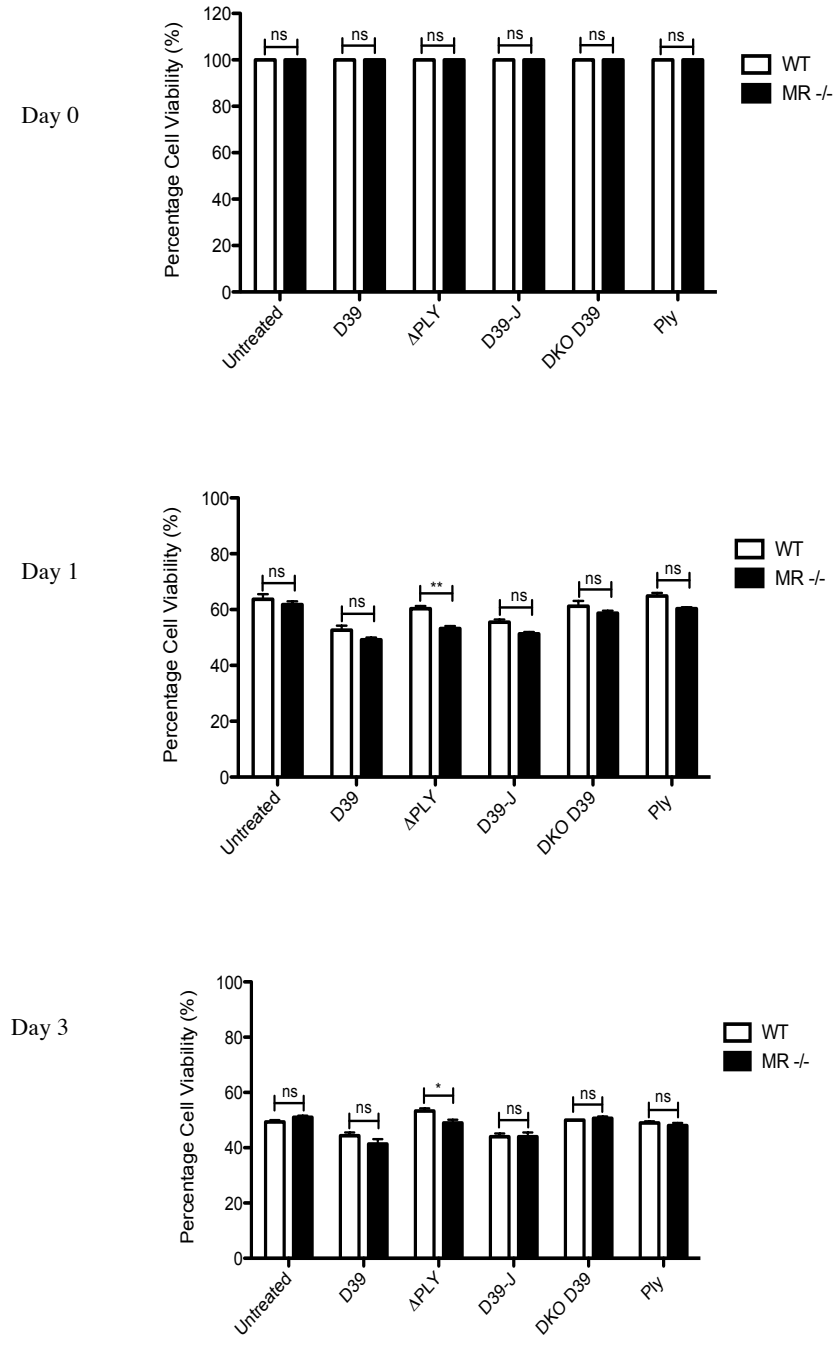


Figure 3.6. MR contributes to macrophage killing of pneumococci. (A) BMDMs from wild type (WT) or $MR^{-/-}$ (MRKO) mice were infected with *S. pneumoniae* D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J and pneumolysin- and capsule-deficient DKO for 72 hours. Data show bacterial colony forming units (CFU) per ml culture media ($MR^{-/-}$ versus WT BMDM). **(B)** Survival and viability of pneumococcal stimulated WT or $MR^{-/-}$ BMDMs. Samples were washed with Dulbecco's phosphate-buffered saline (dPBS), and then stained with PrestoBlue. Samples absorbance was measured at 570 nm using a micro-plate reader. Data are a composite of three experiments and each data point represents a mean of triplicate wells. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.7 Cytotoxicity of pneumococcal infection in WT and $MR^{-/-}$ BMDMs

To evaluate the cytotoxic activity of pneumococcus on WT and $MR^{-/-}$ BMDMs during infection, BMDM were incubated with D39 and ΔPLY for 24 and 72hrs. The effect of pneumococcus on the morphology of WT and $MR^{-/-}$ cells was determined by phase-contrast microscopy (20X) at 0 h (Figure 3.7A), 24 (Figure 3.7B), and 72hrs (Figure 3.7C) post-infection. Untreated WT and $MR^{-/-}$ cells were used as controls (Figure 3.7A). D39 treated WT and $MR^{-/-}$ macrophages were healthy at 0 h, however some sign of cell damage was evident by 24 h of pneumococcal infection, as the cells had begun to round up and lose the characteristic macrophage morphology (Figure 3.7B). More damage was observed in both WT and $MR^{-/-}$ BMDMs at 72 h post-infection. D39 treated $MR^{-/-}$ BMDM showed more cytopathic signs such as rounding of the infected cell and fusion with adjacent cells, as compared to D39 treated WT macrophages. Moreover, ΔPLY treated WT and $MR^{-/-}$ BMDM also displayed some sign of membrane injuries after 24 and 72 h of infection, however the level of damage was reduced in ΔPLY treated WT and $MR^{-/-}$ macrophages compared to D39 treated cells (Figure 3.7C). These data suggest that the pneumococcal toxin pneumolysin is partially responsible for the observed damage. Interestingly, the drop in viability during pneumococcal infections at 24 h and 72 h match the morphology changes observed in BMDMs at the same time points.

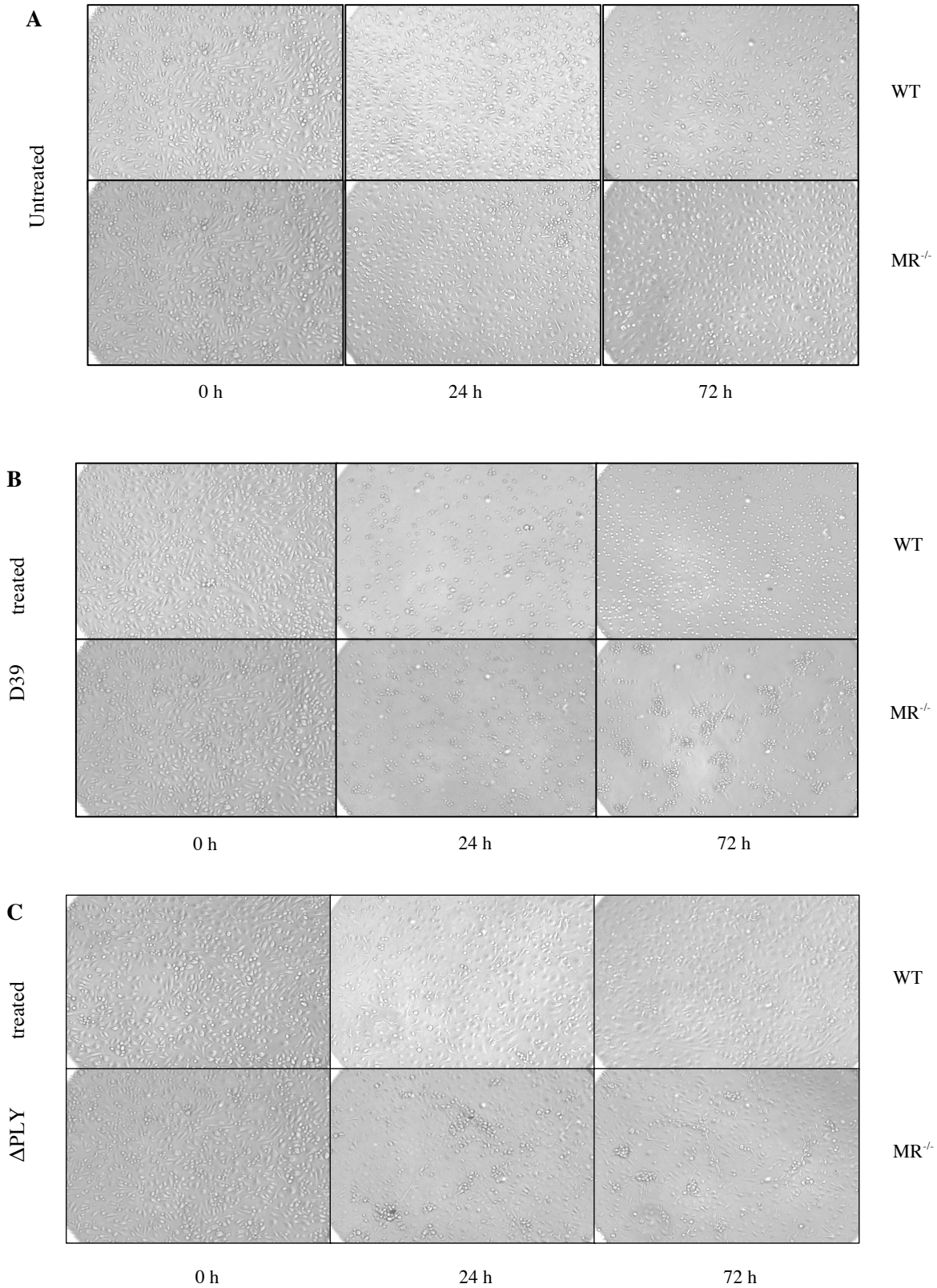


Figure 3.7. Cytotoxic activity of pneumococcal infections in WT and MR$^{-/-}$ BMDMs. Cells were stimulated with D39 and ΔPLY at an MOI of 1:10. Macrophages were photographed by 20x phase-contrast microscopy at 0 h, 24, and 72hrs post-infection. (**A**) Untreated WT and MR$^{-/-}$ Macrophages. (**B**) D39 treated WT and MR$^{-/-}$ BMDMs. (**C**) ΔPLY treated WT and MR$^{-/-}$ cells. Macrophage cell rounding, shrinkage and detachment (Cytopathic signs) are present in D39 and ΔPLY treated WT and MR$^{-/-}$ BMDMs at 24 and 72hrs post-infection.

3.8 Determination of cell death in WT and MR$^{-/-}$ macrophages infected with pneumococcus

To determine if MR plays a role in preventing macrophage lysis or damage during pneumococcal infections, the integrity of the macrophage membrane during pneumococcal infections was determined by measuring the level of lactate dehydrogenase (LDH) in cell culture supernatants. LDH is commonly released during tissue damage and lysis and the levels of it in supernatant correlate with the extent of cell damage. WT and MR$^{-/-}$ BMDMs were infected with D39, ΔPLY, DKO D39 and PLY for 24 h. Culture supernatants were collected at 24 h post infection, and LDH concentrations were determined. LDH levels were markedly increased in infected versus uninfected cells but there were no differences between WT and MR$^{-/-}$ cells (Figure 3.8). Cell damage was markedly reduced during infection with DKO D39 as compared to WT D39, ΔPLY or PLY. These data suggest that macrophage death occurs independently of MR during pneumococcal infections.

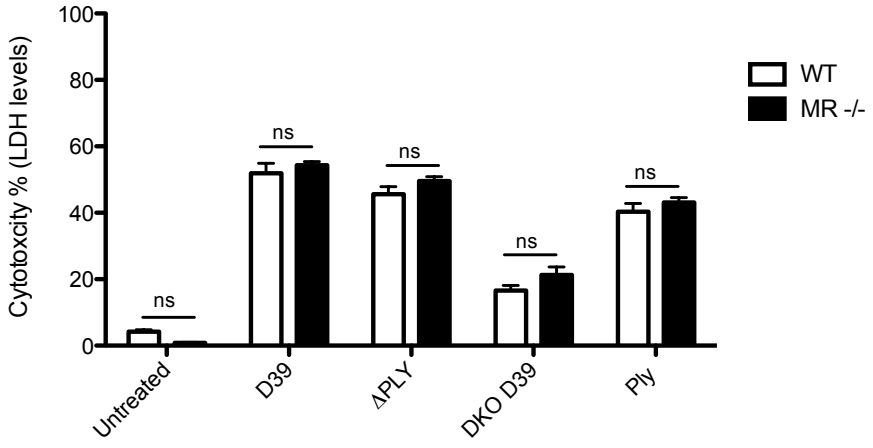


Figure 3.8. The determination of cell death in BMDMs infected with pneumococcus. Supernatants from infected BMDMs were tested for LDH, a marker of cell damage, to quantify cell death. BMDMs were infected with D39, ΔPLY, DKO D39 and PLY for 24 h. Cell death was monitored by the concentration of LDH released from damaged cells. Data are a composite of three experiments and each data point represents a mean of triplicate wells. Error bars represent standard deviations. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.9 MR contributes to pneumococcal-induced macrophage cytokine production

$MR^{-/-}$ BMDM demonstrated a significant attenuation in cytokine production in response to *S. pneumoniae* infection (Figure 3.9). Wild type BMDM stimulated with D39 produced a mixed cytokine response of inflammatory (IL-1β, MIP-2, IL-6 and IL-12) and immunomodulatory (IL-10) cytokines. The production of all these cytokines was reduced when BMDM were stimulated with D39 lacking pneumolysin, capsule or both, confirming the importance of these virulence factors in induction of host responses. When $MR^{-/-}$ BMDM were stimulated with D39 or the mutant bacterial strains, production of IL-1β, IL-6, IL-12 and IL-10, but not MIP-2 was significantly reduced, suggesting MR plays a role in activation of macrophage cytokine signaling pathways. Also, cells stimulated with PLY give no response for IL-1β, IL-6, IL-12 and IL-10, but not MIP-2. However, all cytokines were detected at higher concentrations in pneumococcal-infected $MR^{-/-}$ BMDM than in uninfected controls, demonstrating that MR-independent pathways to pneumococcal induction of macrophage cytokine production exist.

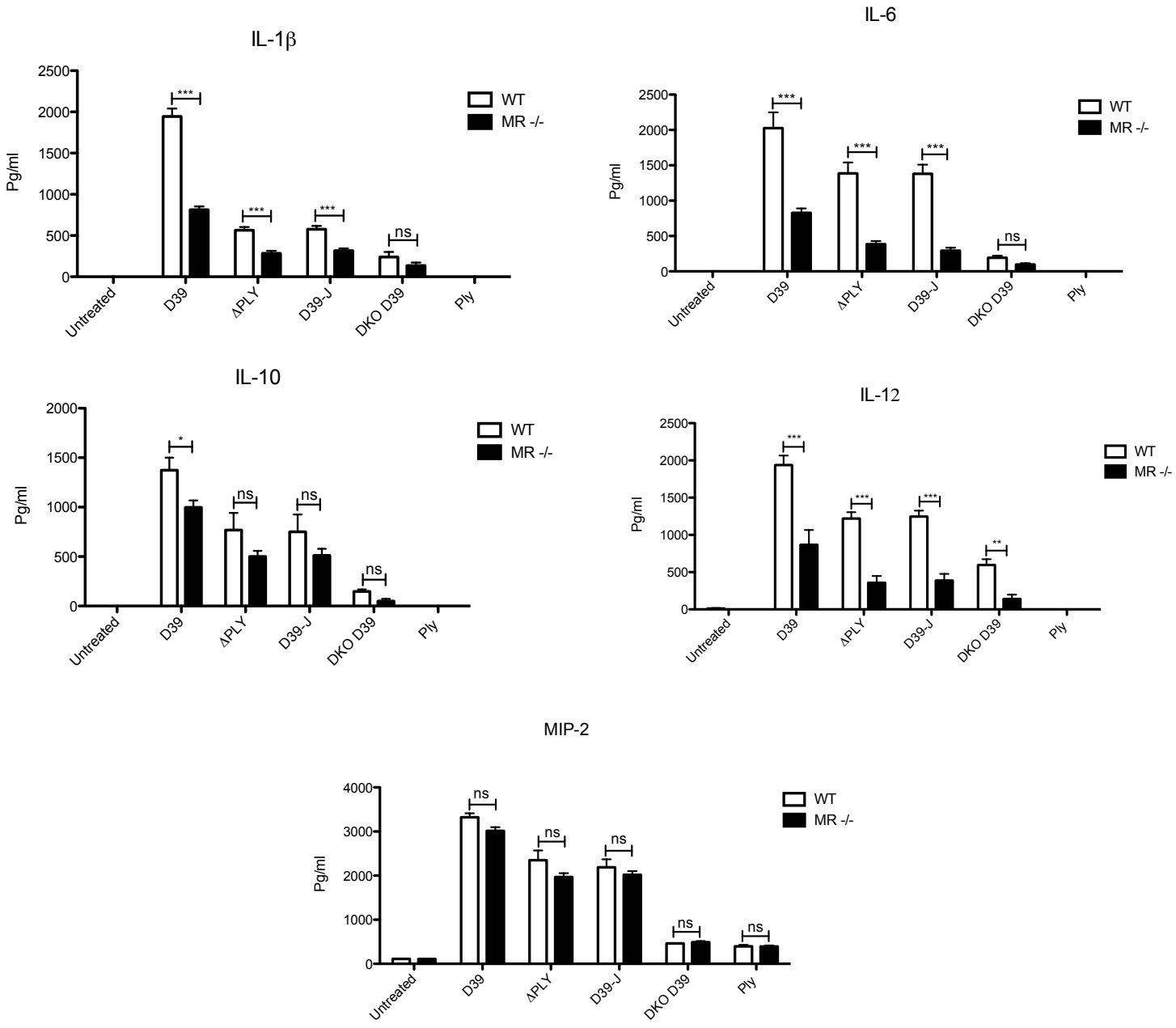


Figure 3.9. MR contributes to pneumococcal-induced macrophage cytokine production. BMDM from wild type (WT) of $MR^{-/-}$ (MR KO) mice were infected with *S. pneumoniae* D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J and pneumolysin- and capsule-deficient DKO for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.10 Domain four of pneumolysin binds mannose receptor

As MR appeared to influence *S. pneumoniae*-induced macrophage cytokine production and as PLY contributed to this process, we next sought to understand the mechanisms that initiate macrophage responses to pneumococcal infection. Binding of MR to PLY was assessed using a solid phase binding ELISA. The aim was to identify if MR could interact directly with PLY. Galactose was used as a negative control (as a sugar that does not bind MR), and Mannan was used as a ligand for the CTLD4-7 and CR domains of the MR (a positive control). The mannose receptor construct antibody (CTLD4-7-Fc) showed strong binding to PLY (Figure 3.10). To identify which domain of PLY was responsible for this interaction with macrophage MR, purified PLY domains 1-3 and domain 4 were utilized. Results revealed that domain 4 was able to bind the MR construct whereas domains 1-3 showed no binding (Figure 3.7). Next, the importance of the hemolytic activity of PLY to MR binding was studied using a genetically inactivated PLY toxoid, PdB. The toxoid is also known as W433F because it has one amino acid change (Tryptophan to phenylalanine) at sequence 433 of PLY. Binding was observed but was significantly reduced as compared to WT PLY (Figure 3.10). These data suggest that cytolytic activity (or the structure of the cytolytic domain) contributes to PLY binding to mannose receptor. These results may explain one mechanism by which *S. pneumoniae* initiates macrophage activation and accumulation during pneumococcal infections via specific binding of MR to PLY.

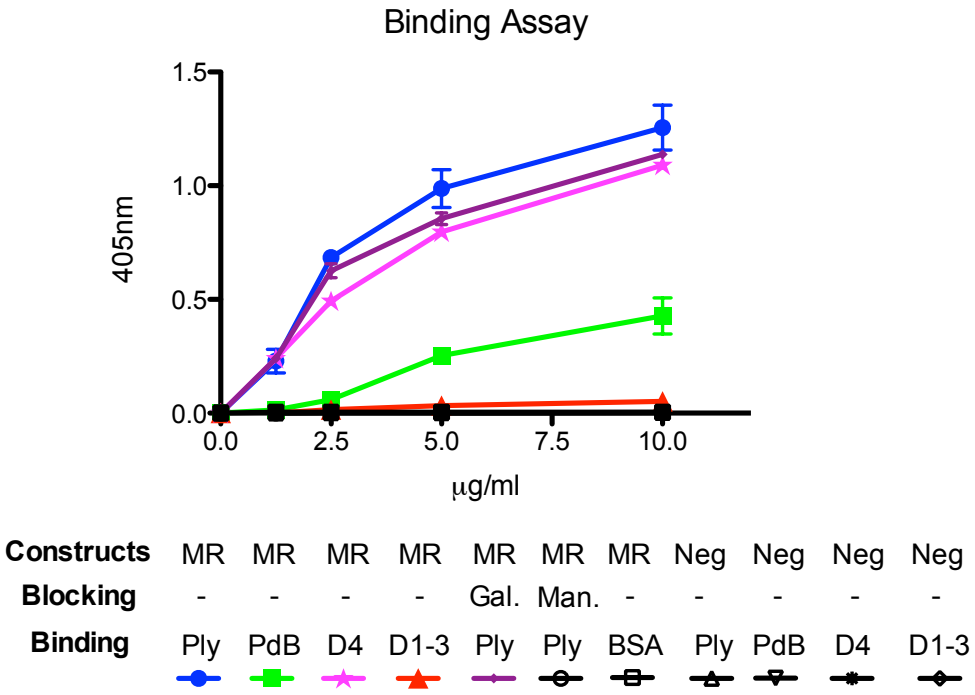


Figure 3.10. Binding of recombinant PLY to macrophage mannose receptor. Mannose receptor (MR) construct antibody (CTLD4-7-Fc), negative (Neg) control construct (CR-FNII-CTLD1-Fc), Galactose (Gal.) and Mannose (Man.) were blocked or incubated in the range of 1.25-10µg/ml with 10µg/ml of PLY, PdB, domain 1-3 and domain 4 for 1hr at 37ºC. Mannan (Man) was used as a specific ligand for CTLD4-7 and CR domains and galactose (Gal) was used as a negative control. Bound proteins and constructs were washed and detected using anti-human IgG Fc-specific, alkaline phosphatase conjugates, and PLY polyclonal antibody. Binding was determined with p-nitrophenyl phosphate substrate or with anti-rabbit IgG alkaline phosphatase. Binding levels were measured at 405nm. Results are mean of three individual experiments (± SD).

As MR was found to mediate macrophage binding to pneumococci via PLY, we next sought to determine whether this interaction facilitated uptake of pneumococci or the toxin. The uptake of whole PLY, domain 4, and domain 1-3 by WT CHO cells and MR-transfected CHO cells was assessed (Figure 3.11). Galactose was used as a negative control, and Mannose was used as a ligand for the CTLD4-7 and CR domains of the MR. As expected, MR-transfected CHO cells were capable of mannose uptake but not galactose uptake. PLY killed both WT and MR-transfected cells, neither cell line internalized domain1-3, and both WT and $MR^{-/-}$ CHO cells

internalized domain 4 with equal efficiency (Figure 3.11). These results suggest that toxin-MR interaction alone is not sufficient to mediate uptake of toxin by cells.

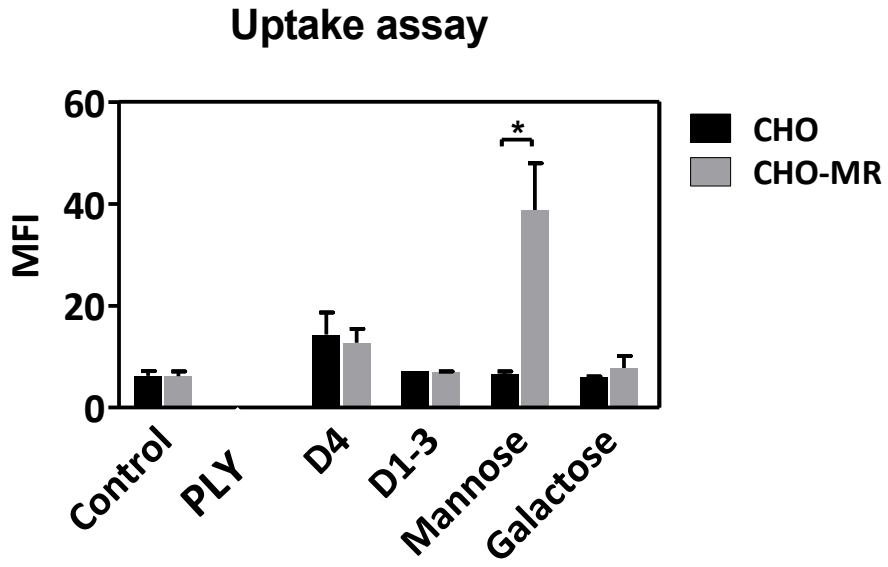


Figure 3.11. The uptake of whole PLY, domain 4 and domain 1-3 by CHO cells. Mannose receptor (MR) construct antibody (CTLD4-7-Fc), negative (Neg) control construct (CR-FNII-CTLD1-Fc), Galactose (Gal.) and Mannose (Man.) were incubated with 5μg/ml of whole PLY, domain 4 and domain 1-3 for 1hr at 37ºC. Mannan (Man) was used as a specific ligand for CTLD4-7 and CR domains, respectively, and galactose (Gal) was used as a control. Uptake was analyzed using BD FACScalibur and CellQuest software. Results are mean of three individual experiments (± SD).

3.11 Pneumolysin and capsule are required for pneumococcal-induced T regulatory, T helper 17 and T helper 1 cell differentiation in vitro

Recent work by our group has revealed that T regulatory cells and macrophages accumulate in the nasopharynx during carriage, and a high portion of macrophages express MR (Neill et al., 2014). This study proposed that alternative activation of macrophages might occur in the nasopharynx of mice during long-term carriage. This observation was supported by the determination of high levels of major histocompatibility complex class II (MHC II) expression on the MR expressing macrophages from low-density (and long-term) WT colonized mice, when compared to high-density (and short-term) colonisation. So, to determine the influence of PLY on macrophage-induced differentiation of CD4+ T cells, WT BMDM were stimulated with D39, ΔPLY, D39-J, DKO D39, or purified PLY for 24 h, and then incubated with naïve CD4+ T cells for 5 days. D39-treated macrophages induced differentiation of 10-15% CD4+ T cells into T reg cells (Foxp3+), and this was significantly reduced when ΔPLY, D39-J or DKO D39-exposed macrophages were used (Figure 3.12). Purified PLY-stimulated macrophages induced no T reg differentiation, suggesting that PLY alone is insufficient to prime macrophages to induce T reg differentiation (Figure 3.12). No differentiation of Th17 or Th2 cells was observed under any condition, but some Th1 differentiation was observed in co-culture of WT D39-exposed BMDM with CD4+ T cells. These data suggest that pneumococci-exposed macrophages regulate the differentiation of CD4+ T cells into T reg cells and that both LPS-PLY and capsule contribute to the process. These observations were confirmed by measuring the levels of lineage-associated cytokines in the supernatants

of the cultured cells (Figure 3.13). T cells stimulated with D39 treated macrophages produced high levels of IL-10 (T reg-associated) and INF-γ (Th1) when compared to ΔPLY, D39-J, DKO, and PLY treated / stimulated cells. Significant IL-17 production was also observed (Figure 13) but given the low levels of Th17 differentiation observed (Figure 3.12), the source of this cytokine is unclear. No IL-4 production was observed under any condition, in line with the lack of Th2 differentiation observed (Figure 3.12).

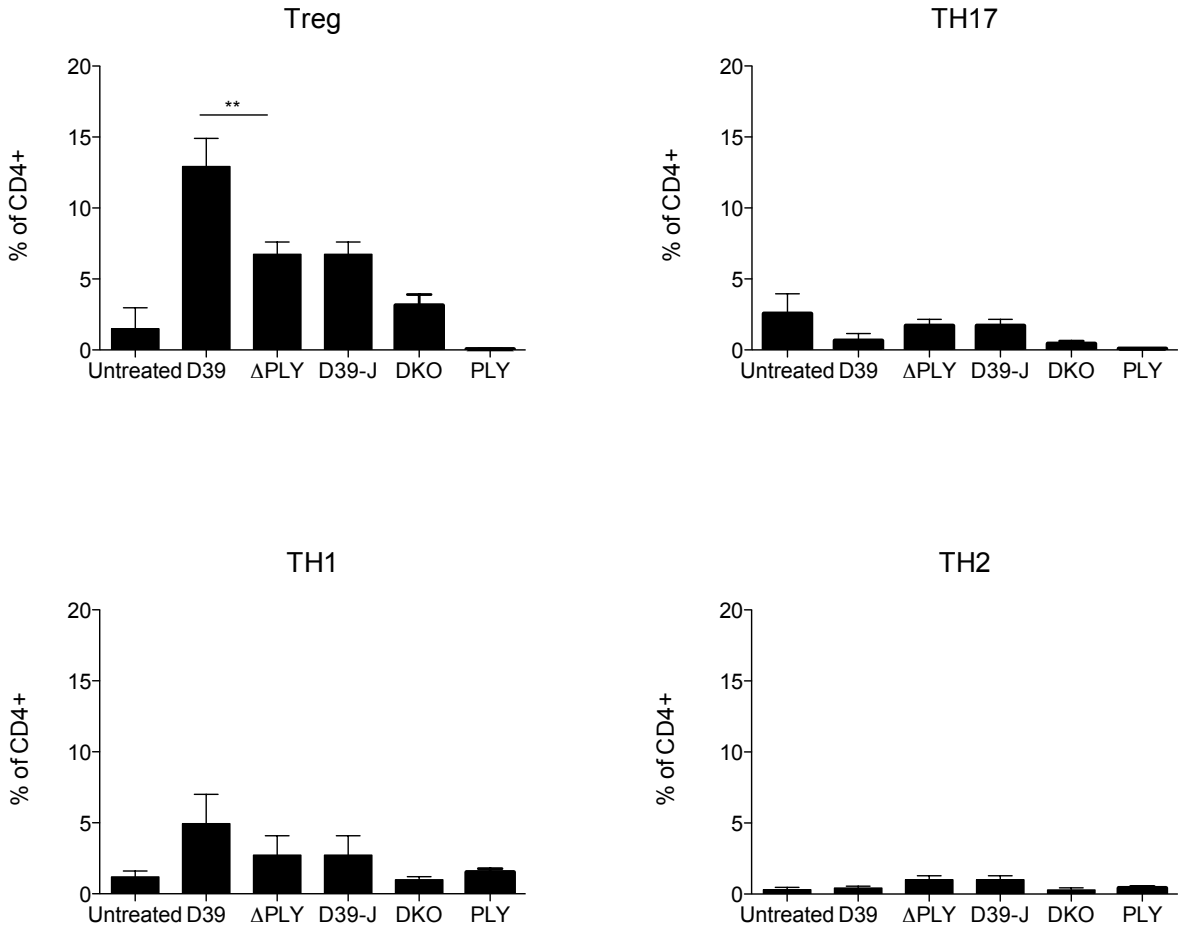


Figure 3.12. D39 induces T regulatory cell up-regulation and differentiation. BMDM were stimulated with *S. pneumoniae* D39, pneumolysin-deficient D39 (ΔPLY), capsule-deficient D39 (D39-J) and D39 lacking both pneumolysin and capsule (DKO) for 24 hours at a 1:10 ratio, activated cells were incubated with naïve CD4+ T cells for 5 days at a 1:15 ratio. **(A)** Percentage of Treg differentiation after 5 days of incubation with D39, ΔPLY, D39-J, DKO, and PLY stimulated macrophages. **(B)** Percentage of Th17 differentiation after 5 days of incubation with D39, ΔPLY, D39-J, DKO, and PLY stimulated macrophages. **(C)** Percentage of Th1 differentiation after 5 days of incubation with D39, ΔPLY, D39-J, DKO, and PLY stimulated macrophages. **(D)** Percentage of Th2 differentiation after 5 days of incubation with D39, ΔPLY, D39-J, DKO, and PLY stimulated macrophages. . Results are representative of three independent experiments of at least 3 wells per condition. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

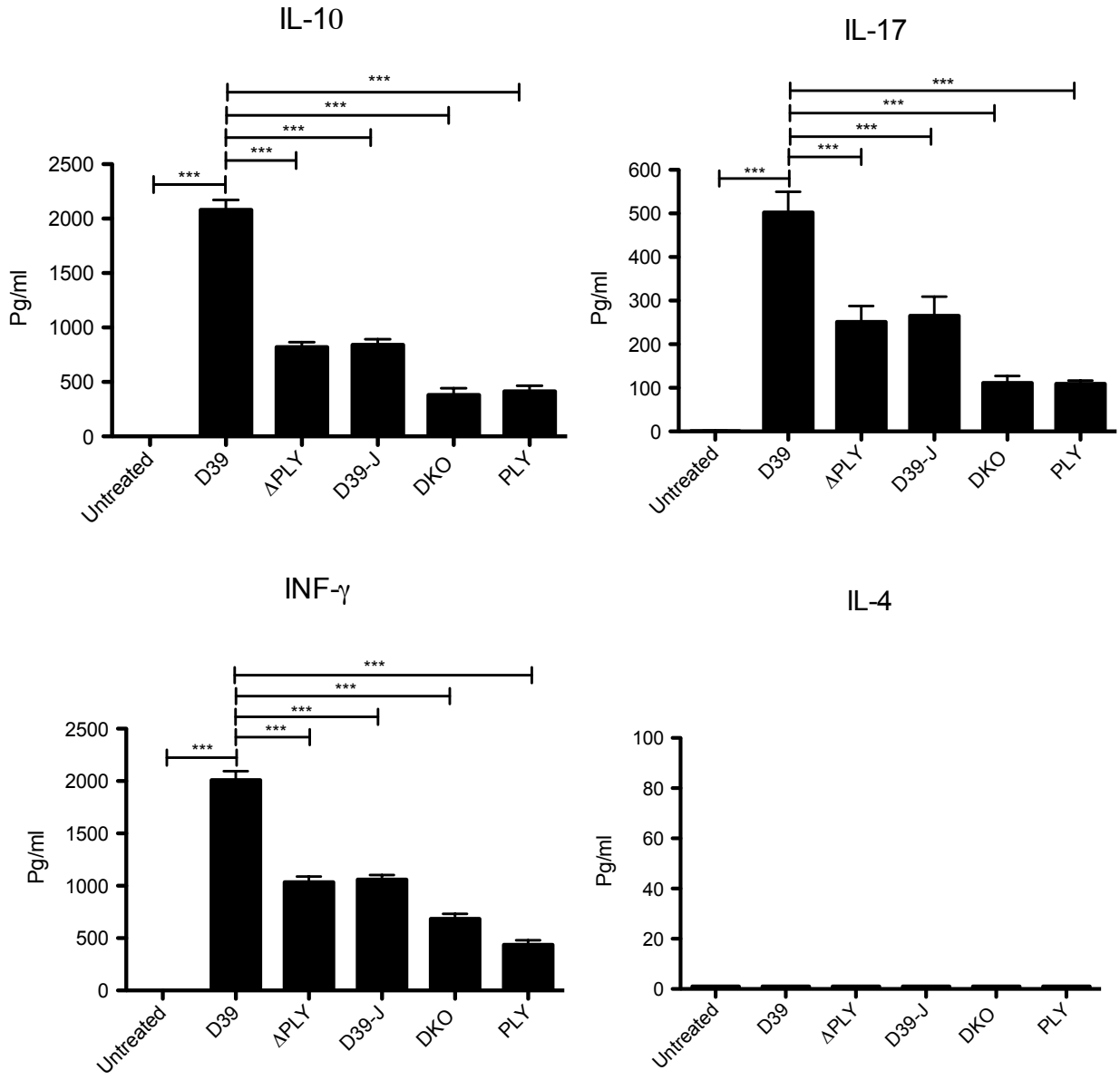


Figure 3.13. D39 induces high levels of IL-10, INF-γ and IL-17 in vitro. BMDM from wild type (WT) mice were infected with *S. pneumoniae* D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO and purified PLY for 24 hours, stimulated cells then incubated with naïve CD4+ T cells for 5 days, cytokine levels in cultures supernatants were measured by ELISA. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.12 Mannose receptor is important for the differentiation of CD4+ T cells into T regulatory cells, and cytokine production in vitro

To determine if the ability of pneumococcal-exposed macrophages to induce the differentiation of T reg cells is MR-dependent, wild type and $MR^{-/-}$ BMDM were incubated with D39, ΔPLY, D39-J, DKO and purified PLY at a 1:10 ratio / 4μg/ml. The presence of MR was associated with the expression of high levels of Foxp3 in T cells (about 20%) stimulated with D39 (Figure 3.14A). In these experiments, levels of Th1 and Th17 differentiation induced by WT BMDM were higher than seen previously, but again, no Th2 differentiation was observed (Figure 3.14). $MR^{-/-}$ cells induced significantly less Th1, Th17 and T regulatory cell differentiation than WT controls. Pneumolysin and capsule contributed to T cell differentiation, and macrophages treated with DKO pneumococci induced very little T cell differentiation. Foxp3 was used as a surface maker for T reg cells, RORgt as a surface marker for Th17 cells, Tbet as a surface marker for Th1 cells, and GATA3 as a surface marker for Th2 cells. CD4+ T cells cultured with pneumococcal-exposed $MR^{-/-}$ macrophages also produced significantly less cytokine than those cultured with WT macrophages (Figure 3.15). Wild type BMDM stimulated with D39 induced the production of inflammatory (IL-17 and INF-γ) and immunomodulatory (IL-10) cytokines from T cells. The production of all these cytokines was reduced when cells were stimulated with pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO and purified PLY. These data suggest a role for MR in the induction of host responses via T cell differentiation and cytokine production. These processes require the presence of PLY and capsule.

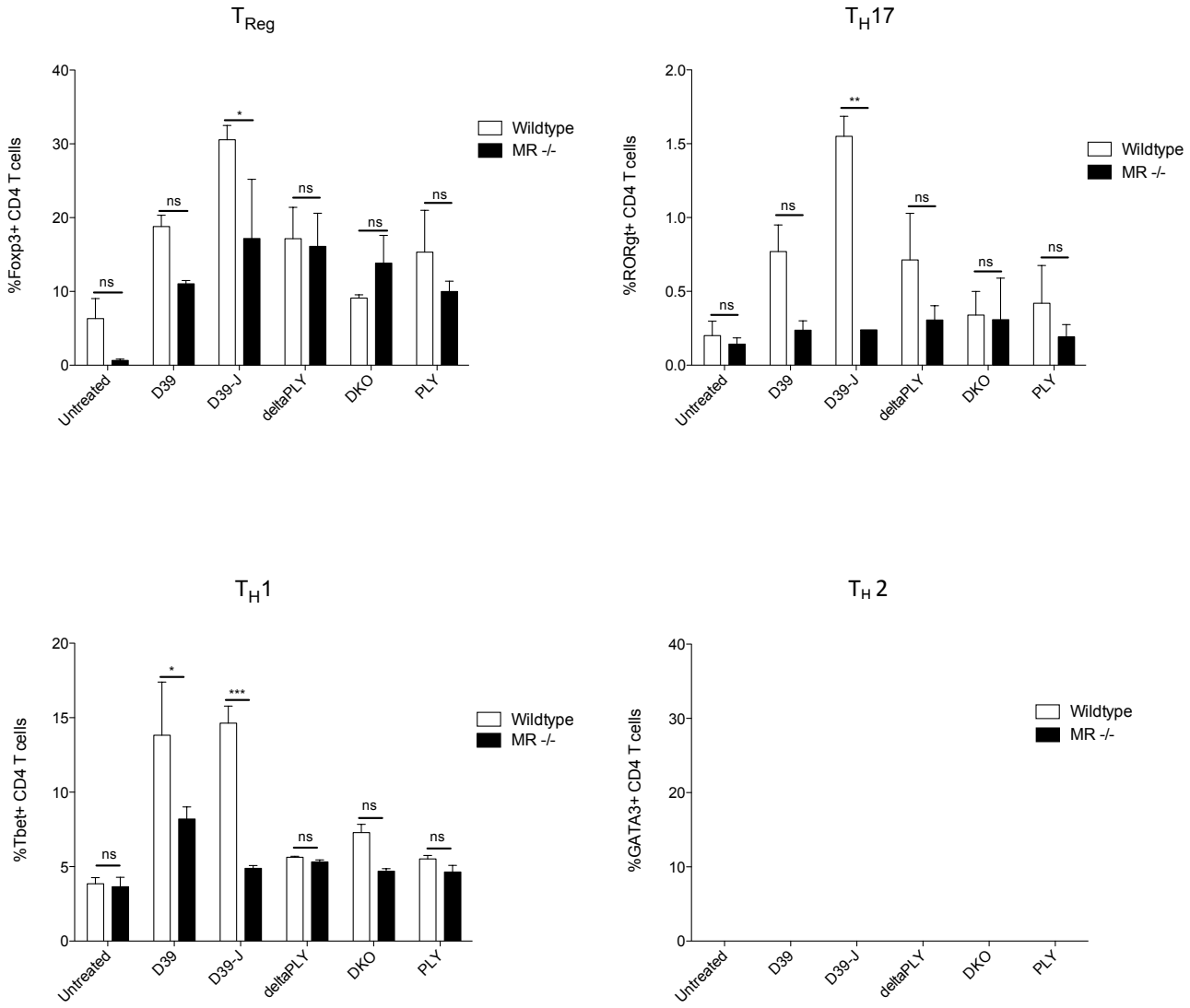


Figure 3.14. Mannose receptor is required for CD4+ T cell differentiation. BMDM were stimulated with *S. pneumoniae* D39, pneumolysin-deficient D39 (ΔPLY), capsule-deficient D39 (D39-J) and D39 lacking both pneumolysin and capsule (DKO) for 24 hours at a 1:10 ratio, activated cells were incubated with naïve CD4+ T cells for 5 days at a 1:15 ratio. **(A)** Percentage of Treg differentiation after 5 days of incubation in the presence of absence of MR, **(B)** Percentage of Th17 differentiation after 5 days of incubation in the presence of absence of MR. **(C)** Percentage of Th1 differentiation after 5 days of incubation in the presence of absence of MR. (**D**) Percentage of Th2 differentiation after 5 days of incubation in the presence of absence of MR. Foxp3, RORgt, Tbet and GATA3 were used as markers for the differentiations of T reg, Th17, Th1 and Th2. Results are representative of three independent experiments of at least 3 wells per condition. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

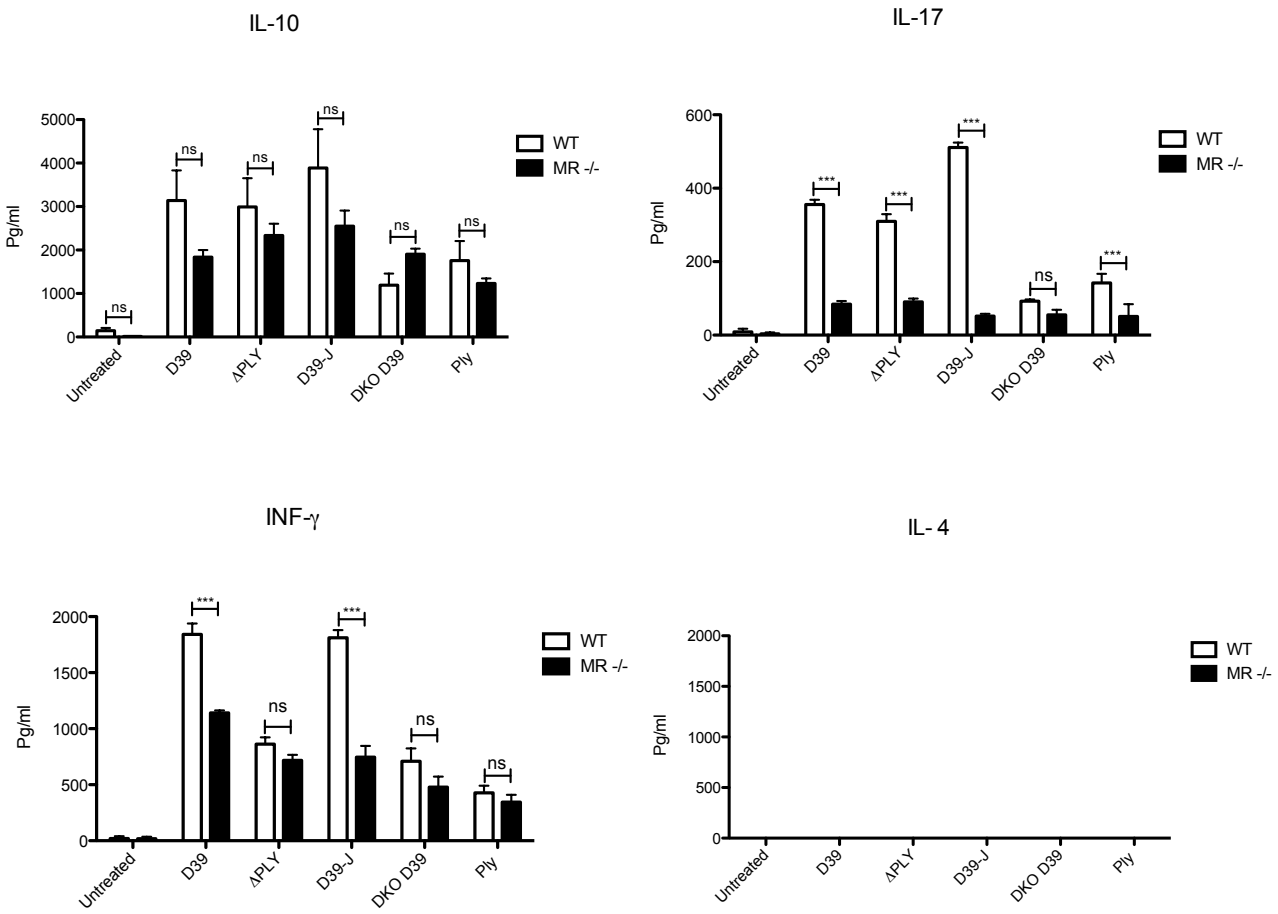


Figure 3.15. Mannose receptor is important for production of CD4+ T cell cytokines in vitro. BMDM from wild type (WT) or MR $^{-/-}$ mice were infected with *S. pneumoniae* D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO and purified PLY for 24 hours. Stimulated cells were then incubated with naïve CD4+ T cells for 5 days, cytokine levels in cultures supernatants were measured by ELISA. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.13 TLR-2 is important for the expression of mannose receptor in response to pneumococcal infections

TLR-2 is a major pattern recognition receptor for Gram-positive bacteria. I have demonstrated here that mannose receptor also plays a key role in response to pneumococcal infection; therefore I sought to determine if the two receptors may have interacting, complementary or redundant roles in anti-pneumococcal immunity. WT BMDMs stimulated with D39 induced the up-regulation of expression of MR (Figure 3.16), while TLR-2 and TLR-4 double knockout BMDMs showed no detectable changes in expression of MR at 24 h post-infection (Figure 3.16). The pneumococcal-induced up-regulation of MR occurs via a TLR2-dependent signaling pathway, as D39 induced up-regulation of MR in TLR4$^{-/-}$ BMDM but no change in MR expression was detected in TLR2-deficient BMDM (Figure 3.16).

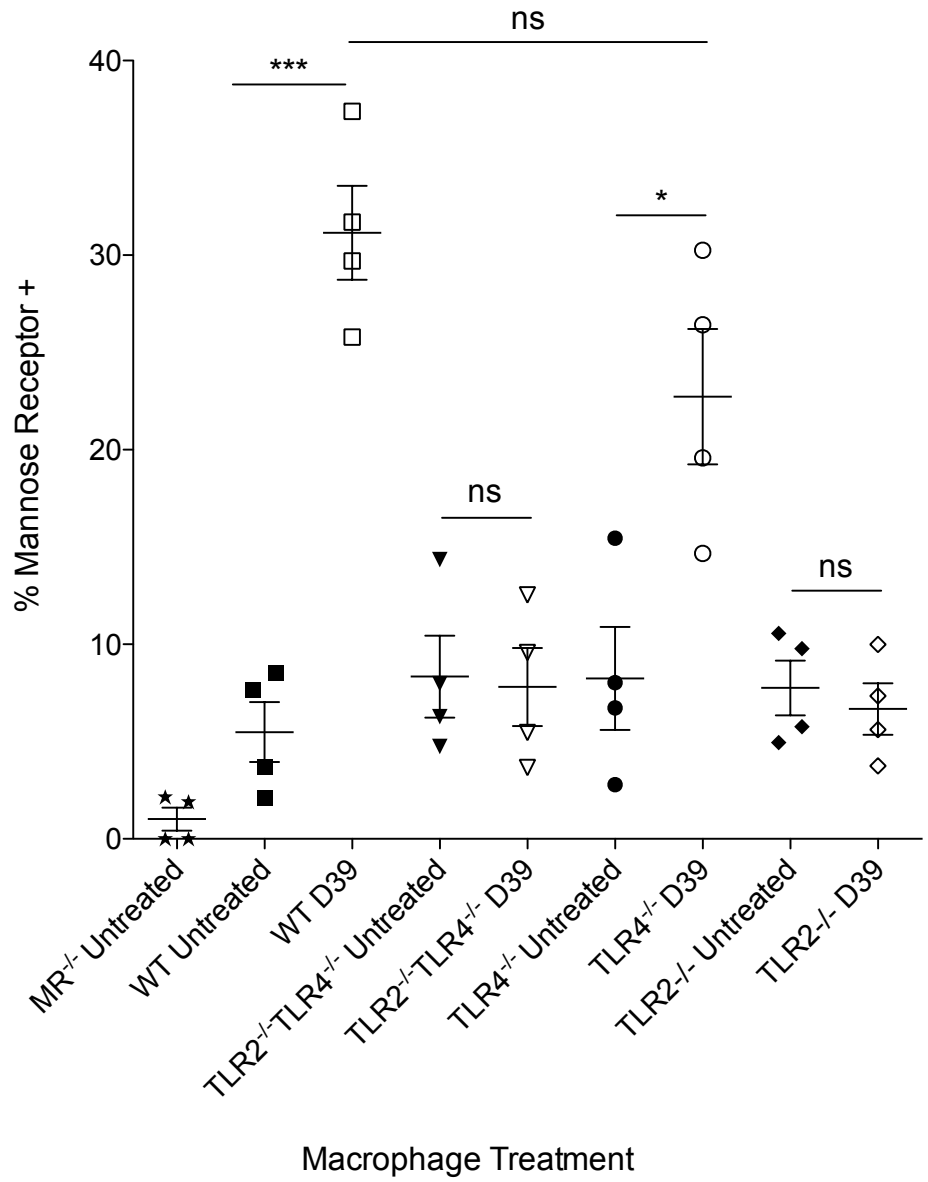


Figure 3.16. TLR-2 has an important role in the induction of MR expression. Wild type (WT), or Toll-like receptor 2 deficient (TLR-2 $^{-/-}$), or toll-like receptor 4 deficient (TLR-4 $^{-/-}$), or double knockouts toll-like receptor 2 & 4 (DKO TLR2 & TLR-4) BMDMs were incubated with *S. pneumoniae* D39 for 24 hours at a 1:10 ratio. Quantification of MR+ BMDM, gated relative to isotype control staining of F4/80+CD11b+ WT BMDM. Results are representative of three independent experiments of at least 3 wells per condition.

3.14 Pneumolysin and TLR-2 are both required for the up-regulation of MR and the production of IL-6

The role of the pneumococcal toxin pneumolysin in the induction of pro-inflammatory cytokines such as IL-6 has previously been described (McNeela et al., 2010). McNeela et al. have shown that PLY and TLR-2 are required for the secretion of IL-6 by dendritic cells (McNeela et al., 2010). In this thesis, I have shown that both TLR-2 and PLY are required for the up-regulation of MR. I have also shown that MR enhances the secretion of IL-6. Therefore, to find out the link between these two phenomena, WT, TLR-2 $^{-/-}$, TLR-4 $^{-/-}$ and DKO TLR-2 & 4 BMDMs were stimulated with D39 and ΔPLY for 24 h. Here I again show that WT BMDMs stimulated with D39 produced a large amount of IL-6 compared to ΔPLY (Figure 3.17), confirming a role for the pneumolysin in inducing IL-6 production. Also, TLR-2 $^{-/-}$ pneumococcus stimulated cells have shown significant reduction in IL-6 amount when compared to either WT or TLR-4 $^{-/-}$ cells, proposing a key role for the TLR-2 in the generation of IL-6 by macrophages. Our data here suggest that PLY and TLR-2 but not TLR-4 are more important for the expression of MR, which has shown to be important for the release of IL-6 by BMDMs, as in the absence of either PLY or TLR-2, very small amounts of IL-6 were detectable. These results may support the findings of McNeela *et al*. that showed no role for TLR-4 in anti-pneumococcal responses.

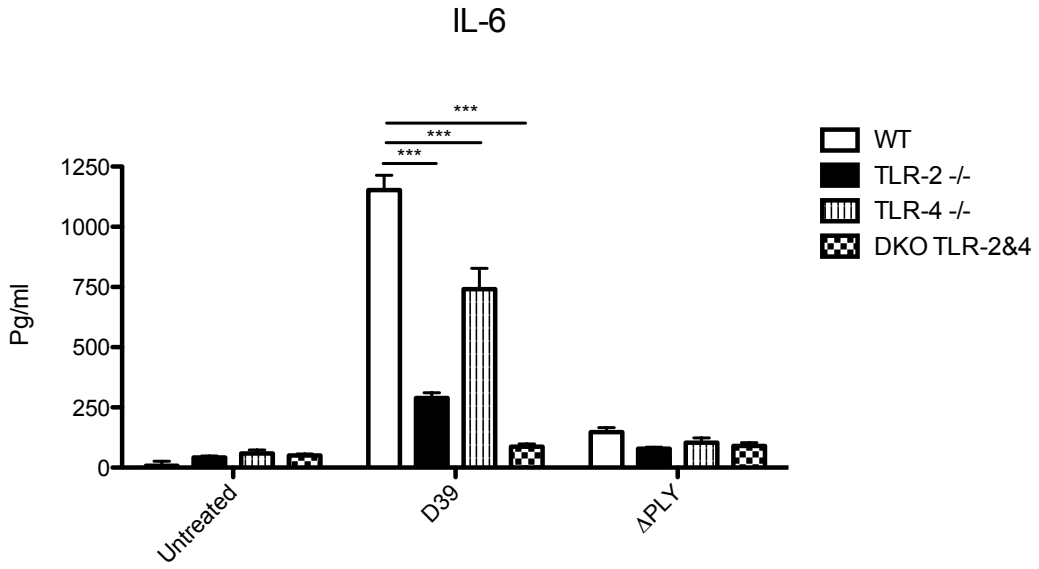


Figure 3.17. PLY and TLR-2 are required for the production of IL-6. BMDMs from WT or TLR-2 $^{-/-}$, TLR-4 $^{-/-}$, or DKO TLR-2 & 4 mice were stimulated with *S. pneumoniae* serotype 2 D39 and pneumolysin deficient ΔPLY for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.15 Pneumococcal pneumolysin is dispensable for pneumococcal-induced changes in the expression of MARCO in vitro

A recent study by Dorrington et al. has revealed that the macrophage receptor with collagenous structure (MARCO), which belongs to the family of class A scavenger receptor molecules, has a key role in defence against pneumococcal infections (Dorrington et al., 2013). The study revealed that mice that lack MARCO were incapable of clearing pneumococcal colonisation as MARCO-deficient mice recruited lower numbers of neutrophils and macrophages to the site of colonisation when compared to wild type mice. However, the influence of the pneumococcal toxin PLY on the expression of MARCO was not studied. As MR and MARCO are related molecules, I hypothesised that *S. pneumoniae* may induce MARCO up-regulation on macrophages via a PLY-dependent pathway. Here I show that D39 or ΔPLY stimulated wild type BMDMs up-regulate MARCO expression at 24 h post-infection when compared to uninfected BMDM (Figure 3.18). These data demonstrate that the pneumococcal toxin PLY is not required for pneumococcal-induced changes in the expression of MARCO.

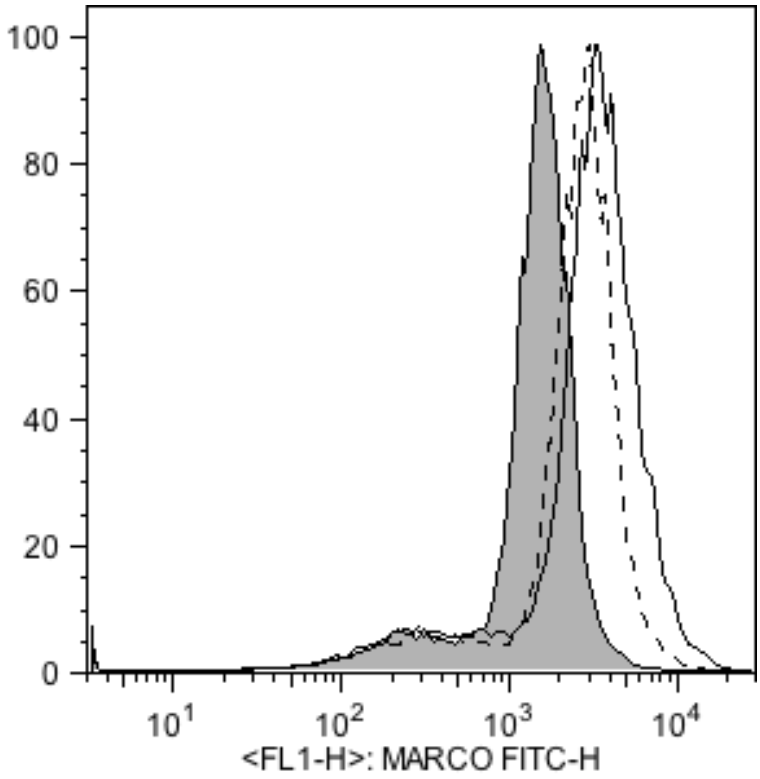


Figure 3.18. PLY is not required for pneumococcal-induced changes in MARCO expression. Wild type (WT) BMDMs were stimulated with *S. pneumoniae* D39 or pneumolysin-deficient D39 (ΔPLY) for 24 hours at a 1:10 ratio. Expression of MARCO on WT BMDMs at 24 h post-infection with D39 (Black line), ΔPLY (Dashed line), and un-stimulated cells (Solid histogram). Result is a representative of one experiment of at least 3 wells per condition; therefore no statistical conclusion can be made.

Moreover, Dorrington et al. has suggested that MARCO is required for TLR-2 mediated immune responses and the clearance of pneumococcal colonisation in murine nasopharynx. My research further demonstrates that TLR-$2^{-/-}$ BMDMs stimulated with D39 do not up-regulate expression of MARCO (Figure 3.19). Intriguingly, TLR-$4^{-/-}$ BMDMs also failed to up-regulate MARCO expression in response to infection (Figure 3.19), demonstrating that the up-regulation of MARCO is dependent upon both TLR-2 and TLR-4 (Figure 3.19).

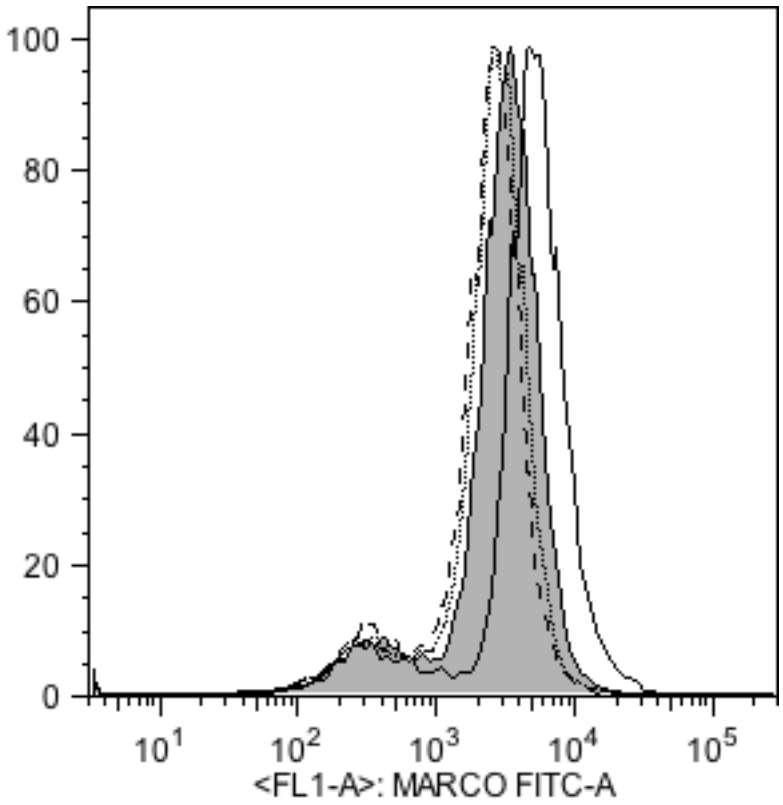


Figure 3.19. TLR-2 is important for the expression of MARCO. Toll-like receptor 2 deficient (TLR-2 $^{-/-}$), or toll-like receptor 4 deficient (TLR-4 $^{-/-}$) or TLR-2 TLR-4 DKO BMDMs were infected with *S. pneumoniae* D39 for 24 hours at a 1:10 ratio. Expression of MARCO on TLR-2 $^{-/-}$ (Dashed line), or TLR-4 $^{-/-}$ (Black line), or TLR-2 TLR-4 DKO (Dotted line), or un-stimulated BMDMs (Solid histogram). Result is a representative of one experiment of at least 3 wells per condition; therefore no statistical conclusion can be made.

3.16 MARCO is required for the production of IL-6 and MIP-2

Since the expression of both MARCO and MR by macrophages increased during pneumococcal infection, I next sought to determine if MARCO expression contributes to pneumococcal-induced cytokine expression in a manner analogous to that of MR. MARCO, like MR, influenced BMDM production of IL-6 during pneumococcal infection (Figures 3.20). MARCO-blocked BMDMs stimulated with D39, ΔPLY, D39-J, DKO, and PLY displayed a remarkable reduction in the production of IL-6 at 24 h post-infection as compared to untreated controls (Figure 3.20). Unlike MR, however, MARCO also contributed to MIP-2 production. MARCO-blocked BMDMs produced significantly less MIP-2 during infection that untreated controls (Figure 3.21). These data suggest that MARCO contributes to the release of IL-6 and MIP-2 by macrophages during infection.

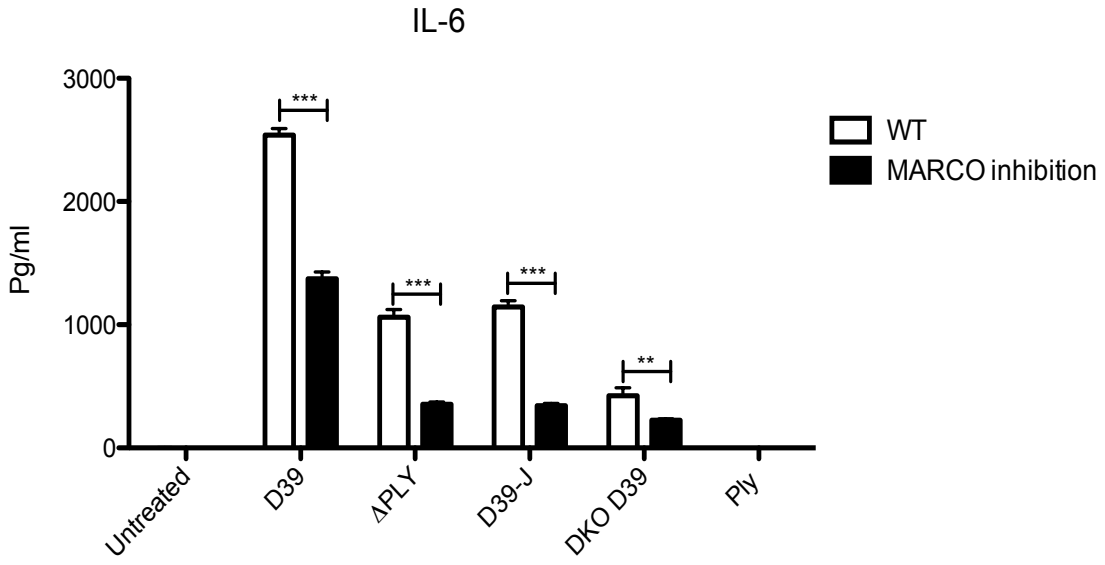


Figure 3.20. IL-6 production requires MARCO and is pneumolysin and capsule dependent. WT or MARCO blocked with dextran sulphate BMDMs were infected with *S. pneumoniae* serotype 2 D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO, and PLY for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

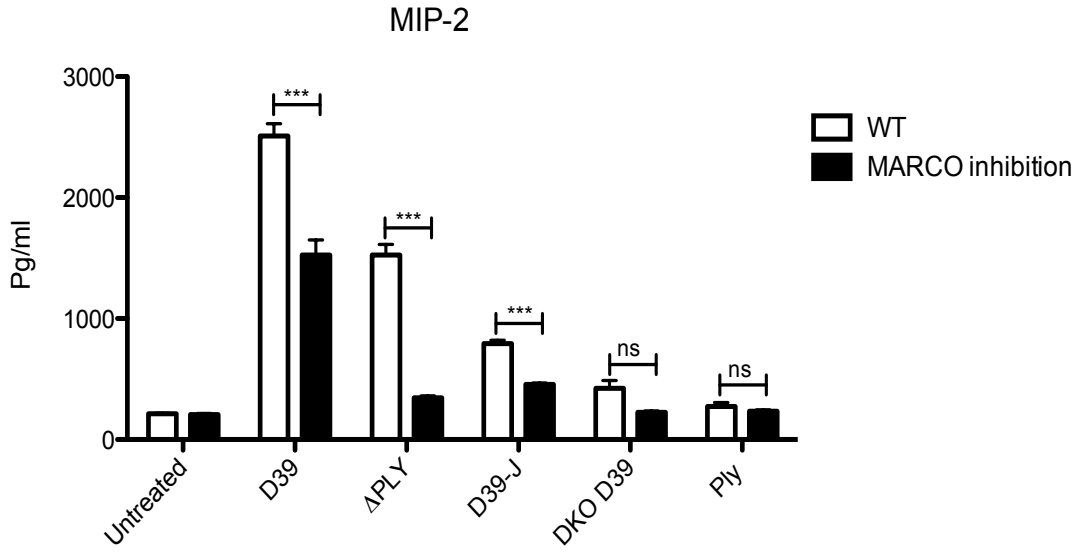


Figure 3.21. MIP-2 production requires MARCO and is pneumolysin and capsule dependent. WT or MARCO blocked with dextran sulphate BMDMs were infected with *S. pneumoniae* serotype 2 D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO, and PLY for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.17 MR and MARCO make significant contributions to pneumococcal-induced up-regulation of IL-6

Our research has revealed that MR or MARCO could play important roles in the recruitment of neutrophils during pneumococcal infection, as they both contribute independently to the enhancement of IL-6 production by macrophages after 24 h of serotype 2 *S. pneumoniae* infections. Abrogation of either pathway failed to completely abolish pneumococcal-induced IL-6 production, so I next sought to determine if combined inhibition of both MR and MARCO would have an additive effect. To do this, MARCO was blocked on $MR^{-/-}$ BMDM and IL-6 responses compared to WT BMDM. MARCO blocked $MR^{-/-}$ cells produced markedly less IL-6 at 24 h post-infection compared to WT BMDMs, but some pneumococcal-induced IL-6 production was still evident (Figure 3.22). Consistent with previous experiments, both PLY and capsule were found to contribute to induction of IL-6 production and stimulation with DKO D39 induced virtually no IL-6 production. However, these experiments confirmed that there are MR and MARCO-independent pathways to pneumococcal-induced IL-6 production.

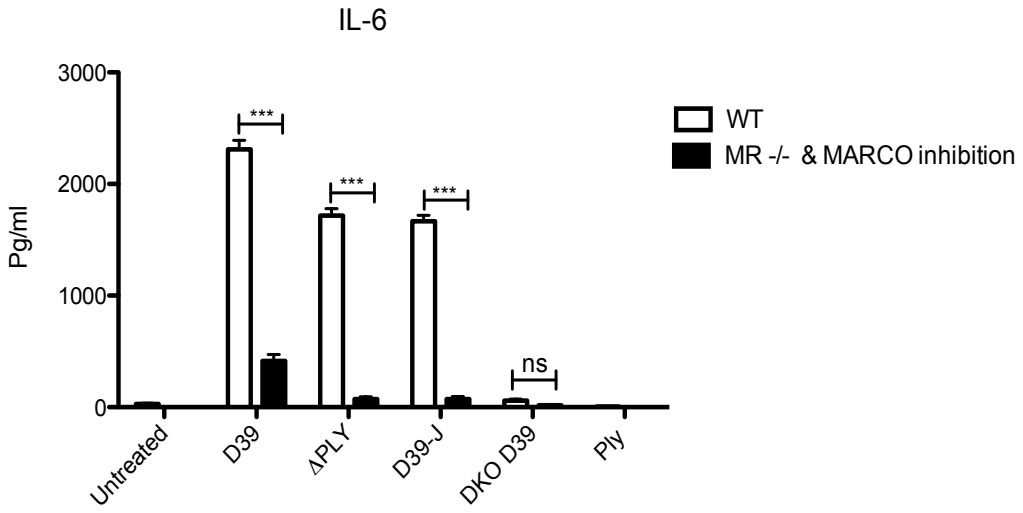


Figure 3.22. MR and MARCO are required for IL-6 production. WT or MR $^{-/-}$ and MARCO blocked BMDMs were infected with *S. pneumoniae* serotype 2 D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J, pneumolysin- and capsule-deficient DKO, and PLY for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.18 MyD88 is required for pneumococcal-induced macrophage IL-6

I have shown that MR and MARCO are involved in the up-regulation of IL-6 production by macrophages following *S. pneumoniae* infection. MyD88 is a common adapter of Toll-like receptor signalling and has been shown to be key for the activation of NF-κB (Yamamoto et al., 2014), and for the production of INF-α (Kawai et al., 2004). To determine whether the MyD88 adapter was involved in the induction of IL-6 production, WT or MyD88$^{-/-}$ BMDMs were treated with D39, Δ PLY, D39-J and DKO for 24 h. MyD88$^{-/-}$ cells did not produce any IL-6 in response to pneumococcal infection (Figure 3.23), while WT BMDMs stimulated with D39 produced high levels of IL-6. MyD88 may be important for the TLR-induced upregulation of MR and MARCO or else for signaling downstream of MR and MARCO binding to pneumococcal ligands.

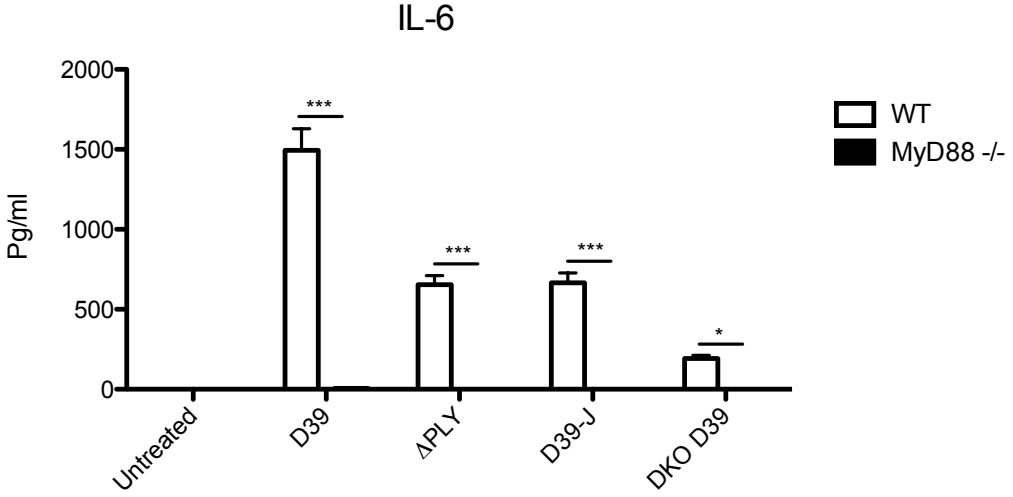


Figure 3.23. MyD88 is essential for the production of IL-6. BMDMs from WT or MyD88 $^{-/-}$ mice were stimulated with *S. pneumoniae* serotype 2 D39, pneumolysin deficient ΔPLY, capsule deficient D39-J, and pneumolysin and capsule deficient DKO D39 for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.19 pneumolysin and capsule are both required for the activation of the NLRP3 Inflammasome

Since my work so far has suggested that both PLY and capsule are required for the induction of macrophages responses, the contribution of these two virulence factors in the initiation of innate immune responses was investigated. Inflammasome activation is a key step in immune response activation following identification of pathogens. The inflammasome triggers the activation of significant pro-inflammatory cytokines including interleukin-1β (IL-1β) (Latz et al., 2013). It is known that PLY is required for the activation of the NLRP3 inflammasome and production of IL-1β (McNeela et al., 2010). To determine whether capsule also contributes to this process, NLRP3 $^{-/-}$ BMDMs were treated with D39, ΔPLY, D39-J, DKO, and PLY for 24 h. NLRP3 $^{-/-}$ cells produced no IL-1β production in response to pneumococcal infection whereas WT BMDM produced the cytokine under all infection conditions (Figure 3.24). However, the production of this pro-inflammatory cytokine from WT cells was reduced in the absence of pneumolysin (ΔPLY), in the absence of capsule (D39-J) and the absence of both pneumolysin and capsule (DKO), confirming that both PLY and capsule contribute to the induction of IL-1β.

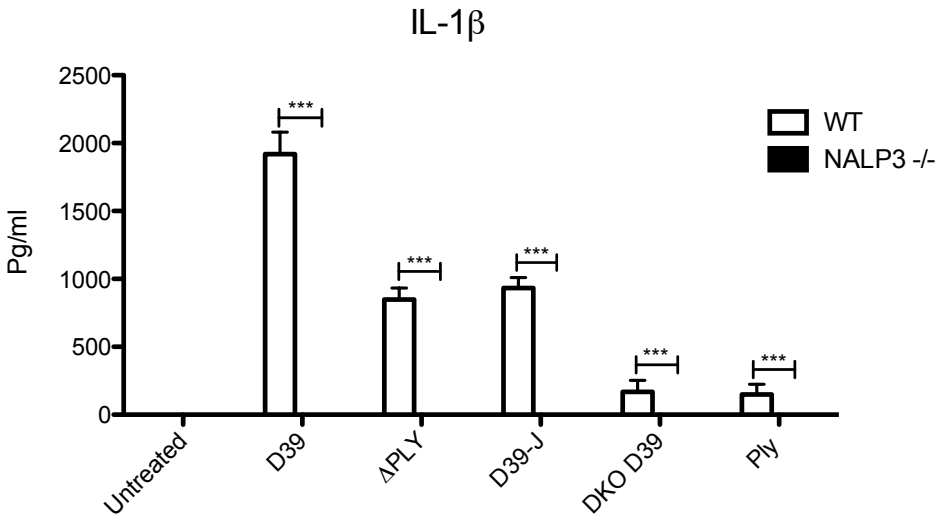


Figure 3.24. Pneumolysin and capsule both are required for the activation of NLRP3. BMDM from WT or NLRP3 $^{-/-}$ mice were infected with *S. pneumoniae* D39, pneumolysin deficient ΔPLY, and capsule-deficient D39-J and pneumolysin- and capsule-deficient DKO for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.20 Inflammasome (NLRP3) is important for the production of IL-6 in response to pneumococcal infections

Next, the importance of NLRP3 for the induction of key pro-inflammatory cytokines such as IL-6 was investigated, since the production of IL-6 by macrophages is important for the recruitment of neutrophils in response to pneumococcal infections. NLRP3 $^{-/-}$ BMDMs stimulated with D39, ΔPLY, D39-J, DKO, and PLY showed a remarkable decrease in IL-6 production at 24 h post-infection as compared to WT BMDM (Figure 3.25). Interestingly however, unlike IL-1β, the production of IL-6 did not critically depend on NLRP3. WT BMDMs stimulated with D39 generate a large amount of IL-6 when compared to other serotype 2 mutants ΔPLY, D39-J, DKO, and PLY, suggesting that the inflammasome and pneumococcal virulence factors contribute to the stimulation of IL-6 production in response to *S. pneumoniae* infection.

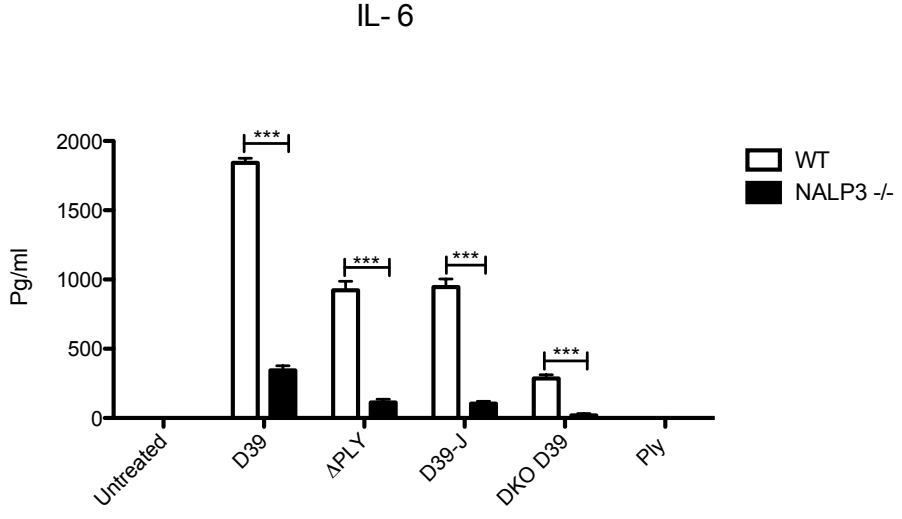


Figure 3.25. NLRP3 is required for the production of IL-6. BMDM from WT or NLRP3 $^{-/-}$ mice were infected with *S. pneumoniae* serotype 2 D39, pneumolysin deficient ΔPLY, capsule-deficient D39-J and pneumolysin- and capsule-deficient DKO for 24 hours. ELISA measured Cytokine levels in cultures supernatants. Results are presented as mean +/-SEM and are representative of three independent experiments. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.005$ and ns = not significant in two-way ANOVA analysis with Bonferroni post-test.

3.21 Discussion:

S. pneumoniae is a pathogen of global significance causing diseases of high morbidity and mortality. The global disease burden due to this pathogen has significant impact on the health care resources of developing and under-developed countries. Nasopharyngeal colonisation is essential for the pneumococcus to initiate localized or systemic infections. The ubiquitous production of pneumolysin by *S. pneumoniae* and the high degree of conservation of the PLY gene between strains suggest that the toxin plays a significant role in key phases of the pathogen life cycle (Rubins et al., 1998, Kadioglu et al., 2008). Using a murine model of long-term nasopharyngeal colonisation our lab has previously shown that pneumolysin is required for successful colonisation, as PLY deficient strains were cleared from the nasopharynx (Kadioglu et al., 2002, Richards et al., 2010). Furthermore, our previous studies have revealed the ability of PLY to induce expression of cytokines in dendritic cells and macrophages (McNeela et al., 2010). In this thesis, I have investigated the role of MR in anti pneumococcal immunity in the presence and absence of pneumococcal pneumolysin. Our lab had previously identified a population of MR-expressing macrophages in the nasopharynx of mice undergoing prolonged pneumococcal carriage (Richards et al., 2010).

3.21.1 Mannose Receptor

Our lab has previously shown that MR-expressing macrophages accumulate in the nasopharynx over 7 days of pneumococcal carriage by a process that is partially pneumolysin dependent (Neill et al., 2014). Here, I have demonstrated a rapid increase (within 24 hours) in the proportion of MR-expressing macrophages in the

nasopharynx, and confirmed that this is sustained up to day 7 post infection. Furthermore, my work showed that MR plays a key role in promoting the phagocytosis of pneumococcus by BMDMs *in vitro*, since $MR^{-/-}$ cells lack the ability to clear D39 within 24 h post-infection. This data was supported by Macedo-Ramos et al., who confirm that MR is involved in the internalization of *S. pneumoniae* by Schwann cells (Macedo-Ramos et al., 2014). Yamamoto and colleagues have previously shown that MR is required for the production of IL-1beta, IL-6, and GM-CSF, but not MIP-2, and KC in response to *Candida albicans* infection (Yamamoto et al., 1997). Similarly, here I demonstrate that WT *S. pneumoniae* induces high levels of IL-6, IL-1β, IL-10, IL-12 and MIP-2 production in WT macrophages, while levels of all these cytokines/chemokines bar MIP-2 were significantly reduced in supernatants from D39-infected $MR^{-/-}$ cells. My study suggests an interesting model in which macrophages become highly activated when infected with pneumococci in the presence of PLY and capsule together, whereas significantly less activation occurs in the absence of either component and cytokine production is almost completely abolished in the absence of both. Furthermore, the absence of IL-6 production in PLY-stimulated macrophages is likely due to the absence of TLR ligands to trigger inflammasome activation and thus IL-6 production (McNeela et al., 2010). However, PLY alone induces noticeable levels of MIP-2 in both WT macrophages and $MR^{-/-}$ cells by a yet to be identified pathway. MR appears to be dispensable for MIP-2-production as no significant differences were observed between WT and $MR^{-/-}$ cells. In contrast, MR seems to play a significant role in induction of macrophage production of IL-6, IL-1β, IL-10 and IL-12, as these factors were markedly reduced in D39-infected $MR^{-/-}$ macrophages when compared to the WT cells. These data support the notion that MR mediates selected cytokine responses to pneumococcus, whereas

some chemokine responses may be facilitated by other macrophage receptors. MR is an essential component of macrophages, providing a critical defense against pathogens including *S. pneumoniae* (Gordon, 2003). PLY is a potent activator for both mucosal and systemic immune responses, directly stimulating macrophages and amplifying their production of pro-inflammatory cytokines (McNeela et al., 2010). The role of PLY in up-regulating macrophage MR expression has been demonstrated for the first time in my studies. I also provide the first evidence for direct binding of PLY to MR, mediated through PLY domain 4. To support this, a recent study has revealed that human L-ficolin, which is a recognition molecule that has structure related to that of mannose receptor, can activate complement by binding to pneumolysin (Ali et al., 2013). My findings suggest that MR is essential to activate macrophages and contribute to their production of pro-inflammatory cytokines in response to *S. pneumoniae* infection.

3.21.2 The Role of Pneumolysin

PLY is crucial for *S. pneumoniae* to asymptomatically colonize the nasopharynx with minimal induction of pro-inflammatory immune responses (Kadioglu et al., 2002) (Richards et al., 2010, Neill et al., 2014), in marked contrast to its ability to drive inflammation in the lungs during pneumonia. Pneumolysin has been identified as the main pneumococcal cytotoxin that is released by the bacterial cells during autolysis (Rubins et al., 1998). The effect of pneumolysin on the morphology of BMDM confirmed its cytotoxicity, as cells stimulated with pneumolysin deficient ΔPLY showed less damage and fewer cytopathic signs than D39-treated cells. It is possible that pneumolysin stimulates tolerogenic immune mechanisms in the nasopharynx in

contrast to its action in the lungs. One potential model, supported by my data, is that during nasopharyngeal colonisation, nasal mucosa-associated macrophages become activated by pneumolysin and subsequently drain to cervical lymph nodes to initiate immune responses that may act to prevent damage to host tissue and systemic pneumococcal dissemination. Such action may be mediated by $Foxp3^+$ T regulatory cells, which have been shown to play key roles in the modulation and inhibition of inflammation in the context of infection (Sakaguchi et al., 2010). These cells are well known to play important roles in limiting infection-associated inflammation, and resolving tissue damage post-infection (Mills, 2004, Barnes and Powrie, 2009) but, the role of T regulatory cells and other immune-modulatory cytokines during *S. pneumoniae* infection is limited and needs further investigations. However, recent studies have provided evidence for the important protective role that T regulatory cells play during invasive pneumococcal pneumonia (Neill et al., 2012) and carriage (Neill et al., 2014). The protective effects of T regulatory cells during carriage were also observed by Pido-Lopez et al. who demonstrated that the response of CD4 T cells to pneumococci increase gradually at the site of colonisation. The presence of regulatory T cells was detected at the peak of CD4 T cell responses, leading to inhibition of anti-pneumococcal CD4 T cell responses (Pido-Lopez et al., 2011). The association of regulatory T cells with carriage was echoed by Zhang and colleagues, who demonstrated that regulatory T cells with suppressive function were present in higher proportions in the nasopharynx of pneumococcal carriers than non-carriers (Zhang et al., 2011). The study suggested that the presence of T reg in nasal-associated lymphoid tissue could play a key role in the persistence of pneumococcus in children.

3.21.3 Macrophages and T cell Differentiation

To further explore the involvement of T regulatory cells and immunomodulatory cytokines in response to *S. pneumoniae* infections, mutant strains of serotype 2 pneumococcus were used to stimulate BMDMs to access contributions of pneumococcal factors to the differentiation of CD4+ T cells into T regulatory cells during pneumococcal infection. BMDMs stimulated with D39 enhanced the differentiation of CD4+ T cells into T regulatory cells and, to a lesser extent, Th17 and Th1 cells. PLY-deficient D39 was less effective at inducing differentiation of all three T cell subsets. The reduced levels of IL-10, IL-17 and INF-gamma in supernatants of ΔPLY-stimulated cells versus D39-stimulated controls supported these conclusions. My data indicate that both PLY and capsule stimulate macrophages to induce the maturation of T regulatory cells. Interestingly, PLY and capsule also stimulated expression and production of RORgt and IL-17 by Th17 and Tbet and INF-gamma by Th1, confirming a key role for both PLY and capsule in the generation of both immune tolerance and inflammatory responses during pneumococcal infection. Similarly, McNeela and colleagues have shown that PLY-expressing pneumococcal strains are able to induce high levels of IL-17 and INF-gamma production by splenocytes *in vitro* when compared to pneumolysin deficient strains (McNeela et al., 2010). The importance of IL-17 in pneumococcal carriage has been described by several studies. A recent study has revealed that Th17 cells have a protective role during pneumococcal carriage in human nasopharynx, due to their ability to produce IL-17 that drives pro-inflammatory responses, recruitment of macrophages and neutrophils, and the clearance of colonisation. This study showed this immune response was promoted by domain 4 and not domain 123 of PLY (Gray et al., 2014).

Moreover, animal studies have also suggested that Th17 cells play a key role in facilitating the clearance of nasopharyngeal colonisation of *S. pneumoniae* (Lu et al., 2008, Zhang et al., 2009). In mouse models, Th17 cells that express IL-17 have been shown to generate protection in response to different respiratory pathogens (O'Connor et al., 2010). Furthermore, an important role for IL-17 signaling in bacterial clearance has been described in murine models for several mucosal pathogens, proposing that the Th17 pathway could be the main mechanism in the clearance of bacteria at mucosal surfaces (Curtis et al., 2009, O'Connor et al., 2010). A recent study has revealed that during pneumococcal carriage in mice, antigen-specific $CD4^+$ Th17 cell immunity reduces colonisation equally by an antigen-expressing strain and a co-colonized antigen-negative strain, consequently minimizing the advantage of escape from this type of immunity by antigenic variation (Li et al., 2012).

3.21.4 Mannose Receptor and T cell Differentiation

Since MR was found to bind the pneumococcal toxin PLY (my data), and pneumococcal capsule (Zamze et al., 2002), the influence of MR on the differentiation of CD4+ T cells into T regulatory cells was studied. MR was found to contribute to the induction of expression of Foxp3, RORgt and Tbet in T cells. Accordingly, MR also contributed to the induction of IL-10, IL-17, and INF-gamma production by T cells. These data validate that MR has a key impact on the generation of T regulatory cells, Th17 and Th1 and associated pro- and anti-inflammatory cytokines. Previously, Chieppa *et al* proposed a direct role for mannose receptor in mediating T regulatory, but not Th1, chemokine and cytokine induction (Chieppa et al., 2003).

MR seems to be more important in providing protection against pneumococcal infections, in particular in stimulating pro-inflammatory responses, since my study revealed that the absence of MR led to significant reduction in the production of IL-17 and INF-gamma by Th17 and Th1 receptively. Therefore, I propose that MR is necessary to generate protective immunity against *S. pneumoniae*, particularly against the pneumococcal toxin and capsule. Here I show that during pneumococcal nasopharyngeal colonisation, nasal mucosa-associated macrophages become activated, via the binding of pneumococcal ligands to both pattern recognition receptors (including TLR-2) and MR (Figure 26). I have shown here that large amounts of IL-6 are produced by the activated macrophages, contributing to the recruitment of PMN to the site of infection. MR-expressing macrophages also contribute directly to control of bacterial colonisation via phagocytosis.

3.21.5 Mannose Receptor and Phagocytosis

Studies investigating the contribution of the MR to phagocytosis have yielded conflicting results. MR has been showed to be involved in the phagocytosis of several pathogens including *Candida albicans* (Marodi et al., 1991) and *Mycobacterium tuberculosis* (Kang et al., 2005). Here I have shown that MR was required for the phagocytosis of serotype 2 D39, pneumolysin-negative ΔPLY, capsule-negative D39-J and pneumolysin and capsule-negative DKO D39. However, some other studies have described different observations and concluded that MR is not important for microbial phagocytosis, since CHO cells expressing MR were not able to phagocytose *Mycobacterium kansasii* (Le Cabec et al., 2005), *Leishmania donovani* and *Leishmania major* (Akilov et al., 2007). In addition, Lee *et al*. demonstrated that

$MR^{-/-}$ mice do not differ from WT mice in both the humoral response to candida antigens or phagocytosis of *Candida albicans* (Lee et al., 2003).

3.21.6 A Model For Mannose Receptor Function

My data suggest that MR-expressing macrophages play dual roles in pneumococcal carriage (Figure 26). In early infection they play a direct antimicrobial role through the phagocytosis of pneumococci and also recruit effector cells such as neutrophils via chemokine and cytokine production. Later, during long-term pneumococcal carriage, activated macrophages may drain to cervical lymph nodes (CLN) to initiate immune responses that act to prevent damage to host tissues and systemic pneumococcal dissemination. This includes the stimulation of differentiation of T cells into regulatory T cells, leading to production of large amounts of IL-10 and TGF-β. These anti-inflammatory cytokines will recruit more regulatory T cells into the nasopharynx leading to maintenance of stable pneumococcal carriage and prevention of damage to host tissues. Stimulation of Th1 and Th17 cell differentiation by MR-expressing macrophages may contribute to control of bacterial proliferation in the nasopharynx. I confirm here that both the pneumococcal toxin pneumolysin and macrophage MR have key roles in encouraging macrophage induction of T cell differentiation. These T cells are likely key to maintenance of a state of immune tolerance in nasopharynx during pneumococcal colonisation.

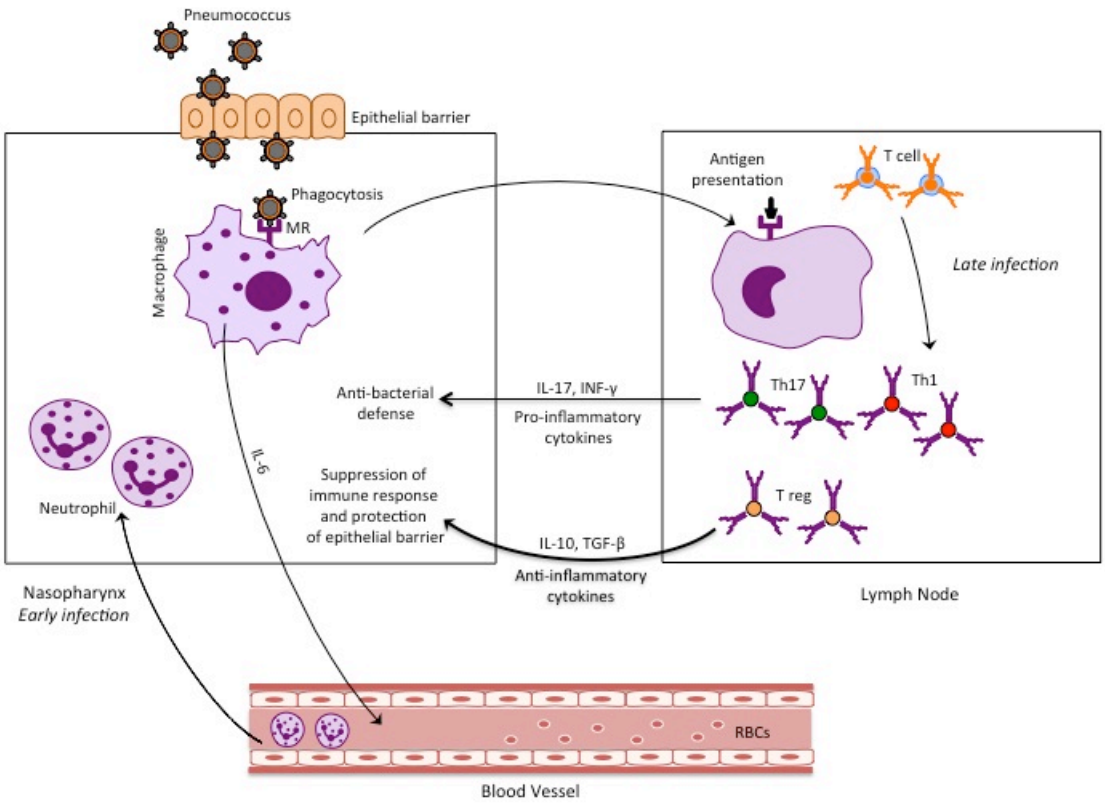


Figure 3.26. The role of MR in generating anti-inflammatory responses during pneumococcal infections. NP = nasopharynx, LN = lymph node.

3.21.7 The Inflammasome

Pneumolysin drives the activation of the NLRP3 inflammasome and the release of IL-1β from macrophages and dendritic cells (McNeela et al., 2010). Here I demonstrate that pneumococcal capsule also contributes to this process as the dual pneumolysin and capsule mutant DKO D39 induced significantly less IL-1β production from macrophages than either single mutant (ΔPLY and D39-J). Moreover, the NLRP3 inflammasome has been shown to be required for protection against invasive pneumococcal pneumonia and for the generation of IL-1β by dendritic cells (McNeela et al., 2010). Here I show that the inflammasome contributes to the induction of IL-6

production in macrophages, which is important for the recruitment of neutrophils in response to infection.

3.21.8 Toll Like Receptors

A recent study has revealed that TLR-2 is required for MARCO to generate sufficient immune response against pneumococcal infections (Dorrington et al., 2013). A separate study demonstrated that MARCO and TLR-2 are required for macrophage cytokine responses to *M. tuberculosis* (Bowdish et al., 2009). I have now shown that the up-regulation of MR by *S. pneumoniae* is similarly TLR-2-dependent, since TLR2-deficient BMDMs stimulated with D39 did not induce the expression of MR, suggesting that MR and TLR-2 may work as co-receptors in response to *S. pneumoniae* infections. In support of such a hypothesis, Tachado et al. have shown that MR interacts with TLR2 during the recognition of *Pneumocystis carinii* (Tachado et al., 2007). Interestingly, I observed that the release of IL-6 by BMDMs that was triggered by D39 was TLR-2 dependent, as TLR-$2^{-/-}$ cells stimulated with ΔPLY released very low levels of cytokine. TLR-4 was found to have no significant involvement in either the expression of MR or the production of IL-6, which is in line with the findings of McNeela et al. who demonstrated that dendritic cells were able to generate pro-inflammatory cytokines in response to PLY independently of TLR-4 (McNeela et al., 2010). The importance of TLR-2 in response to pneumococcal infections has been revealed by several studies. A study by Echchannaoui et al. demonstrated that TLR-$2^{-/-}$ mice infected with serotype 3 pneumococcus succumbed to infection more rapidly that WT mice and had significantly greater bacterial loads in the brain (Echchannaoui et al., 2002). Furthermore, another study by Knapp et al. has

shown that TLR-2 is required for the early inflammatory response to pneumococcal pneumonia in mice (Knapp et al., 2004). MyD88 is a key adaptor molecule for the TLR family and activates the transcription factor NF-κB (Takeuchi et al., 2000, Arancibia et al., 2007). Here I have shown that MyD88 is required for the release of IL-6 by *S. pneumoniae*-infected BMDM.

3.21.9 The Role of MARCO

Both TLR-2 and MARCO have been shown to play a key role in activating macrophage cytokine responses to *M. tuberculosis* (Bowdish et al., 2009), and I have investigated the role of these receptors in response to *S. pneumoniae*. My data shows that both TLR-2 and TLR-4 are required for up regulation of MARCO expression in response to pneumococcal infection. In contrast to MR, PLY does not appear to play a role in the regulation of MARCO expression on macrophages as similar levels of MARCO up regulation were observed following infection with D39 or ΔPLY. Dorrington et al. have revealed the importance of MARCO in the clearance of pneumococcal colonisation in murine nasopharynx (Dorrington et al., 2013). Here I have shown that BMDM stimulated with D39 up-regulate MARCO. A study by Arredouani et al. revealed that Bronchoalveolar lavage (BAL) fluid from MARCO $^{-/-}$ mice infected with serotype 3 *S. pneumoniae* produced higher amounts of MIP-2 and TNF-α when compared to WT mice (Arredouani et al., 2004). By contrast, I have shown that inhibition of MARCO *in vitro* attenuates production of both IL-6 and MIP-2 in BMDMs stimulated with serotype 2 *S. pneumoniae*. The observed differences between the data of Arredouani and colleagues and my results could be due to the use of different serotypes of pneumococcus, the use of different mouse

strains or differences in doses and timings of infection. Collectively, these data point to a significant function for macrophage MARCO in mounting an effective regulated innate immune response against pneumococcal infection. Another significant finding from my study is that both MR and MARCO play key roles in the generation of IL-6, which is known to be involved in the recruitment of neutrophils during *S. pneumoniae* infection (Neill et al, 2014). $MR^{-/-}$ and MARCO-blocked BMDMs produced very small amounts of IL-6 when compared to WT cells. These data clarify that both MR and MARCO are important components of anti- *S. pneumoniae* responses.

3.21.10 Conclusions and Implications

Several previous studies performed by our group have shown that nasopharyngeal colonisation generates some level of host immunity to pneumococcus, however this is either ineffective in clearance of carriage or else other immune mechanisms exist that allow or tolerate colonisation (Richards et al., 2010, Ferreira et al., 2013, Neill et al., 2014). My PhD study shows that macrophage MR is vital for the recognition and phagocytosis of *S. pneumoniae*. However, my hypothesis is that during colonisation of the nasopharynx a state of immune tolerance is generated that is driven by pneumolysin and MR. Nasal mucosa-associated macrophages are key to initiating this tolerogenic state; T- regulatory cells, IL-10 and TGF-β are key to its long-term regulation. Here I have shown that *S. pneumoniae* uses PLY to maintain colonisation in host nasopharynx via induction of macrophage MR. In particular, pneumococcus use PLY to trigger the alternative activation of nasal-associated macrophages via its binding to MR. This type of activation will stimulate the differentiation of T cells into regulatory T cells, which will lead to generation of large amounts of the anti-

inflammatory cytokines IL-10 and TGF-β, leading to suppression of the pro-inflammatory responses at infection sites and therefore maintaining the presence of pneumococcus in host nasopharynx.

I propose that therapeutic blockade of MR might result in the classical activation of macrophages, and thus the induction of pro-inflammatory cytokine production and the recruitment of immune effector cells that would clear pneumococcal infection from host nasopharynx. My findings have implications for the vaccines based around PLY and its derivatives. PLY could be used as a mucosal adjuvant to generate protective immunity against pneumococcal infections. PLY has been shown previously to function as a potent activator of both mucosal and systemic immune responses (McNeela et al., 2010), and to boost IgG and IgA antibodies titres to pneumococcal protein PsaA (Douce et al., 2010). However, these observed phenomena rely on the cytolytic activity of PLY. Therefore, I suggest that PLY could be used in a formulation with carbohydrate antigens (such as Mannan) that could block MR and prevent the alternative activation of macrophages, leading to classical activation and the induction of Th1 and Th17 responses and associated production of protective IFN-γ and IL-17 that are known to stimulate immunity against pneumococcal infections (McNeela et al., 2010). Th1 and Th17 cells have been confirmed to be required for protection in response to both repeated invasive challenges and pneumococcal carriage in mice (Malley et al., 2006).

Chapter 4: Proteomic analysis of pneumococcus-stimulated macrophages

4.1. Introduction

The pneumococcal toxin pneumolysin (PLY) has been shown to activate mucosal and systemic immune response. My previous chapter has revealed interactions between pneumolysin and both innate and adaptive immunity.

The aim of the work described in this chapter was to study the proteome of pneumococcal–stimulated bone marrow derived macrophages (BMDMs) in order to answer some important questions such as; what type of proteins are expressed in response to pneumococcal infection in the presence or absence of pneumolysin, what is the rate of protein production and degradation, how do proteins interact with each other, and which protein pathways are specifically triggered by pneumolysin. The knowledge gained from answering these questions will inform us of correlates of host immunity, to significantly increase our understanding of PLY function and potentially aid future vaccine development.

4.2 Pneumococcus stimulates the expression of a wide range of proteins by macrophages.

Murine BMDMs were stimulated for 24 h with serotype 2 D39 or pneumolysin-negative ΔPLY. Stimulated/infected cells and uninfected control cells were used for label free quantitative proteomics. Figure 4.1 show the heat map for the proteins

expressed by BMDMs at 24 h post pneumococcal infection. The heat-map is an interactive visualization tool that gives an overview of the relative abundance of proteins within a selected set of samples. Here the heat map was used as a tool to compare expression of groups of proteins under different infection conditions.

In Figure 4.1, the expressed proteins are grouped together using hierarchal clustering (using the GENE-e software). Similar patterns of proteins abundance are grouped together. For example, the proteins that go up in one group of samples are grouped together. Here my data show an overview comparison between un-stimulated BMDMs, BMDMs stimulated with D39, and BMDMs stimulated with ΔPLY at 24 h. The colours represent the relative abundance of the expressed proteins, a protein with the highest intensity (relative to the other samples) is red and a protein with lowest intensity is blue. Distinct expression patterns can be seen for untreated and D39-infected cells, with the pattern for ΔPLY more variable between repeat samples, but still more similar to each other than to either untreated or D39-infected cells (Figure 4.1).

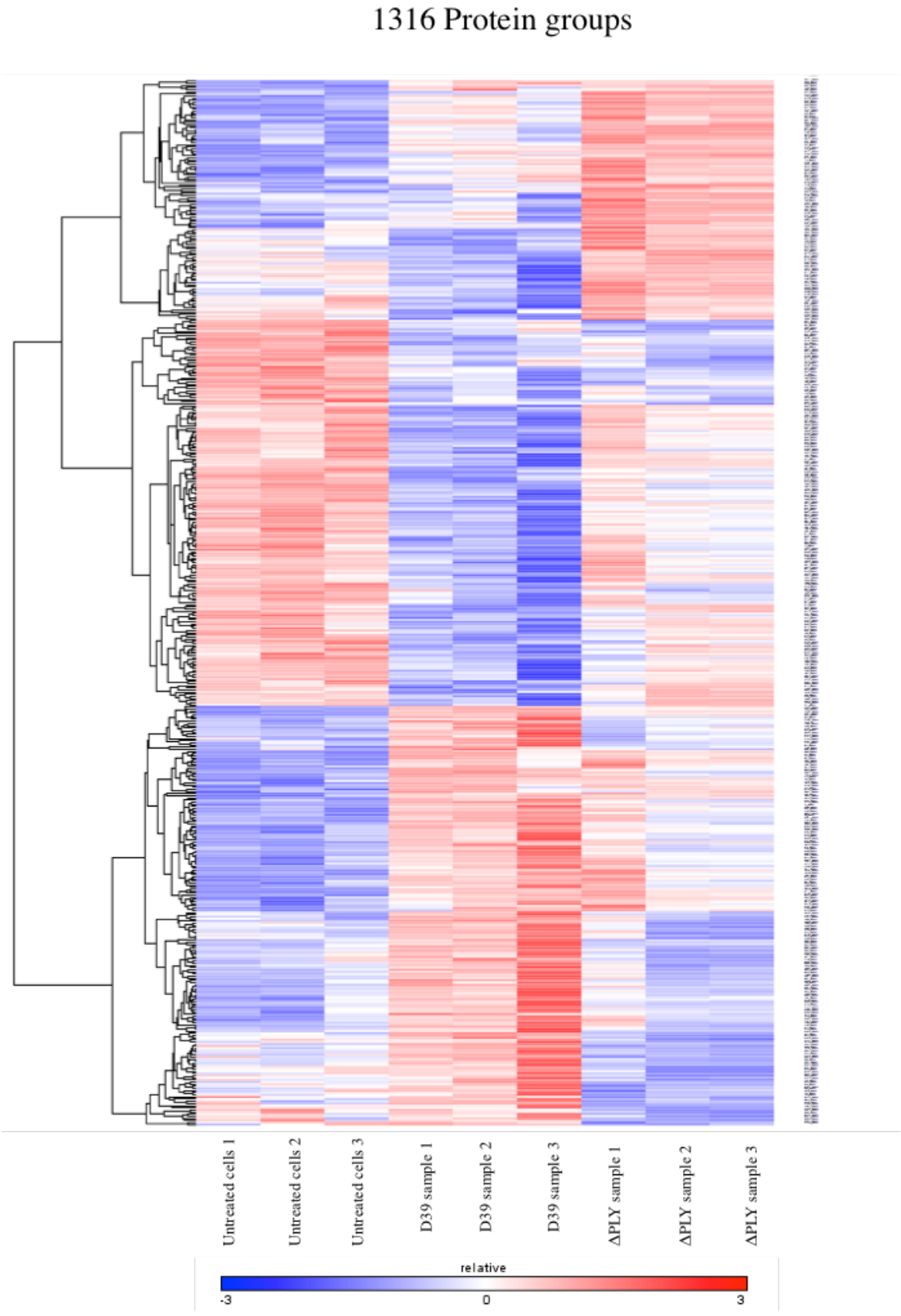
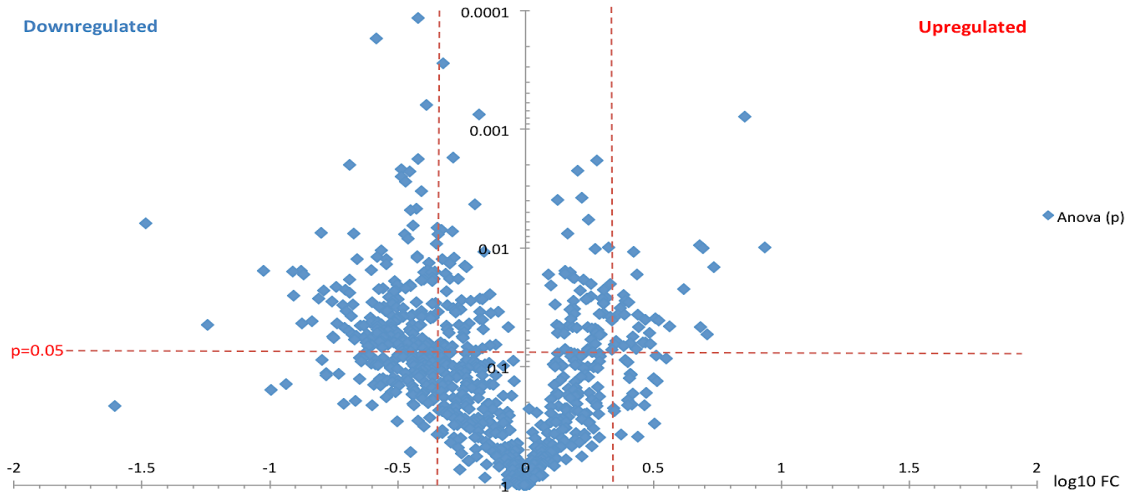


Figure 4.1. Heat map depiction of protein groups evident as being regulated due to pneumococcal infections of BMDMs. BMDMs were stimulated for 24 h with D39 or ΔPLY (at a ratio of 1:10). Protein groups were selected for presentation based intensity measurements.

Furthermore, the volcano plots in Figure 4.2A and B show an overall view of the differences between untreated cells and D39, and between D39 and ΔPLY. A full list of differentially expressed proteins can be found in Appendix 1 and 2 of this thesis. The volcano plot is beneficial for displaying significant differences in expression together with fold change. The vertical axis corresponds to statistical significance (− log base 10 (p value)) and the horizontal axis represents average fold change between conditions. Data points in the top right and left rectangles correspond to proteins with both small p-values and large fold changes. This information together with biological information about each protein is useful for identifying the important proteins for further investigation.

A



B

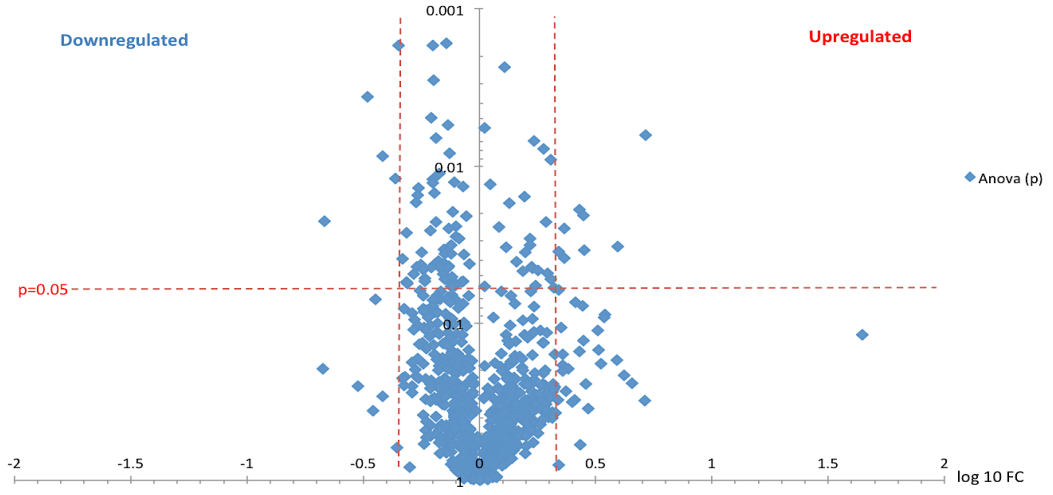


Figure 4.2. Volcano plots showing fold changes and statistically significant differences in expression of BMDM proteins following pneumococcal infection. Statistical significance versus fold change for (A) untreated cells and D39 (B) D39 and ΔPLY. The vertical axis represents –log base 10 of the p-value and the horizontal axis denotes average fold change on the log base 2 scale. The horizontal reference line corresponds to a p-value cut-off of 0.05, the Bonferroni adjusted significance criteria. The two vertical lines correspond to a 2-fold change.

Next, to identify and determine the importance of these differentially expressed proteins, a ternary plot of normalized protein abundances in macrophages treated with D39 or ΔPLY *Streptococcus pneumoniae*, compared to untreated cells was created. Figure 4.3 shows a wide range of protein abundances in macrophages stimulated with D39 and pneumolysin-negative ΔPLY.

Both D39 and ΔPLY induced up-regulation and down-regulation of expression of a wide range of different proteins by macrophages at 24 h post-infection. Up-regulated proteins were divided into 3 groups; proteins up-regulated in D39 infection only (red circles in Figure 4.3), proteins up-regulated in ΔPLY infection only (Blue) and proteins up-regulated with both D39 and ΔPLY (Purple).

These proteins are listed with their functions in Table 4.1. Down-regulated proteins were also divided into 3 groups; proteins down regulated in D39 infection (Orange), proteins down regulated in ΔPLY infection (Yellow) and proteins down regulated in both D39 and ΔPLY infection (Green). These proteins are also listed with roles in Table 4.2.

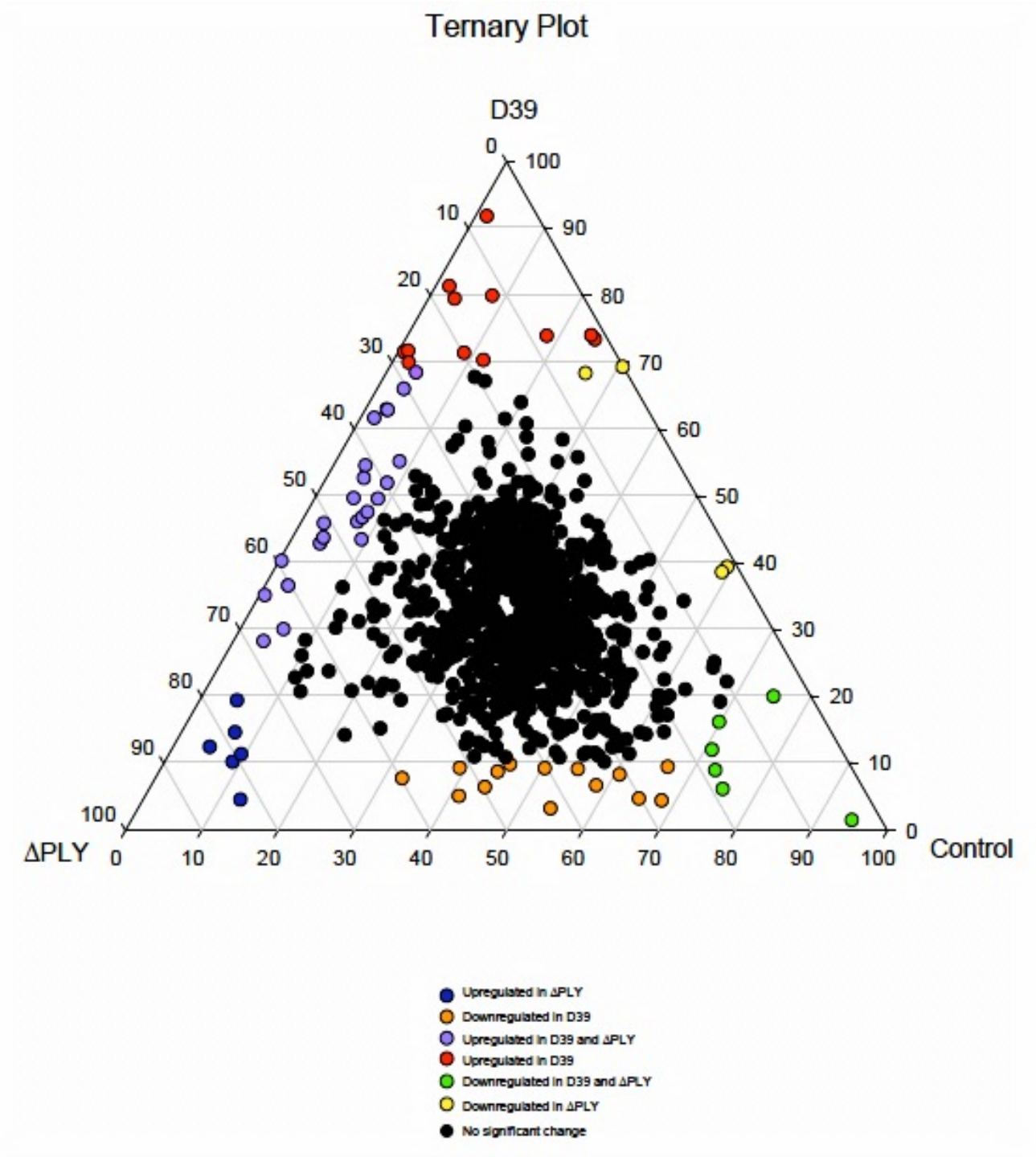


Figure 4.3. Ternary plot of normalized protein abundances in macrophages treated with D39 or ΔPLY *Streptococcus pneumoniae* compared to control. Protein abundance was measured using a label free proteomic approach. Only proteins with > 2 unique peptides and a q –value <0.05 were included in the analysis. Highly up-regulated or down-regulated proteins (normalised abundance >70, <70 >20) are highlighted by solid colours (see key). Proteins with no significant changes between conditions are displayed in black. The numbers along the three sides of the triangle represent the proportion of the total signal that belongs to each of the three groups. For each point the summed value should equal 100.

Group	Protein ID	Protein name	Fold-change	P value
Up-regulated in D39	P29477	Nitric oxide synthase, inducible	1185.429408	0.015203081
Up-regulated in D39	P10889	C-X-C motif chemokine 2	1185.429408	0.014117147
Up-regulated in D39	P50396	Rab GDP dissociation inhibitor	1185.429408	0.013176004
Up-regulated in D39	P50446	Keratin, type II	929.1203468	0.016466426
Up-regulated in D39	P00920	Carbonic anhydrase 2	838.4744593	0.017433144
Up-regulated in D39	O35744	Chitinase-like protein 3	763.9433962	0.017329313
Up-regulated in D39	Q05769	Prostaglandin G/H synthase 2	763.9433962	0.016462847
Up-regulated in ΔPLY	Q05117	Tartrate-resistant acid phosphatase type 5	2369.408276	0.016971881

Up-regulated in ΔPLY	Q8K4B2	Interleukin-1 receptor-associated kinase 3	2042.593341	0.02036519
Up-regulated in ΔPLY	P16056	Hepatocyte growth factor receptor	2042.593341	0.02036519
Up-regulated in ΔPLY	Q61549	Adhesion G protein-coupled receptor E1	1795.00627	0.023758143
Up-regulated in ΔPLY	P18581	Cationic amino acid transporter 2	1795.00627	0.023758143
Up regulated in D39 and ΔPLY	P10810	Monocyte differentiation antigen CD14	1152.43833	0.022467051
Up regulated in D39 and ΔPLY	Q91XB0	Three-prime repair exonuclease 1	61.92289593	0.021218882
Up regulated in D39 and ΔPLY	P42230	Signal transducer and activator of transcription 5A	1152.43833	0.020102098
Up regulated in D39 and ΔPLY	P56394	Cytochrome c oxidase copper chaperone	29.25381444	0.02121448
Up regulated in D39 and ΔPLY	P53690	Matrix metalloproteinase-14	29.25381444	0.020365901

Up regulated in D39 and ΔPLY	Q9Z2H6	C-type lectin domain family 4 member D	1152.43833	0.019096994
Up regulated in D39 and ΔPLY	Q05915	GTP cyclohydrolase 1	26.27554637	0.019582597
Up regulated in D39 and ΔPLY	Q9CQR2	Interleukin-1 beta	932.9262673	0.023566755
Up regulated in D39 and ΔPLY	P10749	Guanylate-binding protein 5	477.8402832	0.027264445
Up regulated in D39 and ΔPLY	Q8CFB4	Z-DNA-binding protein 1	28.4982512	0.026324292
Up regulated in D39 and ΔPLY	Q9QY24	Endothelial protein C receptor	932.9262673	0.029681794
Up regulated in D39 and ΔPLY	Q64695	Palladin	26.27554637	0.030831831
Up regulated in D39 and ΔPLY	Q9ET54	Interferon-induced protein with tetratricopeptide repeats 3 OS=	28.4982512	0.029925013
Up regulated in D39 and ΔPLY	Q9D154	Leukocyte elastase inhibitor A	783.6580645	0.029070012

Up regulated in D39 and ΔPLY	P01582	Interleukin-1 alpha	932.9262673	0.031788732
Up regulated in D39 and ΔPLY	P54987	Cis-aconitate decarboxylase	73.50328166	0.032597066

Table 4.1. Proteins up-regulated during pneumococcal infection. The table shows proteins up regulated by *S. pneumoniae* D39 or ΔPLY, which may contribute to the pathogenesis of pneumococcus or may play immune roles in response to pneumococcal infection.

Group	Protein ID	Protein name	Fold-change	P value
Down-regulated in D39	P09405	Nucleolin	563.5648005	0.038757849
Down-regulated in D39	Q9EQU5	Protein SET	409.1634853	0.036312847
Down-regulated in D39	P09581	Macrophage colony-stimulating factor 1 receptor	524.0163934	0.036384641
Down-regulated in D39	Q9EPK2	Protein XRP2	409.1634853	0.034110601

Down-regulated in D39	Q9EST5	Acidic leucine-rich nuclear phosphoprotein 32 family member B	368.7522769	0.032104095
Down-regulated in D39	Q8C0P5	Coronin-2A	351.3992285	0.032648691
Down-regulated in D39	Q9Z1T1	AP-3 complex subunit beta-1	321.1713379	0.030930339
Down-regulated in D39	Q61239	Protein farnesyltransferase/geranylgeranyltransferase type-1 su	20.2649208	0.035666615
Down-regulated in D39	P22366	Myeloid differentiation primary response protein 88	524.0163934	0.033968205
Down-regulated in ΔPLY	Q9CR09	Ubiquitin-conjugating enzyme 1	304.4285714	0.011042398
Down-regulated in ΔPLY	Q3UVL4	Vacuolar protein sorting-associated protein 51 homolog	220.7688113	0.01411326

Down-regulated in ΔPLY	Q3V3R1	Mono-functional C1-tetrahydrofolate synthase, mitochondrial	183.4294775	0.015797896
Down-regulated in D39 and ΔPLY	P02088	Hemoglobin subunit beta-1	369.9502538	0.019693972
Down- regulated in D39 and ΔPLY	Q8BVF2	Phosducin-like protein 3	342.1605634	0.015627666
Down- regulated in D39 and ΔPLY	A2AGT5	Cytoskeleton-associated protein 5	318.2541485	0.022483393
Down-regulated in D39 and ΔPLY	Q07113	Cation-independent mannose-6-phosphate receptor	307.5113924	0.019482527
Down- regulated in D39 and ΔPLY	P16546	Spectrin alpha chain, non-erythrocytic 1	191.2866142	0.017481556

Table 4.2. Proteins down-regulated during pneumococcal infection. Proteins down-regulated in macrophages during pneumococcal D39 or ΔPLY infection, which may contribute to the pathogenesis of pneumococcus or may play immune roles in response to pneumococcal infection.

4.3 Deep analysis for the up-regulated proteins

Using the protein lists identified in the groupings of my ternary plot, I next sought to highlight the differences in response between D39- and ΔPLY-stimulated BMDMs, and to define the molecular functions for the up-regulated proteins. The aim of this analysis was to get a 'snapshot' inside the macrophage during pneumococcal infection. To do this, I grouped proteins that were up regulated (relative to untreated cells) during D39 infection (Figure 4.4A), ΔPLY infection (Figure 4.4B) and during both D39 and ΔPLY infections (Figure 4.4C) into different clusters based upon their molecular functions. These groups were; binding activity, catalytic activity, enzyme regulator activity, nucleic acid binding/transcription factor activity, receptor activity, structural molecule activity and transporter activity. Most of the up-regulated proteins displayed catalytic and binding activities.

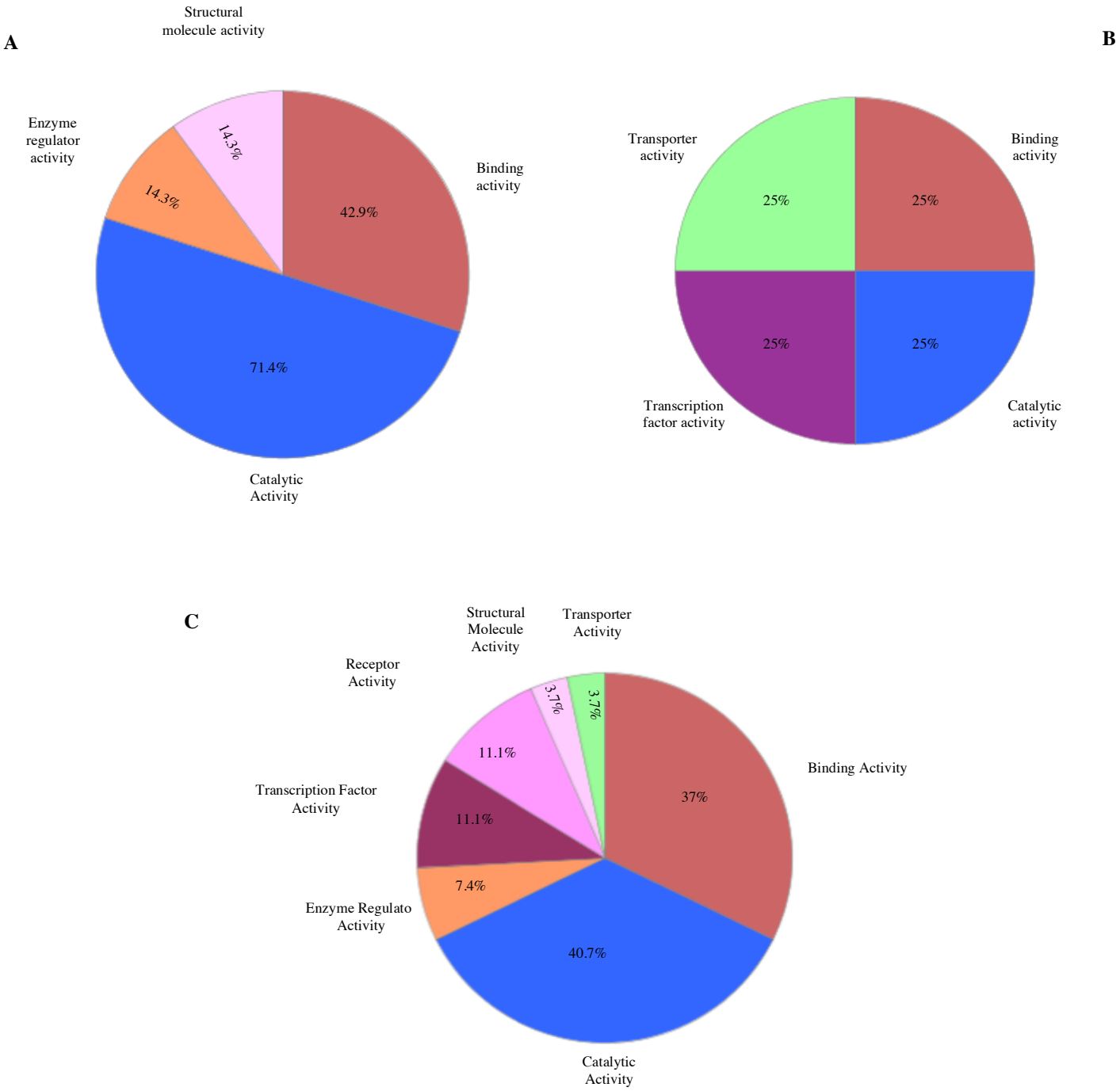


Figure 4.4. The molecular functions for the proteins up regulated in BMDM during pneumococcal infection. (A) The pie chart shows the molecular functions for the up-regulated proteins with D39. (B) The molecular functions for the up-regulated proteins with ΔPLY (C) the molecular function in both D39 and ΔPLY.

Next, I aimed to define proteins that were up regulated significantly with D39 (Table 4.3), ΔPLY (Table 4.4) or in both conditions (Table 4.5).

4.4 Proteins up regulated during D39 infection

Protein Code	Protein Name	Stimulus
Chi3l3	Chitinase-like protein 3,	
Gdi1	Rab GDP dissociation inhibitor alpha,	
Cxcl2	C-X-C motif chemokine 2,	
Nos2	Nitric oxide synthase, inducible	**D39**
Car2	Carbonic anhydrase 2	
Ptgs2	Prostaglandin G/H synthase 2	
Krt6a	Keratin, type II cytoskeletal 6A	

Table 4.3. D39 up-regulated proteins. Protein codes and names of proteins that were up regulated by BMDMs during D39 infection. The data were generated from the Panther database.

Chi3l3 is known as chitinase 3-like protein 3, a lectin that binds to a wide range of particles. It is composed of 398 amino acids. It has the ability to bind saccharides with a free amino group (such as glucosamine or galactosamine), oligomeric saccharides, chitin and heparin. It has chemotactic activity for T-lymphocytes, eosinophils and bone marrow cells and, plays a significant role in inflammation and allergy (Chang et al., 2001, Harbord et al., 2002).

Gdi1 is known as a guanosine diphosphate (GDP) dissociation inhibitor 1. It is composed of a 447 amino acids that work to regulate the GDP/GTP exchange reaction of most Rab proteins through blocking the dissociation of GDP from them, and the subsequent binding of GTP to them. It may also play a role in cell motility regulation (Gupta et al., 2013).

Cxcl2 is a chemokine (C-X-C motif) ligand 2 that consists of 100 amino acids. It is Chemotactic for human polymorphonuclear leukocytes (Tekamp-Olson et al., 1990), however it does not induce chemokinesis or an oxidative burst (Shao et al., 1998).

Nos2 is known as nitric oxide synthase 2. It consists of 1144 amino acids and is able to promote the production of nitric oxide (NO). The importance of this protein in macrophages has been described, since NO mediates tumoricidal and bactericidal activities (Kone et al., 1995). The protein is also involved in inflammation, enhancing the synthesis of pro-inflammatory mediators such as IL6 and CXCL8 (Kim et al., 2005).

Car2 is known as Carbonic anhydrase 2. It consists of 259 amino acids. The protein is crucial for osteoclast differentiation and bone resorption. It also contributes to the regulation of intracellular pH in the duodenal upper villous epithelium during proton-coupled peptide absorption (Simpson et al., 2010).

Ptgs2 is prostaglandin synthase 2. It is composed of 604 amino acids. It mediates the formation of prostaglandins, which functions as a main mediator of inflammation (Wang et al., 2005). Its up-regulation has been correlated with improved epithelial adhesion, phenotypic changes, resistance to tumor angiogenesis and apoptosis. In cancer cells, changes in the protein increase production of prostaglandin E2, which has

essential roles in modulating proliferation, motility and resistance to apoptosis (Manieri et al., 2012).

Krt6a is known as keratin 6A and is composed of 564 amino acids. It is epidermis-specific type I keratin, which is involved in wound healing (Rotty and Coulombe, 2012). The protein is also involved in the activation of follicular keratinocytes after wounding, however it does not has a major role in the proliferation of keratinocytes or their migration (Wojcik et al., 2000).

4.5 Proteins up regulated during ΔPLY

Protein Code	Protein Name	Stimulus
Irak3	Interleukin-1 receptor-associated kinase 3	**ΔPLY**
Slc7a2	Low affinity cationic amino acid transporter 2,	
Acp5	Tartrate-resistant acid phosphatase type 5	
Met	Hepatocyte growth factor receptor	

Table 4.4. ΔPLY up regulated proteins. Protein codes and names of proteins that were up regulated by BMDMs during infection with pneumolysin-negative pneumococci. The data were generated from the Panther database.

IRAK3 is a 596 amino acids protein known as interleukin-1 receptor-associated kinase 3 or IRAK-M and is a negative regulator of Toll-like receptor (TLR) signaling (Kobayashi, 2002). It has the capability to inhibit the dissociation of IRAK1 and

IRAK4 from the TLR signaling complex (Rosati and Martin, 2002). This inhibition process could occur by either blocking the phosphorylation of IRAK1 and IRAK4 or stabilizing the receptor complex (Kobayashi et al., 2002). TLR-mediated signaling processes identify various substances from microbial and non-microbial sources, and relay signals downstream to trigger the expression of several pro- and anti-inflammatory cytokines (Li, 2004). The signaling of TLR is regulated throughout a series of intra-cellular proteins involving IRAKs. There are four different IRAK proteins; IRAK1, IRAK2, IRAK M, and IRAK 4) (Janssens and Beyaert, 2003), between which IRAK-4 is significant for activating transcription factor NFκB and thus activating the inflammasome (Li et al., 2002) (Man and Kanneganti, 2015). Up regulation of the inhibitory IRAK-3 suggests the TLR signaling is negatively regulated during D39 infection.

Slc7a2 is a protein composed of 698 amino acids. It is known as solute carrier family 7 (cationic amino acid transporter, y+ system), member 2. It is a low-affinity, high capacity permease, which is involved in the transport of the cationic amino acids such as lysine ornithine and arginine macrophages activation (Yeramian et al., 2006).

Acp5 is known as Tartrate-resistant acid phosphatase type 5 and is composed of 327 amino acids. Its may have a role in the process of bone resorption (Huttlin et al., 2010).

Met is the Hepatocyte growth factor receptor. It is a receptor tyrosine kinase, which transduces signals from the extracellular matrix into the cytoplasm via binding to hepatocyte growth factor ligand (Prat et al., 1991). Met also regulates several physiological processes involving proliferation, morphogenesis, scattering and survival. The protein has a significant role in wound healing, organ regeneration and

tissue remodeling. During embryonic development, Met signaling plays a key role in the maturation and migration of muscles and neuronal precursors, and kidney formation (Bladt et al., 1995).

4.6 Proteins up regulated during both D39 and ΔPLY infections

Protein Code	Protein Name	Stimulus
IL1a	Interleukin-1 alpha,	**D39 and ΔPLY**
Serpinb1a	Serine protease inhibitor 1A,	
Cd14	Monocyte differentiation antigen CD14,	
Stat5a	Signal transducer and activator of transcription 5A	
Clec4d	C-type lectin domain family 4 member D	
IL1b	Interleukin-1 beta	
Procr	Endothelial protein C receptor	
Palld	Palladin	
Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	

Table 4.5. Proteins up regulated in both D39 and ΔPLY. Protein codes and names of proteins that were up regulated by BMDMs during infection with both D39 and ΔPLY. The data were generated from the Panther database.

IL1a is interleukin 1 alpha (composed of 271 amino acids). It is produced by stimulated macrophages. IL-1A encourages the proliferation of thymocyte by the

release of IL-2, maturation and proliferation of B-cells, and stimulates fibroblast growth factor activity (Nicklin et al., 1994). The protein is involved in the inflammatory response, and has been identified as an endogenous pyrogen (Hu et al., 2003).

Serpinb1a is a serine peptidase inhibitor, clade B, member 1a. It is composed of 379 amino acids and has a role in regulating neutrophil protease activity and therefore forms complexes with elastase, cathepsin G, chymotrypsin and proteinase-3 (Benarafa et al., 2002).

CD14 is known as Monocyte differentiation antigen CD14. It functions as a co-receptor with TLR4 to mediate the response of innate immune cells to bacterial lipopolysaccharide (LPS) (Drage et al., 2009). It acts via MyD88, TIRAP and TRAF6 leading to the activation of NF-kappa-B, cytokine production and the inflammatory responses (Jiang et al., 2005). It is also a co-receptor for TLR2 in response to diacylated and triacylated lipopeptides (Zhao et al., 2011).

Stat5a is signal transducer and activator of transcription 5A. It consists of 794 amino acids. It may mediate the cellular responses to Stem cell factor (SCF) cytokines and other growth factors (Muraoka-Cook et al., 2006). The protein has two important functions including signal transduction and activation of transcription (Jones et al., 1999).

Clec4d is a C-type lectin domain family 4 and consist of 215 amino acids. It works as an endocytic receptor that is involved in antigen presenting cells during the uptake of antigen, clearance of the antigen, and presentation to T cells (Balch et al., 1998).

IL1b is Interleukin-1 beta. It is a pro-inflammatory cytokine. The protein was initially

discovered as the major endogenous pyrogen that is able to induce the synthesis of prostaglandins, influx and activation of neutrophils, T-cell activation and cytokine production, activation of B-cells and antibody secretion, and fibroblast proliferation and collagen production (Mariathasan et al., 2006, Qu et al., 2007). Pneumolysin activates the NLRP3 inflammasome and triggers the release of IL-1β from macrophages and dendritic cells (McNeela et al., 2010). Also, the NLRP3 inflammasome has been shown to be essential for protection against pneumococcal pneumonia and for the generation of IL-1β by dendritic cells (McNeela et al., 2010).

Procr is a protein C receptor that binds activated protein C. It is composed if 238 amino acids. It enhances protein C activation by the thrombin-thrombomodulin complex. The protein plays a significant role in the protein C pathway controlling blood coagulation (Liang et al., 1999).

Palld is known as a palladin, which is a cytoskeletal-associated protein. It is composed of 1123 amino acids. This protein is essential for organization of normal actin cytoskeleton (Parast and Otey, 2000). It has an important role in establishing cell morphology, cell adhesion, motility, and cell-extracellular matrix interactions in a wide range of cell types (Liu et al., 2007).

Ifit3 is known as interferon-induced protein with tetratricopeptide repeats 3. The protein acts as an inhibitor for viral processes, proliferation, cell migration, signaling, and viral replication (Lee et al., 1994).

4.7 Involvement of the up regulated proteins in biological processes

Next, I aimed to examine the involvement of these up-regulated proteins in BMDM biological processes such as; immune system processes, developmental processes and metabolic processes.

The contributions of these proteins to BMDM biological processes are presented in Figure 4.5. A number of processes were represented, but metabolic and cellular pathways were particular prominent. Cellular processes include cell transport, cells diffusion, the Krebs cycle and homeostasis.

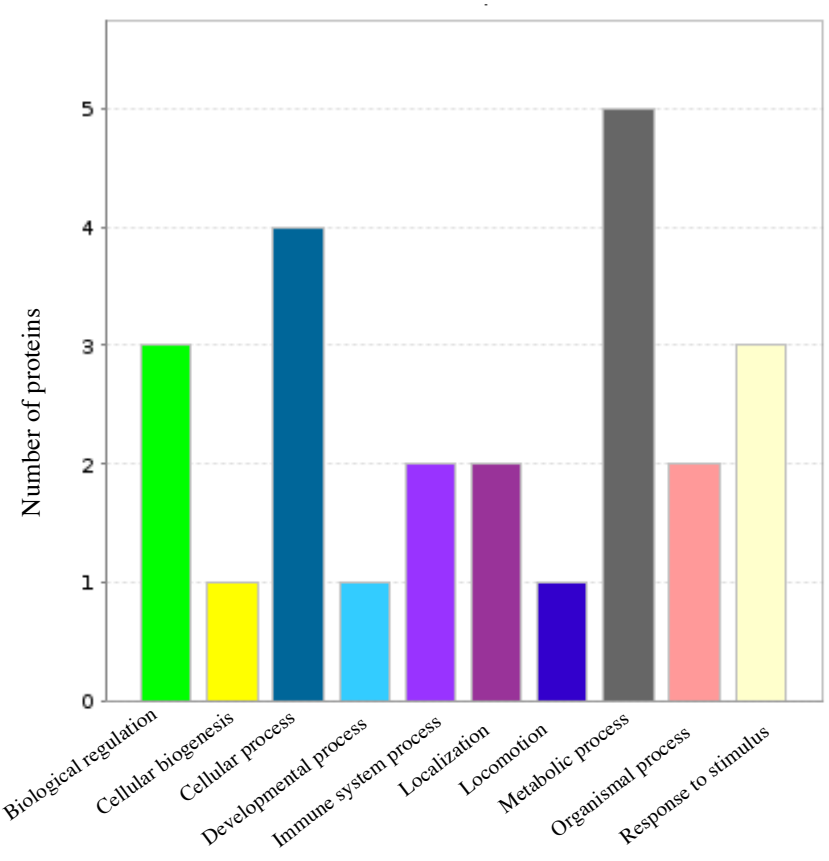


Figure 4.5. The involvement of D39 up-regulated proteins in the biological process of BMDMs. BMDMs were stimulated with D39 at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

Furthermore, I have showed that during ΔPLY infection, proteins involved in metabolic pathways were again up regulated (Figure 4.6).

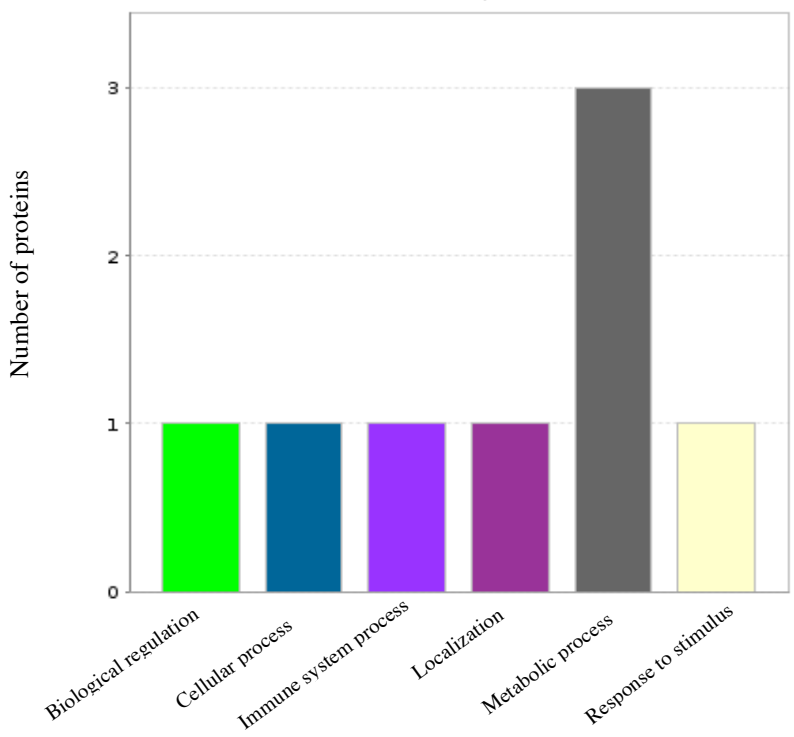


Figure 4.6. The involvement of ΔPLY up-regulated proteins in the biological process of BMDMs. BMDMs were stimulated with ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

Proteins up-regulated in both D39 and ΔPLY infection are involved in diverse biological processes (Figure 4.7). However, once again, metabolic processes feature heavily, as do cellular processes and the response to stimulus. Biological regulation is known as any process that regulates the occurrence, rate or level of any biological process function. While, biological process is a known sequence of events or molecular functions.

A process is a group of molecular events that have defined start and end. Cellular processes include cell transport, cells diffusion, the krebs cycle and homeostasis. In summary, Figure 4.8, a composite of Figures 4.5, 4.6 and 4.7, shows the collective response of BMDMs to pneumococcal infection at 24 h.

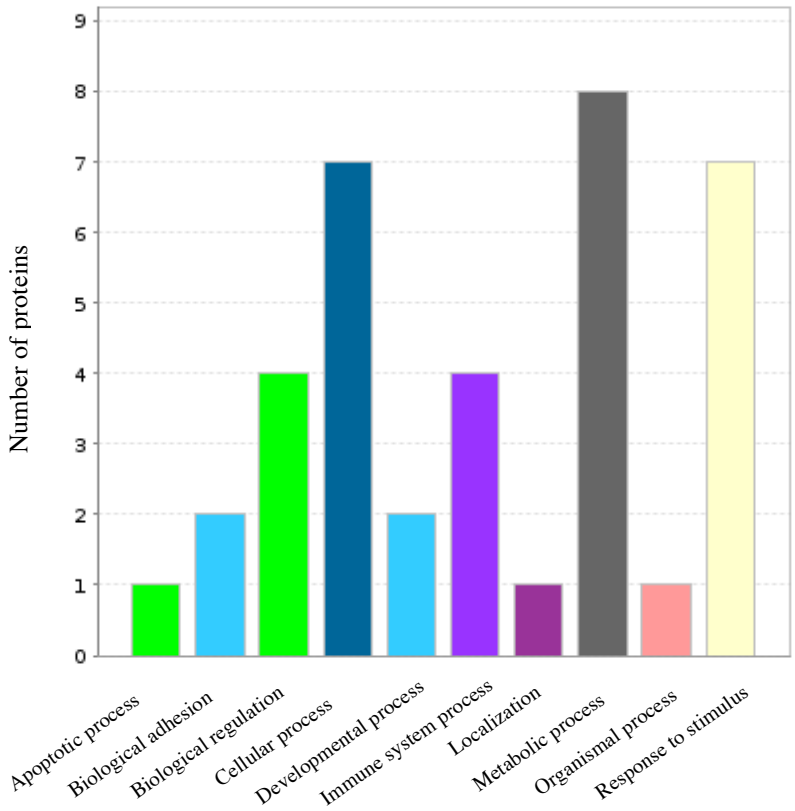


Figure 4.7. The involvement of up-regulated proteins with D39 and ΔPLY in the biological process of BMDMs. BMDMs were stimulated with either D39 or ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

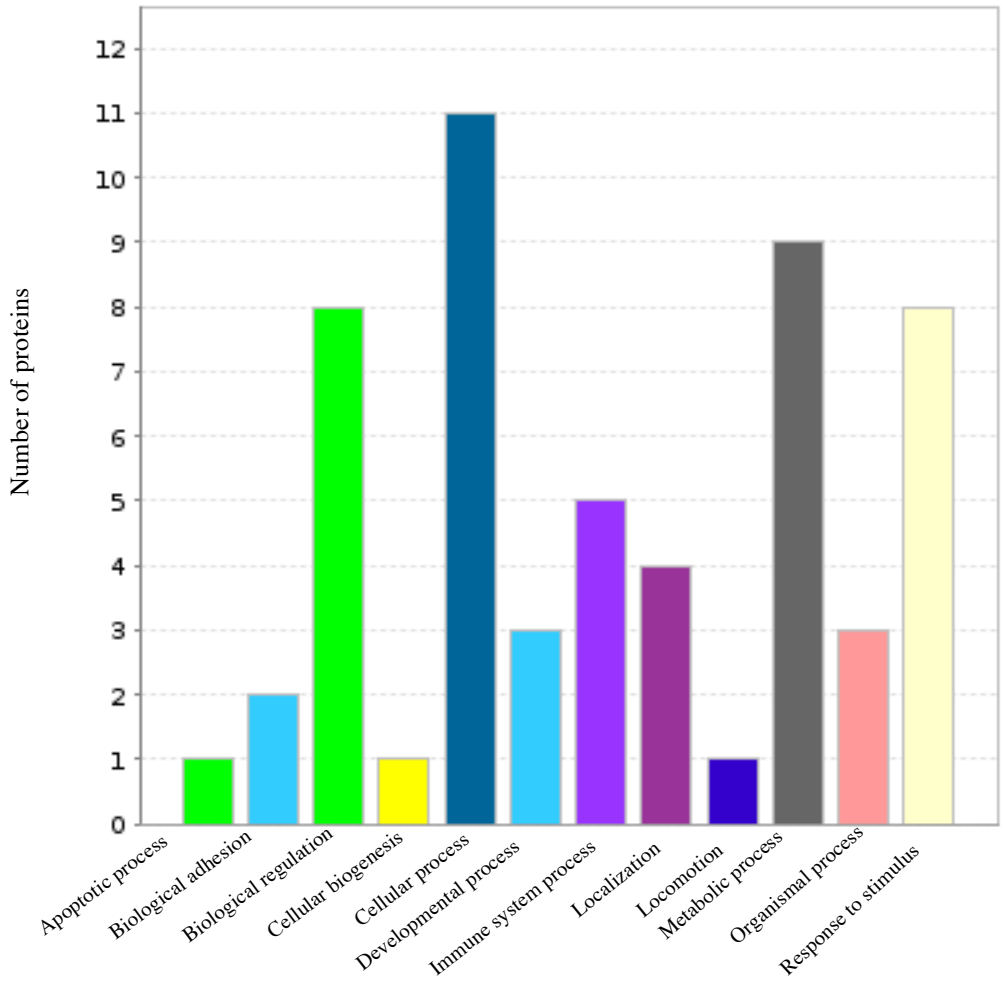


Figure 4.8. The collective response of macrophages to pneumococcal infections. BMDMs were stimulated with either D39 or ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

4.8 Interactions between up regulated proteins

Next, I identified the described interactions between proteins up regulated with either D39 or ΔPLY, as it is essential to understand how these proteins interact with each other inside BMDMs in the presence or absence of pneumolysin during *S. pneumoniae* infection.

In Figure 4.9, the interactions between proteins that were up regulated following stimulation with D39 (Figure 4.9A) or ΔPLY (Figure 4.9B) were examined. Data in Figure 4.9A, suggest that some of the up-regulated proteins during D39 infection may have direct interactions with each other and their expression may rely on each other. Binding activity has been described between Nos2 and Ptgs2 proteins (Blue line, Figure 4.9A) Co-expression has been identified for Nos2 and Chi3l3, and Ptgs2 and Cxcl2 (Gray line, Figure 4.9A). Figure 4.9B reveals that proteins up regulated during ΔPLY infection have no direct link to each other and therefore may function in different pathways. However, co-expression has been observed for Irak3 and Slc7a2 (Gray line, Figure 4.9B).

I also examined the relationships between proteins up regulated in both D39 and ΔPLY infection. In Figure 4.10, the interactions between proteins are shown. Here the data demonstrate a direct relationship between Il1a and Il1b resulting in binding, catalysis and reaction activities. Also, the data here has shown that Cd14 is responsible for the activation of Clec4d, which is known to function as an endocytic receptor during the uptake of antigen, clearance of the antigen, and presentation to T cells

(Balch et al., 1998). However, no direct correlation has been identified with the other proteins described in Figure 4.10, although co-expression has been identified with Clec4d, Il1b and Cd14 (Gray line, Figure 4.10).

Figure 4.11 show a summary of the interactions between all the proteins up regulated by BMDMs during infection with serotype 2 *S. pneumoniae* at 24 h. Co-expression has been observed between Chi3l3 and Nos2, Chi3l3 and Serpinb1a, Serpinb1a and Ptgs2, Ptgs2 and Met, Ptgs2 and Cxcl2, Ptgs2 and Il1b, Ptgs2 and Il1a, Il1a and Il1b, Il1b and Irak3, Il1a and Irak3, Slc7a2 and Irak3, Il1b and Clec4d, Cxcl2 and Clec4d, Cxcl2 and Cd14, and Cd14 and Clec4d (Gray lines, Figure 4.11). Also, catalysis activities were identified between Stat5a and Met, Il1a and Irak3, Il1b and Irak3, Il1a and Il1b, and Irak3 and Cd14 (Purple lines, Figure 4.11).

Furthermore, binding relationships were determined between Il1b and Irak3, Il1a and Il1b, and Irak3 and Cd14 (Blue lines, Figure 4.11). Reaction has been observed between Il1b and Irak3, Stat5a and Met, Il1a and Il1b, and Irak3 and Cd14 (Black lines, Figure 4.11). Finally, Ptgs2 and Stat5a regulate each other expression, as do Il1a and Irak3 (Yellow lines Figure 4.11).

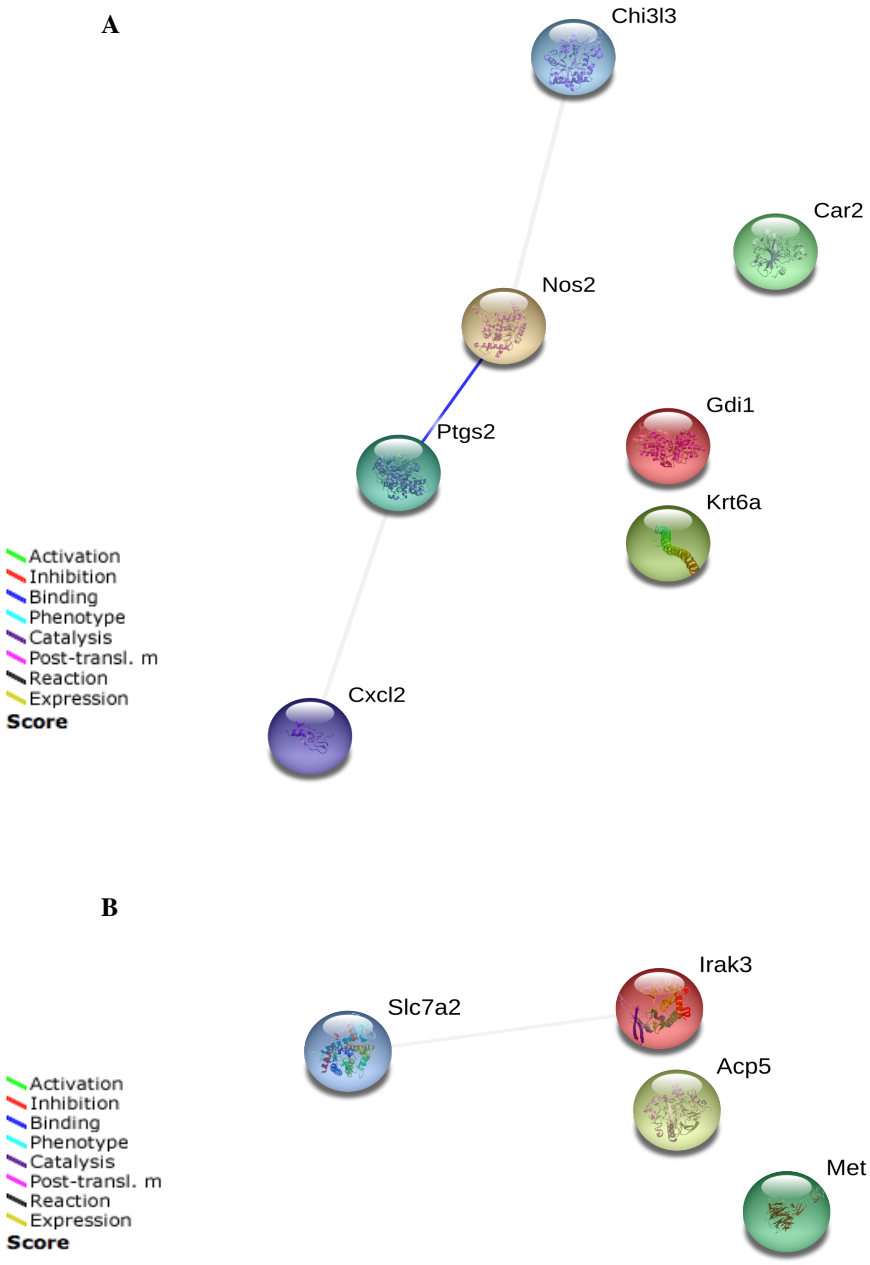


Figure 4.9. Proteins up-regulated during *S. pneumoniae* infection. BMDMs were stimulated with D39 or ΔPLY at a ratio of 1:10 for 24 h. (**A**) The interaction between proteins up-regulated with D39. (**B**) The interaction between proteins up-regulated with ΔPLY. String protein–protein interaction display with known and predicted functional partners, presenting direct correlation between up-regulated proteins. Data generated by String data base system.

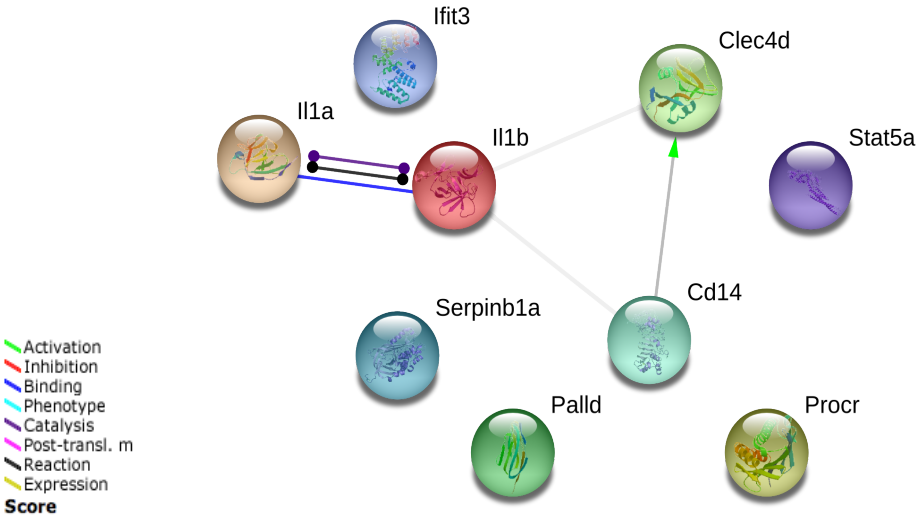


Figure 4.10. Proteins up-regulated with both D39 and ΔPLY. BMDMs were stimulated with D39 or ΔPLY at a ratio of 1:10 for 24 h. String protein–protein interaction display with known and predicted functional partners, presenting direct correlation between up-regulated proteins. Data generated by String data base system

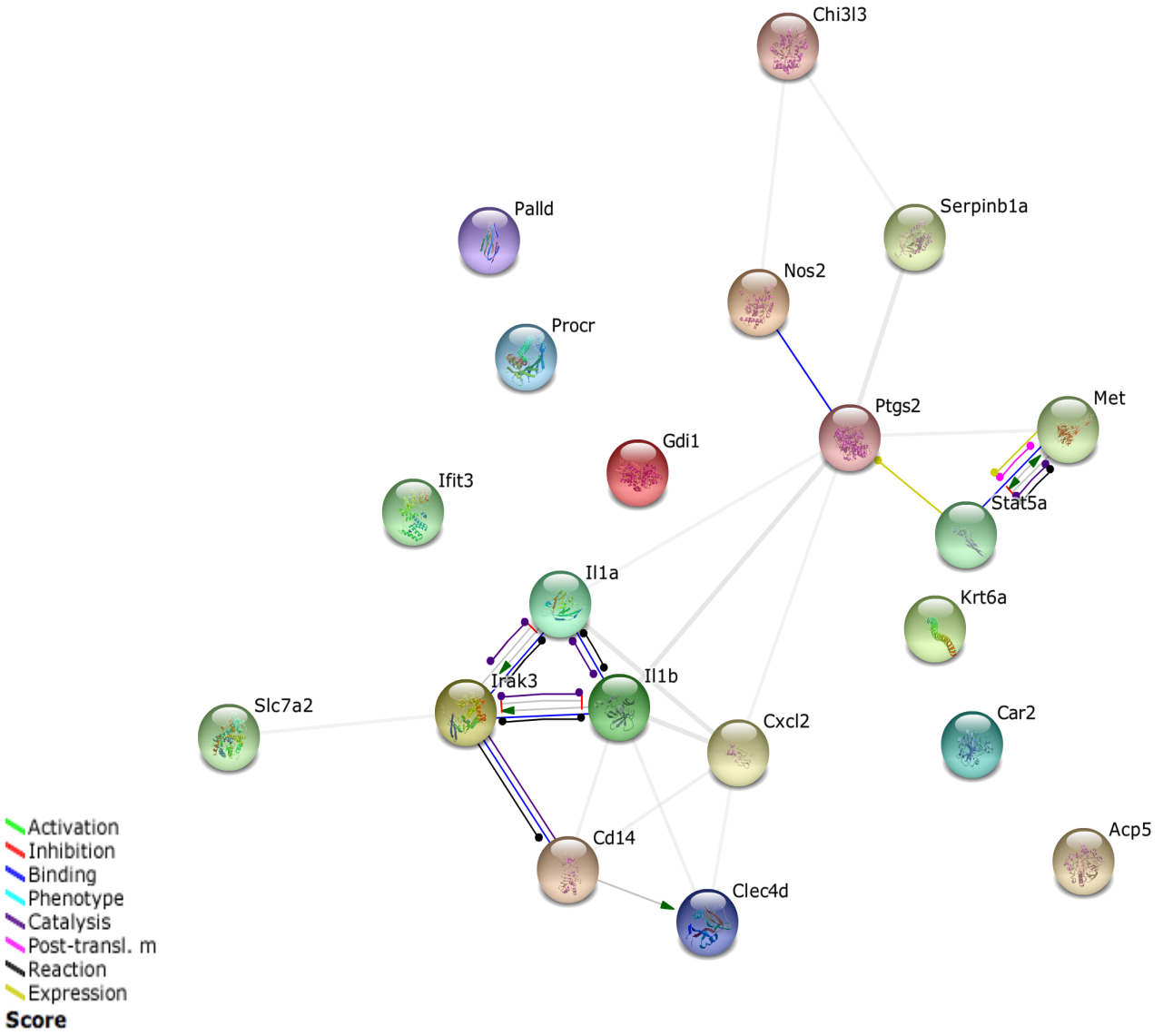


Figure 4.11. All proteins up regulated during pneumococcal infection. BMDMs were stimulated with D39 or ΔPLY at a ratio of 1:10 for 24 h. String protein–protein interaction display with known and predicted functional partners, presenting direct correlation between up-regulated proteins. Data generated by String data base system.

4.9 Deep analysis for the down-regulated proteins

In Figure 4.12 the molecular functions for the proteins down-regulated during D39 or ΔPLY infection are presented. These proteins are responsible for 5 different molecular functions (Figure 4.12A-C); binding activity, catalytic activity, enzyme regulator activity, receptor activity and structural molecule activity. About 59% of the down-regulated proteins had catalytic or binding activity. Identifying the proteins that contribute to these significant molecular functions is necessary to understand how macrophages response to pneumococcus.

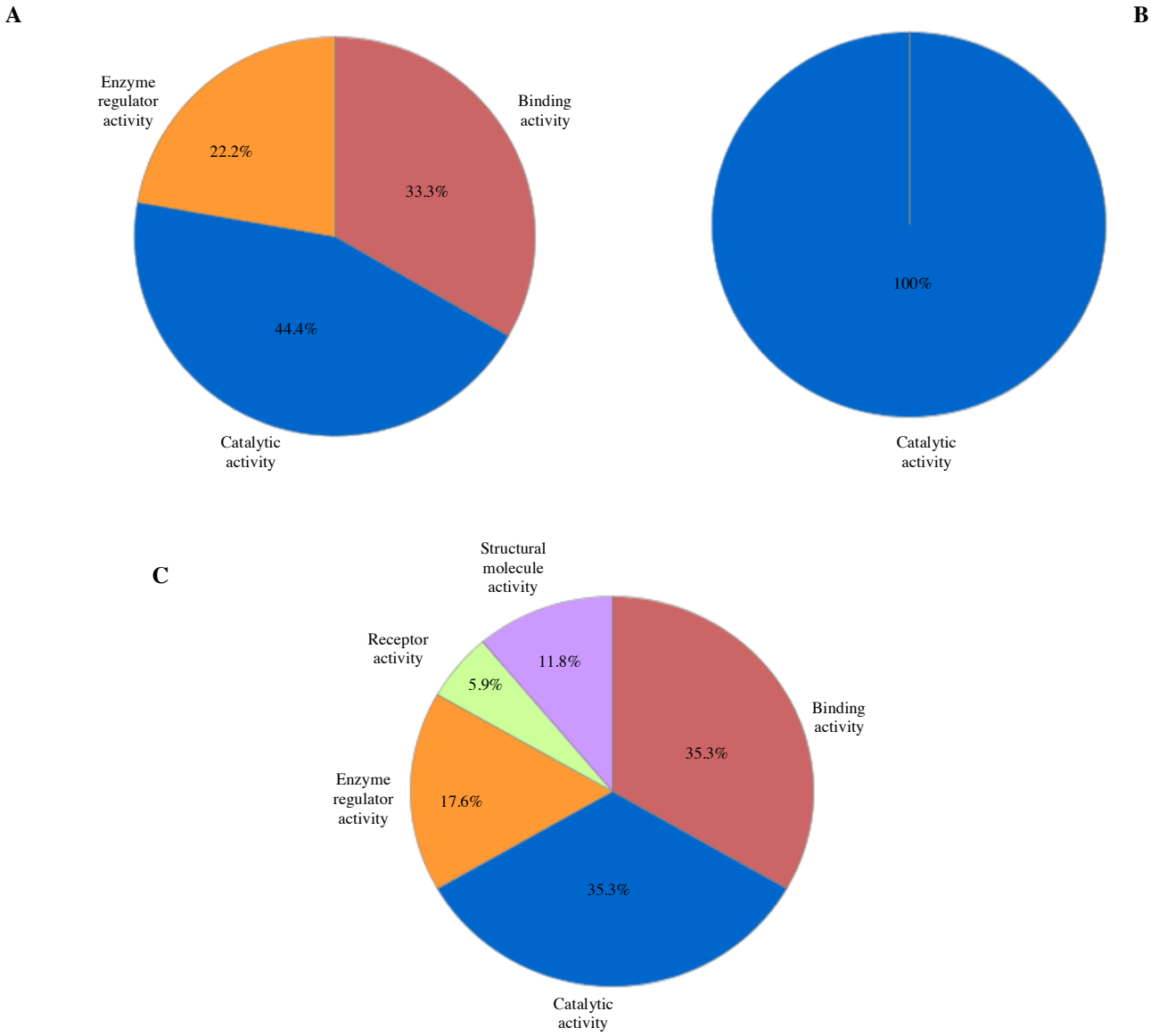


Figure 4.12. The molecular functions for the proteins down-regulated during pneumococcal infection. (A) The pie chart shows the molecular functions for the down-regulated proteins with D39. (B) The molecular functions for the down-regulated proteins with ΔPLY (C) the molecular function for proteins down regulated in both D39 and ΔPLY.

Next, I aimed to define proteins that were down regulated significantly with D39 (Table 4.6), ΔPLY (Table 4.7) or in both conditions (Table 4.8).

4.10 Proteins down regulated during D39 infection

Protein Code	Protein Name	Stimulus
Ncl	Nucleolin,	**D39**
Set	Protein SET	
Csf1r	Macrophage colony-stimulating factor 1 receptor	
Rp2	Protein XRP2	
Anp32b	Acidic leucine-rich nuclear phosphoprotein 32 family member B	
Ap3b1	AP-3 complex subunit beta-1	
Fnta	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha	
Myd88	Myeloid differentiation primary response protein MyD88	

Table 4.6. D39 down regulated proteins. Protein codes and names of proteins down-regulated during D39 infection. The data were generated by Panther database.

Ncl, known as Nucleolin, is a major nucleolar protein that is present in eukaryotic cells. It is believed to have a key role in pre-rRNA transcription and ribosome assembly. Also, it has been shown to play a role in the process of transcriptional elongation (Yang et al., 1994).

Set is identified as SET nuclear oncogene. It is a multitasking protein involved in

biological processes such as transcription, apoptosis, nucleosome assembly and histone chaperoning (Rogowski et al., 2009). The protein is composed of 289 amino acids. Set protein is part of a complex that inhibits apoptosis following attack by cytotoxic T lymphocytes upon T cell receptor stimulation (Fan et al., 2003).

Csf1r is a Tyrosine-protein kinase that functions as a cell-surface receptor for colony stimulating factor (CSF) 1, which plays an important role in the proliferation, regulation of survival and differentiation of hematopoietic precursor cells, in particular mononuclear phagocytes, such as monocytes and macrophages (Dai et al., 2002). This protein is also able to promote the production of pro-inflammatory chemokines in response to CSF1, and thus plays a significant role in inflammatory processes and innate immunity (Wei et al., 2010).

Rp2 is identified as Protein XRP2. It acts as a GTPase-activating protein (GAP) involved in trafficking between the Golgi and the ciliary membrane. The protein is composed of 347 amino acids. It is also involved in protein localization (Huttlin et al., 2010).

Anp32b is acidic (leucine-rich) nuclear phosphoprotein 32 families, member B and is a multifunctional protein composed of 272 amino acids. It functions as a cell cycle progression factor as well as a cell survival factor (Matilla and Radrizzani, 2005). It is an anti-apoptotic protein that functions as a caspase-3 inhibitor (Huttlin et al., 2010).

Fnta is known as farnesyltransferase, CAAX box, alpha; that contributes to the transfer of a farnesyl or geranyl-geranyl moiety from farnesyl or geranyl-geranyl pyrophosphate to a cysteine at the fourth position from the C-terminus of proteins containing the C-terminal sequence Cys-aliphatic-aliphatic-X leading to regulate the

catalytic activity such as cellular metabolism and motility, inside cells (Bon et al., 2011). The alpha subunit is believed to participate in a stable complex with the substrate. This protein is composed of 377 amino acids (Luo et al., 2003).

Myd88 is a key adapter protein involved in the Toll-like receptor and IL-1 receptor signaling pathways during the response of the innate immune system to infection (Burns et al., 1998). The protein acts via IRAK1 and IRAK2, leading to activation of NF-kappa-B, to trigger the secretion of cytokines and the inflammatory response (Janssens et al., 2002). Also, it increases the transcription of IL-8 and is involved in the signaling pathway of IL-18 (Adachi et al., 1998). Furthermore, MyD88 has been shown to mediate signaling in intestinal epithelial cells since it is required for maintenance of gut homeostasis and controls the expression of the anti-microbial lectin in the small intestine (Vaishnava et al., 2011).

4.11 Proteins down regulated during ΔPLY

Protein Code	Protein Name	Stimulus
Krt14	Keratin, type I cytoskeletal 14,	**ΔPLY**
Ufc1	Ubiquitin-fold modifier-conjugating enzyme 1,	
Vps51	Vacuolar protein sorting-associated protein 51 homolog,	
Mthfd1l	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	

Table 4.7. ΔPLY down regulated proteins. Protein codes and names of proteins down-regulated during ΔPLY infection. The data were generated by Panther database.

Krt14, Keratin 14 type 1 is responsible for stimulating KRT5-KRT14 filaments to self-organize into large bundles (Huttlin et al., 2010), leading to enhancement of the mechanical properties that are involved in resilience of keratin in-between filaments *in vitro* (Lee et al., 2012).

Ufc1 is known as Ubiquitin-fold modifier-conjugating enzyme 1. The protein is involved in the tranferase activity of Eukaryotic cells, since it has the ability to form an intermediate via a thioester linkage with ubiquitin-like protein 1 (UFM1), which is required for a number of cellular processes (Carninci et al., 2005).

Vps51 functions as part of the GARP complex, which is known to be involved in the retrograde transport from early and late endosomes to the trans-Golgi network (TGN)

(Carninci et al., 2005). The GARP complex is important for the maintenance of protein retrieval from endosomes to the TGN, lysosome function, acid hydrolase sorting, endosomal cholesterol traffic and autophagy (Huttlin et al., 2010).

Mthfd1l connects the mitochondria and the cytoplasm in the mammalian model of one-carbon folate metabolism. The protein consists of 977 amino acids (Christensen et al., 2005).

4.12 Proteins down regulated during both D39 and ΔPLY

Protein Code	Protein Name	Stimulus
Hbb-b1	Hemoglobin subunit beta-1	**D39 and ΔPLY**
Pdcl3	Phosducin-like protein 3	
Ckap5	Cytoskeleton-associated protein 5	
Igf2r	Insulin-like growth factor 2 receptor	
Sptan1	Spectrin alpha chain, non-erythrocytic 1	

Table 4.8. Proteins down regulated in both D39 and ΔPLY. Protein codes and names of proteins down-regulated during both D39 and ΔPLY infection. The data were generated from the Panther database.

Hbb-b1 is the Hemoglobin subunit beta-1 protein that is composed of 147 amino acids. It is involved in oxygen transport process from the lung to the other peripheral

tissues (Carninci et al., 2005).

Pdcl3 is composed of 240 amino acids. It is known as phosducin-like 3. This protein has the ability to modulate the activation of caspases during apoptosis (Wilkinson et al., 2004).

Ckap5 is known as cytoskeleton associated protein 5 and is composed of 2032 amino acids. The protein binds to the plus end of microtubules in order to regulate microtubule dynamics and organization. Also, it stimulates the nucleation and elongation of cytoplasmic microtubule (Huttlin et al., 2010).

Igf2r is insulin-like growth factor 2 receptor and is composed of 2483 amino acids. The main function for this protein is to transport lysosomal acid hydrolase precursors from Golgi apparatus to lysosome (Braulke et al., 1999).

Sptan1 is a protein composed of 2478 amino acids. It is involved in cell adhesion as it interacts with calmodulin in a calcium-dependent manner to mediate movement of the cytoskeleton at the membrane (Huttline et al., 2010).

4.13 Involvement of the down regulated proteins in Biological processes

My next aim was to study the contribution of these down-regulated proteins in BMDMs biological processes (Figures 4.13, 4.14. and 4.15). During D39 infection, proteins involved in a range of biological processes were downregulated but metabolism again featured strongly (Figure 4.13).

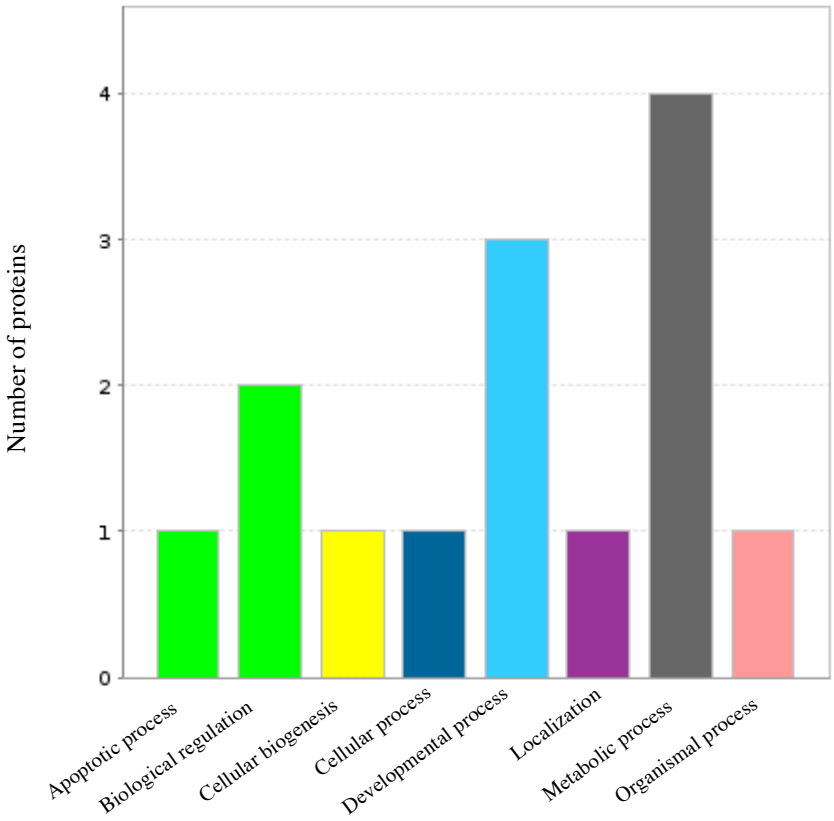


Figure 4.13. The involvement of D39 down-regulated proteins in the biological process of BMDMs. BMDMs were stimulated with D39 at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

Many of the proteins downregulated either in ΔPLY infection (Figure 4.14) or in both infections (Figure 4.15) were involved in cellular processes. Figure 4.16 shows a summary for all proteins down regulated by BMDMs during infection with serotype 2 *S. pneumoniae*.

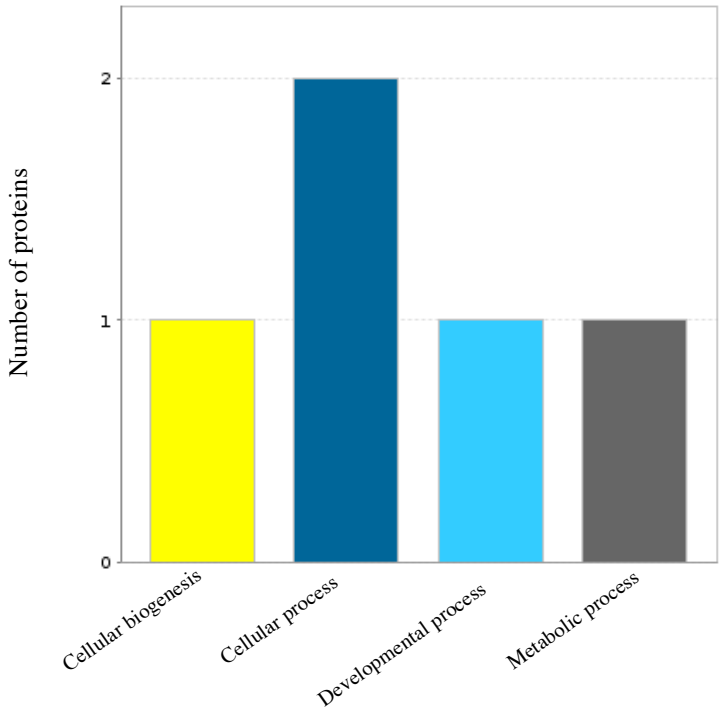


Figure 4.14. The involvement of ΔPLY down regulated proteins in the biological process of BMDMs. BMDMs were stimulated with ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

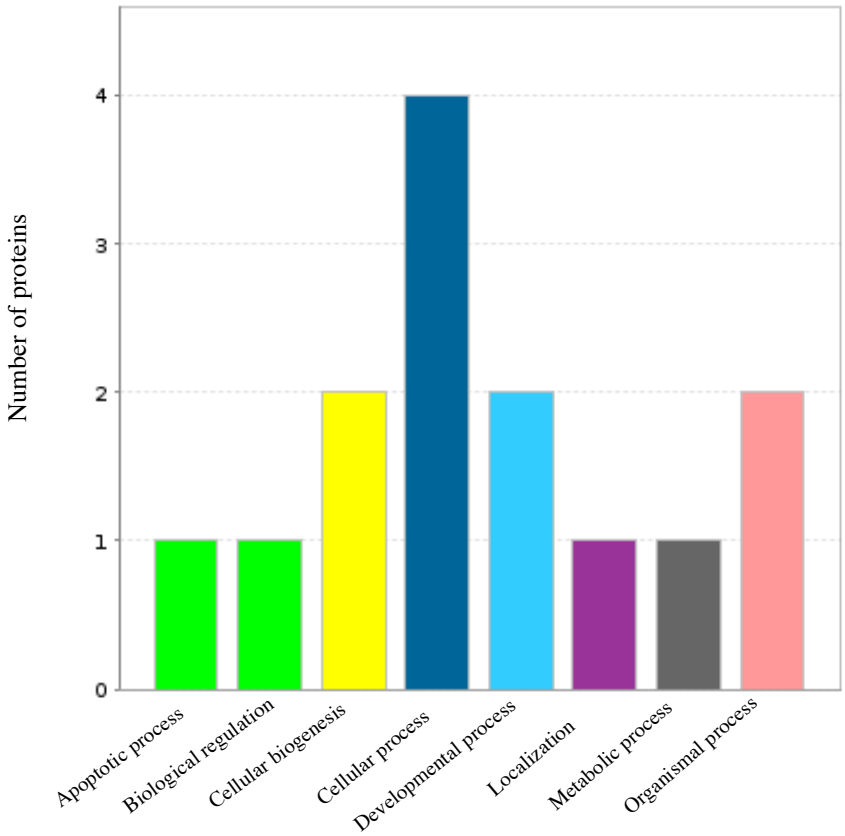


Figure 4.15. The involvement of the down regulated proteins with D39 and ΔPLY in the biological process of BMDMs. BMDMs were stimulated with either D39 or ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

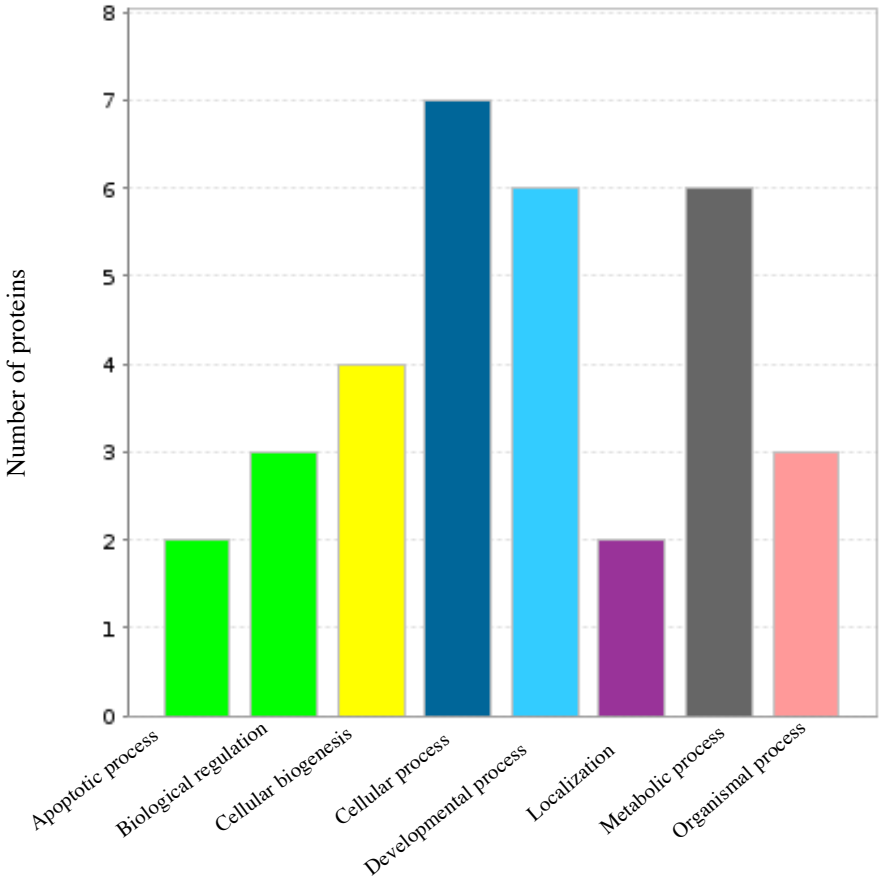


Figure 4.16. All down regulated proteins by BMDMs during infection with serotype 2 S. pneumoniae at 24 h. BMDMs were stimulated with either D39 or ΔPLY at a ratio of 1:10 for 24 h. Bar charts show the number of proteins involved in each biological process. Data generated by Panther classification system.

4.14 Interactions between up regulated proteins

Next, I aimed to analyse the interactions between proteins down regulated during either D39 or ΔPLY infection. Figure 4.17 shows the interactions between proteins that had been stimulated with D39 (Figure 4.17A) or ΔPLY (Figure 4.17B).

Co expression has been observed between Set and Ncl (Gray line, Figure 4.17A). Also, Figure 4.17A shows that Anp32b and Set, both of which are downregulated during D39 infection, have been described to react together (Black line) and to bind one another (Blue line). Figure 4.17B and 4.17C demonstrate that no direct links or interactions have been established between proteins downregulated during ΔPLY infection or those downregulated in both D39 and ΔPLY infection.

Figure 4.18 show a summary for all the down-regulated proteins during infection with pneumococcus at 24 h.

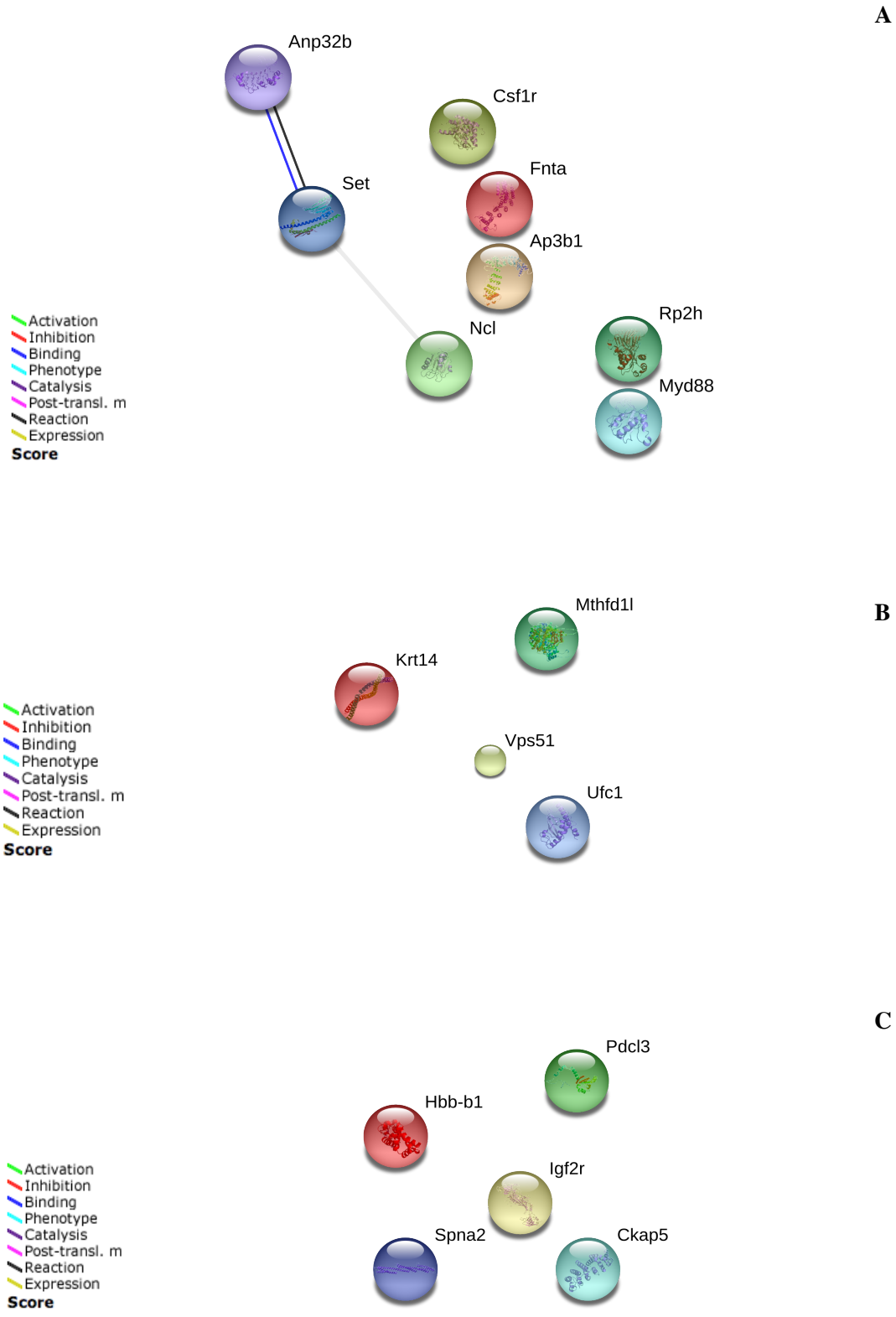


Figure 4.17. Proteins down regulated during *S. pneumoniae* infection. BMDMs were stimulated with D39 or ΔPLY at a ratio of 1:10 for 24 h. (**A**) The interaction between proteins down regulated with D39. (**B**) The interaction between proteins down regulated with ΔPLY. (**C**) Proteins down regulated with both D39 and ΔPLY. String protein–protein interaction display with known and predicted functional partners, presenting direct correlation between up-regulated proteins. Data generated by String data base system.

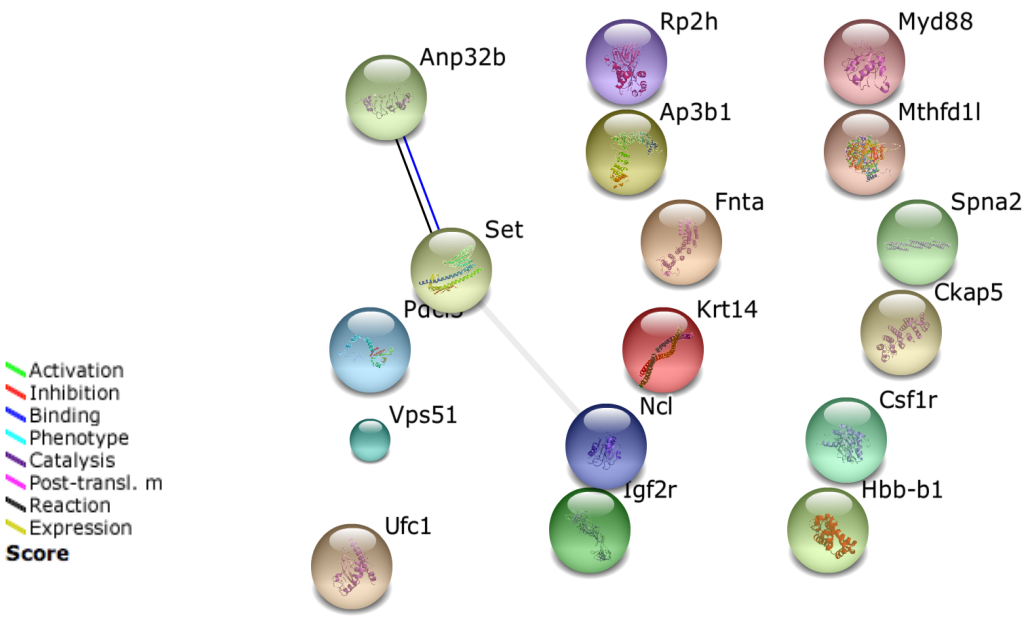


Figure 4.18. A summary for down regulated proteins by BMDMs during *S. pneumoniae* infection. BMDMs were stimulated with D39 or ΔPLY at a ratio of 1:10 for 24 h. String protein–protein interaction display with known and predicted functional partners, presenting direct correlation between up-regulated proteins. Data generated by String data base system.

4.15 Discussion

S. pneumoniae is a common pathogen responsible for diseases of high morbidity and mortality and one which has a major influence on the health care resources of developing and under-developed countries. Colonisation of the nasopharynx is important for the pneumococcus to initiate localized or systemic infections. The near universal production of pneumolysin by *S. pneumoniae* clinical isolates and the high degree of conservation of the PLY gene between pneumococcal strains suggest a significant role for this toxin in the pathogen life cycle (Kadioglu et al., 2008). Furthermore, the pneumococcal toxin pneumolysin has been demonstrated to activate mucosal and systemic immune response (Rubins et al., 1998). In my previous chapter I have described a series of interactions between pneumolysin, innate and adaptive immunity. In this chapter I was aiming to define the type of proteins expressed in response to serotype 2 *S. pneumoniae* infections in the presence or absence of pneumolysin, to highlight the differences between proteins up-regulated/down regulated with D39 or ΔPLY, to examine the contribution of these proteins to BMDMs molecular functions and biological processes, and to study the interactions of these differentially expressed protein with one another. I have presented proteome data that give an overview of what is happening inside the BMDMs during infection with serotype 2 *S. pneumoniae* in the presence or absence of pneumolysin. Both my heat map data and volcano figures have revealed that, by 24 hours post-infection, BMDM had significantly altered expression of a wide range of proteins.

Furthermore, many of the proteins for which expression increased or decreased differed between D39 and ΔPLY infection, suggesting that PLY influences host protein expression. Interestingly, several of the differentially expressed proteins have

been shown to be associated with the activation of the immune system in response to infection.

4.15.1 Proteins up regulated during pneumococcal infections

Chi3l3 is a lectin protein that binds a wide range of particles. Several studies have demonstrated a key role for this protein in inflammation. In particular, Chi3l3 has been shown to have chemotactic activity for T-lymphocytes, eosinophils (Owhashi et al., 2000), macrophages (Lee, 2009) and bone marrow polymorphonuclear leukocytes *in vitro* (Chang et al., 2001, Harbord et al., 2002). Many of the chitinase family proteins such as Chi3l3 are expressed in macrophages and epithelial cells of the lung, functioning as host first-line defense against exogenous agents, particularly chitin-containing pathogens (Homer et al., 2006, Mizoguchi, 2006). Interestingly, Wohlkönig et al. have revealed the chemical similarity between chitin and peptidoglycan (a major component of bacterial cell walls), since chitinases can cleave peptidoglycan (Wohlkönig et al., 2010). A recent study by Lee has revealed that exogenous chitin stimulates the response of macrophages and regulates adaptive type 2 allergic inflammation. This study demonstrates that chitin, or perhaps structurally similar peptidoglycan, could activate macrophages by interacting with surface receptors such as macrophage mannose receptor (MR) and toll-like receptor 2 (TLR-2) (Lee, 2009). My data suggest that pneumolysin is required for the up regulation of Chi3l3 protein. This process may lead to increased macrophage responsiveness to infection and lead to downstream recruitment of T lymphocytes or neutrophils. Ptgs2 was also up regulated during D39 infection. It acts as an enzyme that is involved in the production of prostaglandin in response to mucosal damage. It is also known to

affect downstream responses such as proliferation and apoptosis (Wang et al., 2005). During inflammation, damage signals and cytokines signal via Myd88 to trigger the activation of NF-κB and to induce Ptgs2 transcription (Wu et al., 2005). A study by Manieri et al. showed that Ptgs2 is required for wound repair, since Ptgs2 knockout mice had defects in wound repair when compared to WT mice (Manieri et al., 2012). During D39 infection, pneumolysin-driven damage in BMDMs may drive Ptgs2 production in order to trigger the wound healing response. Here I suggest that pneumolysin play a key role in the up regulation of Ptgs2 by BMDMs. Cxcl2 is another protein that I found to be up regulated during D39 infection. The protein has been shown to be chemotactic for human polymorphonuclear leukocytes (Tekamp-Olson et al., 1990), however it does not induce chemokinesis or an oxidative burst (Shao et al., 1998). A recent study by Roualt et al. has revealed that during human obesity the hypertrophied white adipose tissue (WAT) generates several inflammatory mediators, including cytokines such as IL-6 and TNF-α, and chemokines such as chemokine ligand 2 (CXCL2) and IL-8. The expression of CXCL2 mRNA was greater in macrophages when compared to other WT cells and positively associated with macrophages inflammatory markers IL-6 and TNF-α. Another finding from this study was that CXCL2 activated the adhesion of the neutrophils, its selective cell targets, to endothelial cells *in vitro* (Rouault et al., 2013). My data here propose that the pneumococcal toxin pneumolysin contributes to the up regulation of Cxcl2 and therefore is likely to increase recruitment and adhesion of neutrophils to infection sites. Nos2, up regulated by BMDMs stimulated with D39, stimulates the production of nitric oxide (NO). A study by Kone et al. has shown that NO has bactericidal activity (Kone et al., 1995) and enhances the synthesis of pro-inflammatory mediators such as IL6 and CXCL8 (Kim et al., 2005). Two recent studies have revealed that

during mycobacterial infection, the non-covalently attached mycobacterial cell wall glycolipid di-*O*-acylated trehalose (DAT) down-regulates the expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) in macrophages, which are important components in the response of the immune system against mycobacteria, leading to inhibition of two important features of adaptive immunity; the production of cytokines and the proliferation of T cells (Saavedra et al., 2006) (Espinosa-Cueto et al., 2015). A study by Marriott et al. has shown also that phagocytosis of pneumococci by human monocyte-derived macrophages stimulated the up regulation of iNOS and the production of NO (Marriott et al., 2006), which have shown previously to functions as potential mediators for both antimicrobial host defense and apoptosis induction during macrophage response to pneumococci (Bogdan, 2001). My data here show that pneumolysin is vital for the up regulation of Nos2 protein by macrophages, since this protein was up regulated only with D39 and not with ΔPLY. Here I may suggest that both Nos2 and pneumolysin are required for the activation of macrophages and the production of pro-inflammatory cytokines.

IRAK3 was up regulated by BMDMs during infection with ΔPLY but not D39. Rosati and Martin have revealed that IRAK3 play a key role in blocking the dissociation of IRAK4 from the Toll- like receptor signaling complex, which is necessary for the activation of both the transcription factor NFκB and the NLRP3 inflammasome (Rosati and Martin, 2002). Inflammasomes are defined as molecular proteins that are commonly activated upon cellular infection leading to the maturation of interleukin-1β (IL-1 β) and recruitment of immune cells such as neutrophils and macrophages to site of infections (Schroder and Tschopp, 2010). It is well know that pneumolysin has the ability to trigger the activation of the NLRP3 inflammasome and the release of IL1β from macrophages and dendritic cells (McNeela et al., 2010).

IRAK3 up regulation in the absence of pneumolysin suggests a weakened induction of inflammatory responses. Met, up regulated during ΔPLY, but not D39 infection, has a significant role in wound healing, organ re-generation and tissue re-modeling (Bladt et al., 1995). Met has also been revealed to play a key role in B cell differentiation (Van der Voort et al., 2000). Met up regulation, taken together with the high expression of IRAK3 and the lower levels of CXCL2 and Nos2 in ΔPLY infected BMDM suggests that the macrophage response in the absence of pneumolysin is less geared towards inflammation and more towards healing and repair. Interleukin 1 alpha (IL-1α) and interleukin beta (IL-1β) are critically important cytokines that are up regulated by BMDMs during both D39 and ΔPLY infection. Both IL-1α and IL-1β are commonly produced by activated cells including macrophages, epithelial cells and neutrophils (Nicklin et al., 1994, Qu et al., 2007).

IL-1 family cytokines are known to promote immunity. The role of inflammasome activation and the stimulation of IL-1 family cytokines including IL-1β in *S. pneumoniae* diseases have been examined. An early study revealed that macrophage death results in pneumolysin-dependent release of Il1b, indicating that the inflammasome has been activated (Cookson and Brennan, 2001). Furthermore, McNeela et al. showed that pneumolysin activates the NLRP3 inflammasome and triggers the production of IL-1β from dendritic cells and macrophages (McNeela et al., 2010). IL-1β has been shown to be critical to protection against pneumococcal pneumonia (McNeela et al., 2010), by encouraging the production of IL-17A, which was previously showed to provide protection against pneumococcal infection (Lu et al., 2008). Moreover, a recent study by Lemon et al. showed that mice lacking interleukin-1 receptor type 1 have fewer neutrophils early after infection, fewer

macrophages later in carriage, and persistent pneumococcal colonisation. Furthermore, mice treated intranasally with Il-1β clear colonised pneumococci (Lemon et al., 2015). My data show here, showing up regulation of IL-1 family cytokines during D39 infection is in line with previous findings that pneumococcus activates the inflammasome. Interestingly, the same cytokines were up regulated during infection with ΔPLY (in the absence of pneumolysin), which may propose that another pneumococcal protein might have the ability to stimulate the activation of inflammasomes. CD14 was also up regulated during both D39 and ΔPLY infection. The importance of this protein was revealed when several studies demonstrated that lipopolysaccharides from gram-negative bacteria and peptidoglycan from gram-positive bacteria were able to activate cytokine production by monocytes following interaction with CD14, leading to activation of Toll-like receptors (Kusunoki et al., 1995, Gupta et al., 1999, Schwandner et al., 1999). Another study by Yoshimura et al. has shown that CD14-dependent NF-κB translocation occurred upon infection of cells with *Staphylococcus aureus* (Yoshimura et al., 1999). This study demonstrated that gram-positive bacteria have a key role in triggering intracellular pathways after their interaction with CD14 and TLRs. Furthermore, a study by Ebong et al. has shown that CD14 is significant for the production of pro-inflammatory and anti-inflammatory cytokines, since CD14 knockout mice produce reduced levels of IL-1β, TNF, IL-6 and IL-10 during sepsis induced by cecal ligation and puncture when compared to wild-type mice (Ebong et al., 2001). Increased expression of CD14 by macrophages during pneumococcal infection may increased their responsiveness to peptidoglycan present in the cell wall of *S. pneumoniae*, thus aiding their activation and leading to the production of important cytokines such as IL-1β, TNF, IL-6. Stat5a, up regulated during both D39 and ΔPLY infection has been shown to play a vital role in a range of

cellular functions, including differentiation, proliferation, and survival. Stat5 plays a significant role in the maintenance of immune function and homeostasis, both of which are regulated by IL-2 cytokine signaling (Rani and Murphy, 2015). An early study has shown that Stat5 proteins are activated by granulocyte macrophage-colony stimulating factor (GM-CSF) in early myeloid differentiation (Yamaoka et al., 1998, Lehtonen et al., 2002). GM-CSF is known to play a key role in the maturation and activation of monocyte and macrophage functions, which is important for the production of pro-inflammatory responses in response to infections (Biethahn et al., 1999, Lehtonen et al., 2002). Furthermore, another study by Litherland et al. has showed that the dysfunction of Stat5 leads to the dysregulation of GM-CSF signaling and gene activation in autoimmune macrophages and monocytes (Litherland et al., 2005). As a result, the study suggest that Stat5 and GM-CSF-induced dysfunction in myeloid differentiation and macrophage/monocyte activation has the potential to affect the progress and role of myeloid cells as antigen presenting cells (APC) and their main function in maintaining immune self-tolerance. Increased Stat5 production during D39 and ΔPLY infections likely indicates an activated macrophage state and a step on the pathway towards production of key immune defense cytokines such as IL-2. The C-type lectin receptor Clec4d was up regulated during D39 and ΔPLY infections. It has been shown to function as a pattern recognition receptor (PRR), shaping immune responses by identifying pathogen associated molecular patterns (PAMPs) including peptidoglycan of Gram positive bacteria (Rabinovich et al., 2012). A study by Stiechen et al. has shown that Clec4d has a protective role in resolution of pneumonia caused by *Klebsiella pneumoniae* (Steichen et al., 2013). WT mice infected with a sub-lethal dose of bacteria were able to resolve the infection, whereas Clec4d knockout mice were highly susceptible to infection with a huge

accumulation of neutrophils in lungs when compared to WT mice. Interestingly, this study has revealed that the Clec4d knockout neutrophils did not show any defect during the clearance of bacteria, suggesting that Clec4d has a key role in resolution of inflammation, probably by helping neutrophil turnover in lungs (Steichen et al., 2013). Furthermore, a recent study by Wilson et al. has demonstrated that Clec4d functions as a key molecule in anti-mycobacterial host defense (Wilson et al., 2015), since Clec4d knockout mice infected with *M. tuberculosis* display greater bacterial burdens and increased mortality during infection. The same mice have acute pulmonary inflammation, characterized by increased neutrophil recruitment, and reduced mycobacterial uptake by pulmonary leukocytes when compared to WT mice (Wilson et al., 2015). High levels of production of Clec4d by BMDMs during D39 and ΔPLY infections may suggest that Clec4d is required to control inflammation during pneumococcal infection, perhaps by regulating neutrophil recruitment and turnover. Control of the magnitude of inflammatory responses during pneumococcal infection is crucial to disease resistance (Neill et al., 2010).

My data highlighted key interactions between the proteins that were up regulated by BMDMs during pneumococcal infection. These include Chi3l3, Nos2, Ptgs2, Met, IL-1α, IL-1β, Cxcl2, Cd14, Clec4d, Stat5a and Irak3. My data showed that these proteins were variously co-expressed, involved in catalysis of one another, bound one another, and reacted with and regulated one another. Some of these proteins were not up regulated in the absence of pneumolysin, suggesting involvement in toxin-induced signaling. Interestingly, a cluster of these interacting proteins (including IL-1α, IL-1β, Cd14, IRAK3, Cxcl2) were described previously to be involved in the signaling pathway of Toll-like receptors (TLRs), which is a key mechanism by which the immune system identifies the molecular patterns that are expressed by pathogens

including *S. pneumoniae* (Akira and Takeda, 2004). The activation of TLRs induces the expression of a wide range of proteins that may have key roles in promoting the activation of important immune cells such as macrophages (Akira and Takeda, 2004). It has been shown previously that the activation of TLR-2 on monocytes during mycobacterial infection was required to trigger the activity of IL-1 receptor and IL-1beta (Liu et al., 2009). The induction of IRAK3 protein by tumor cells was also described previously to be mediated by TLR-4 (del Fresno et al., 2005). Furthermore, the production of Cxcl2 by monocytes/macrophages has also shown previously to be correlated with the expression of TLR-4 (Tsujimoto et al., 2005) and Nos2 has also been identified as contributing to the expression of TLR-4 during sepsis (Zhu et al, 2006). A recent study by Vogel et al. has showed that the expression of Met protein is influenced and controlled by TLR-4 (Vogel et al., 2014). Moreover, another study has showed also that Stat5a has an important role in the regulation of TLR-2 expression (Musikacharoen et al., 2001). Another study has revealed that during the stimulation of macrophages with yeast zymosan, Ptgs2 expression was associated with the presence of TLR-2 (Hellmann et al., 2015). These data suggest that many of the up-regulated proteins identified in my study may be related to macrophage recognition of infection by pattern recognition. Macrophages have been considered as an important immune cells due to their involvement in the initiation and resolution of tissue inflammation (Gordon et al., 2002). Also, macrophages have shown the ability to regulate the host inflammatory response to infection through production of antimicrobial molecules, the generation of cytokines and chemokines, and the presentation of microbial antigens on MHC, therefore connecting innate to adaptive immunity (Gordon et al., 2002). Taken together, my data here suggest that during pneumococcal infection BMDMs function as a first line defense against infection by

expressing a wide range of proteins that act at the first stages of induction of immunity to initiate and coordinate responses. The up regulated proteins would likely induce downstream signaling that would stimulate other immune processes including phagocytosis and the recruitment of other immune cells such as neutrophils to sites of infections.

4.15.2 Proteins down regulated during pneumococcal infection

Csf1r was expressed at lower levels in D39 infected BMDM than in uninfected macrophages. This protein plays a key role in the proliferation, regulation of survival and differentiation of monocytes and macrophages (Dai et al., 2002). A recent study by Huynh et al. has revealed that Csf1r was required for the proliferation and regulation of macrophages functions in the large intestine of mice during acute inflammation induced by dextran sulfate sodium (Huynh et al., 2013). This protein has also been shown to promote the production of important cytokines. Another recent study has showed that Csf1r was required for the production of CSF-1 and IL-34 cytokines, which are known to stimulate the development of macrophages and monocytes and promote their responses to infection (Yamane et al., 2014). My data here showed that Csf1r has down regulated by BMDMs during infection with D39, but not ΔPLY, which may point to a role for pneumolysin in prevention of the proliferation or activation of macrophages and inhibition of their functions in response to infection. Anp32b, down regulated during infection with D39, has been reported to function as an inhibitor for caspase-3, and thus act as a negative regulator for apoptosis (Shen et al., 2010). Caspase-3 is required to activate death protease, catalyzing the specific cleavage of numerous cellular proteins (Huttlin et al., 2010).

Apoptosis is a genetic mechanism used by a wide range of immune cells to eliminate infected, or damaged cells, to maintain homeostasis tissue, and to provide an effective immune response (Adams, 2003). The down regulation of Anp32b by BMDMs during D39 infection would reduce the inhibition of caspase 3 and thus stimulate apoptosis. BMDMs may utilize this mechanism to trigger apoptosis of infected cells to aid in containment of infection. Myd88 was also down regulated during D39 infection and is known to be involved in Toll-like receptor and IL-1 receptor signaling pathways during the response of the innate immune system to infection (Burns et al., 1998). MyD88 has previously been described as a marker of macrophage differentiation (Hardiman et al., 1996). A study by Von Bernuth et al. has shown that Myd88 knockout mice infected intranasally with *S. pneumoniae* were more susceptible to infection when compared to WT mice (Von Bernuth et al., 2012). Another study has revealed that MyD88 is important for signaling via all TLRs, except TLR3, and for signaling via the interleukin 1 receptor (IL-1R), which is required for the activation of NF-κB and inflammasome (Kawai and Akira, 2010). To support this, in my previous chapter I have showed that MyD88 is required for the production of IL-1β and activation of the inflammasome. Here, my data suggest that Myd88 is down regulated during D39 infection, which may reflect a pathogen-driven inhibition of host immune responses. A previous study by McNeela et al. demonstrated that pneumolysin is required for the activation of inflammasome and for the production of IL-1 β (McNeela et al., 2010) and so its down regulation here is contrary to expectations.

Pdcl3 is known to modulate the activation of caspases during inflammation and apoptosis. Caspase expression is required for the maturation of lymphocytes (Wilkinson et al., 2004). My data here show that Pdcl3 was down regulated during pneumococcal infections with both D39 and ΔPLY. This phenomenon may suggest

either that the pneumococcus plays a key role in promoting the activation of caspases, to trigger apoptosis of host effector cells or else that it is a host-driven response to infection as an attempt to clear infected cells and prevent the damaging inflammation associated with necrotic cell death.

4.15.3 Proteomic study limitations:

Proteomic is an expensive method to study large scale of proteins, since it biased against certain groups of proteins involving hydrophobic and low abundance proteins only. Furthermore, its labeling processes have some limits, because it lacks the ability to label proteins without lysine, and therefore require particular equipment for visualization, which are very expensive. Analyzing proteomic data is another important issue, since it requires the use of different complicated protein resources to identify the obtained proteins and their molecular and biological functions. Also, the use of proteins classification systems is not easy and may need special training to analysis and understand the obtained data.

4.15.4 Conclusions and Implications

Several previous studies performed by our group have shown that nasopharyngeal colonisation promotes host immunity to pneumococcus, however this is either ineffective in clearance of colonisation or else other immune mechanisms exist that allow or tolerate carriage (Richards et al., 2010, Ferreira et al., 2013, Neill et al.,

2014). My data in this chapter shows that macrophages produce a wide range of proteins in response to infection that may play key roles in defense against *S. pneumoniae*. Here I have suggested that *S. pneumoniae* pneumolysin induces the up regulation/down regulation of several proteins to subvert the responses of macrophages. However, pneumolysin has also been shown to be required for the production of protective pro-inflammatory responses. In particular, PLY triggers the production of IL-1 β and the activation of the inflammasome, which has been described previously to be important for protection against pneumococcal infection (McNeela et al., 2010). Also, I have shown that pneumolysin is required for the production of several significant proteins such as Chi3l3, Ptgs2, Cxcl2 and Nos2 proteins, which have been shown to be required to trigger the activity of key immune cells (such as T-lymphocytes and macrophages) and the production of pro-inflammatory response (such as IL-6 and TNF).

My data in this chapter have provided an overall picture for the reaction of macrophages in response to pneumococcus. Since infected macrophages express a wide range of proteins, my data here suggest that during pneumococcal infection macrophages use several mechanisms to deal with pneumococcus. Some proteins were responsible for the activation of macrophages; leading to induction of the production of important pro-inflammatory cytokines, other proteins had involvement in the apoptosis process, aiding macrophage clearance of infection. However, it is not clear to what extent the pneumolysin-induced changes in protein expression benefit the host and to what extent they benefit the bacteria. More research in this area is required. My data do suggest, however, that pneumolysin drives the production of factors, which will lead to downstream recruitment of T lymphocytes and neutrophils. The macrophage response in the absence of pneumolysin is less geared towards

inflammation and more towards healing and repair. My data support the proposal that pneumolysin could be used as a mucosal adjuvant to produce protective immunity against pneumococcal infections, since it drives the expression of key immune-activating factors. These data, combined with previous reports that pneumolysin functions as a strong activator of both mucosal and systemic immune responses (McNeela et al., 2010) and boosts IgG and IgA antibody titres to pneumococcal protein PsaA (Douce et al., 2010), suggests its inclusion in vaccine formulations would be advantageous.

Chapter 5: Conclusion

5.1 Overall summary

The work presented in this PhD thesis is part of a wider aim to elucidate host-pathogen interactions in pneumococcal infection, with the long-term goal of informing future vaccine design. The pneumococcus is a significant contributor to global morbidity and mortality, and treatment and management of infection comes at a huge financial cost to society. In particular, pneumococcal disease places a great strain on the health care resources of developing and under-developed countries.

Nasopharyngeal colonisation is a vital precursor to localized or systemic pneumococcal infection. The pervasive production of pneumolysin by *S. pneumoniae* and the high degree of conservation of the PLY gene between pneumococcal strains suggest a significant role for the toxin in key phases of the pathogen life cycle

(Kadioglu et al., 2008). Murine models of long-term nasopharyngeal colonisation have revealed that pneumolysin is required for successful colonisation, since PLY deficient strains are cleared from the nasopharynx (Kadioglu et al., 2002) (Richards et al., 2010). However, in addition to its role as a key virulence factor, pneumolysin is also a potent stimulator of host immunity, and has been demonstrated to induce cytokine expression in dendritic cells and macrophages (McNeela et al., 2010. A recent study by Bewely et al. has shown that PLY contributes to macrophage activation and production of cytokines including IL-1β (Bewley et al., 2014).

The data in this PhD thesis highlight the key role of pneumolysin in shaping immune responses during pneumococcal infection, particularly through interactions with macrophages. I have explored the role of MR in anti-pneumococcal immunity in the presence and absence of the pneumococcal toxin pneumolysin, since a previous study by our lab had identified a population of MR-expressing macrophages in the nasopharynx of mice undergoing prolonged pneumococcal carriage (Neill et al., 2014). My work demonstrates that macrophage MR is important for the recognition and phagocytosis of *S. pneumoniae*. Furthermore, I propose that during nasopharyngeal colonisation a state of immune tolerance is generated that is driven, in part, by pneumolysin-MR interactions. Nasal mucosa-associated macrophages are key to inducing this tolerogenic state; T- regulatory cells, IL-10 and TGF-β are key to its long-term regulation. *S. pneumoniae* may use PLY to maintain colonisation in the host nasopharynx through interactions with macrophage MR, encouraging macrophage-induced differentiation of regulatory T cells, leading to production of anti-inflammatory cytokines including IL-10 and TGF-β that are key to long-term maintenance of carriage (Neill et al., 2014). Thus, PLY contributes to the suppression

of pro-inflammatory responses to infection and hence facilitates maintenance of asymptomatic nasopharyngeal carriage.

My data suggest that therapeutic blockade of MR could encourage classical activation of macrophages, the production of pro-inflammatory cytokines and the recruitment of effector immune cells that would clear pneumococcal infection from host nasopharynx. Such a strategy would, however, run the risk of tipping the delicate immune balance in the nasopharynx in favor of damaging inflammation that might facilitate bacterial dissemination. Further work in this area is required to determine whether a therapeutic approach directed towards encouraging classical macrophage activation would be efficacious. My study results also have implications for vaccines based around PLY and its derivatives. The pneumococcal toxin has been proposed as a candidate mucosal adjuvant to generate protective immunity against pneumococcal infections, as it has been shown previously to act as a potent activator of both mucosal and systemic immune responses (McNeela et al., 2010), and to boost IgA and IgG antibodies titres to pneumococcal protein PsaA (Douce et al., 2010). However, these observed phenomena rely on the cytolytic activity of toxin. Therefore, I suggest that PLY could be used in a formulation with carbohydrate antigens (such as Mannan) that could block MR and thus promote classical activation and the induction of Th1 and Th17 responses and associated production of protective IL-17 and IFN-γ, which are known to stimulate immunity against pneumococcal infections (McNeela et al., 2010). However, my proteomic data highlights the complexity of pneumolysin-induced signaling in immune cells. The activation of several pro-inflammatory and apoptotic pathways in macrophages appeared to be partially PLY-dependent, suggesting that any inclusion of PLY or its derivatives as vaccine adjuvants would require careful formulation.

My proteomic data also demonstrate that macrophages use several mechanisms to deal with pneumococcal infection. Some upregulated proteins were associated with the activation of macrophages and the production of key pro-inflammatory cytokines, whilst other proteins form part of apoptotic pathways and may reflect a 'sacrificial' approach used by macrophages to clear infection. It is possible, however, to peer through the complexity of pneumococcal-induced protein expression changes in macrophages and see some key elements of host defense emerging. Several of the up-regulated proteins, such as Chi3l3, would likely yield increased macrophage responsiveness to infection and lead to downstream recruitment of T lymphocytes or neutrophils. Furthermore, there is evidence of macrophages responding to pneumolysin-induced cell damage through the up regulation of proteins associated with the wound healing response, including Ptgs2. In the absence of pneumolysin, macrophages showed a reduction in the induction of several key immune defense proteins, including IRAK3 and Met, and the overall picture was of a macrophage less geared towards inflammation and more towards healing and repair. Interestingly, a number of proteins up regulated in the presence of pneumolysin form part of the signaling pathway of Toll-like receptors, supporting previous observations that pneumolysin is important in the process of immune cell pattern recognition during pneumococcal infection (McNeela et al, 2012). My data support the notion that pneumolysin is an important trigger of key pro-inflammatory cytokines such as IL-1β and that it aids the activation of NF-κB pathways that are required for the activation of the inflammasome.

Taken together, my studies highlight a dichotomy in pneumolysin-induced host responses. On the one hand, PLY triggers the production of pro-inflammatory cytokines and the induction of proteins that have key roles in the activation of

immune defense against pneumococcal infections. The cytolytic activity of PLY is often key to the initiation of these immune actions. On the other hand, however, the binding of PLY to MR seems to be linked with the alternative activation of macrophages, leading to reduction in the production of pro-inflammatory mediators and increased differentiation of T regulatory cells. Again, the cytolytic domain of PLY seems to facilitate binding to MR, since PdB showed reduced levels of binding compared to PLY.

The dual role of pneumolysin is matched by a dual role for MR-expressing macrophages during pneumococcal carriage. In early infection macrophages play a direct antimicrobial role through the phagocytosis of pneumococci via a process that is partially MR-dependent. They also recruit effector cells such as neutrophils via the production of MR-dependent chemokines and cytokines. Subsequently, during long-term pneumococcal carriage, activated macrophages may drain to cervical lymph nodes to trigger immune responses that act to inhibit host tissue damage and systemic pneumococcal dissemination. This involves the stimulation of T cell differentiation into regulatory T cells, leading to production of large amounts of IL-10 and TGF-β. These anti-inflammatory cytokines will recruit more regulatory T cells into the nasopharynx, leading to maintenance of stable pneumococcal carriage and prevention of damage to host tissues. MR expression appears to be key to the ability of macrophages to induce T regulatory cell differentiation following exposure to pneumococci. However, stimulation of Th1 and Th17 cell differentiation by MR-expressing macrophages was also observed and this may contribute to control of bacterial proliferation in the nasopharynx. I verify here that both the pneumococcal

toxin pneumolysin and macrophage MR have important roles in encouraging macrophage induction of T cell differentiation.

In conclusion, my data highlight the complexity of host-pathogen interactions, especially when the pathogen concerned is also a some-time commensal. A 'one size fits all' approach to immune defense is not appropriate when the need to control bacterial proliferation must be balanced with the need to maintain tolerance and homeostasis in the microbe-rich niche of the nasopharynx. Consequently, the interaction between pneumococcus and macrophage is not as simple as an attempt by one to kill the other; rather it reveals the delicate balance that exists in the nasopharynx, where bacterial colonisation is controlled by resident macrophages but is not, on the whole, met with a robust attempt at clearance. This balance is clear in the ability of macrophages to phagocytose and kill pneumococci, however at the same time to stimulate the differentiation of T regulatory cells whose actions maintain bacterial carriage. The same balance can be observed in the induction of both pro- and anti-inflammatory host signaling by pneumolysin.

5.2 Future prospects

My work raises some key unanswered questions and paves the way for future research. The observation that PLY binds to MR is novel but requires further characterization. I have demonstrated that domain 4 is instrumental in this binding activity, but the precise binding site has not been characterized. However, the weak binding observed with PdB suggested that the substitution of tryptophan for phenylalanine in the cytolytic domain disrupts the binding site. Exploring the binding

of PLY to MR and understanding the mechanism that PLY uses to trigger the alternative activation of macrophages would aid production of PLY derivatives for use in future vaccine development.

Despite the apparent importance of MR for macrophage responses to pneumococci *in vitro*, $MR^{-/-}$ mice displayed only a transiently increased susceptibility to pneumococcal carriage. Elucidating which pathways compensate for the MR-deficit *in vivo* would aid our understanding of redundancy within the immune system. Furthermore, performing carriage experiments using WT and $MR^{-/-}$ mice with different pneumococcal serotypes and with clinical isolates may expose new phenotypes. My data reveal the importance of MR during pneumococcal carriage, where its ability to bind PLY leads to induction of host signaling and cytokine production. It would be of interest to examine the role of MR during acute infection (pneumococcal pneumonia, sepsis or meningitis), as macrophages resident in different tissues often display very different phenotypic characteristics. So, performing pneumonia, sepsis and meningitis experiments using WT and $MR^{-/-}$ mice with different serotype of *S. pneumoniae* may further aid elucidation of the role of macrophages MR during pneumococcal infections.

Although my proteomic data has demonstrated the ability of macrophages to produce a wide range of proteins in response to pneumococcal infection, the exact role of the implicated signaling pathways is not clear and requires further investigation. Ablation of expression of implicated genes, such as Nos2 and Ptgs2, in macrophages may help reveal their functions during carriage and disease.

The cytolytic activity of PLY is required for MR binding, for the generation of immune tolerance and for the induction of cytokine production during pneumococcal

infection. Interestingly however, some serotype 1 isolates produce a non-haemolytic pneumolysin but are still capable of inducing invasive diseases and have been associated in particular with empyema (Jefferies et al., 2007). Examining the interaction between MR and serotype 1 that produce non-haemolytic PLY using invasive dose of bacteria with WT and $MR^{-/-}$ mice could be beneficial and may shown other important roles for MR during pneumococcal infections. Also, exploring the contribution of the pneumococcal capsule or other pneumococcal virulence factors may shed further light on complex disease processes.

MR has previously been described as a marker for the alternative activation of macrophages, however my data highlight a broader role for this receptor in macrophage activation and in directing responses to pneumococcal infection. PLY has long been known to be a potent trigger of innate immunity and my data reveal that PLY contributes to the activation of macrophages and to interactions with MR. These observations indicate that there is a strong justification for the inclusion of PLY or its derivatives in future pneumococcal vaccines and that the induction of MR signaling pathways by vaccine components should be considered.

Appendices

Appendix 1

List of proteins showing fold changes and statistically significant differences in expression of BMDM proteins between untreated (Unt) cells and D39.

Protein ID	Protein Name	Fold-Change	P-Value	Highest mean condition	Lowest mean condition
Q9DBG3	AP2B1_MOUSE	2.642094804	0.000115212	Unt	D39
P57776	EF1D_MOUSE	3.830880606	0.000172432	Unt	D39
Q91VC3	IF4A3_MOUSE	2.099253882	0.000276994	Unt	D39
Q9WV80	SNX1_MOUSE	2.444609948	0.000620401	Unt	D39
P08905	LYZ2_MOUSE	1.522645975	0.000752341	Unt	D39

P54987	IRG1_MOUSE	7.179098567	0.000778451	D39	Unt
P84078	ARF1_MOUSE	1.921879929	0.001737475	Unt	D39
P61161	ARP2_MOUSE	2.624891073	0.001767278	Unt	D39
P08113	ENPL_MOUSE	1.894164913	0.00183959	D39	Unt
O08808	DIAP1_MOUSE	4.876600063	0.001986346	Unt	D39
P60766	CDC42_MOUSE	3.062520569	0.002185952	Unt	D39
P20029	GRP78_MOUSE	1.601034316	0.002239283	D39	Unt
P62334	PRS10_MOUSE	2.845010272	0.002278164	Unt	D39
P10605	CATB_MOUSE	3.050879484	0.002519153	Unt	D39
Q60854	SPB6_MOUSE	2.947031808	0.002778146	Unt	D39
Q11136	PEPD_MOUSE	2.550697703	0.003314946	Unt	D39
P67778	PHB_MOUSE	1.658629662	0.003793509	D39	Unt
Q8BFR5	EFTU_MOUSE	1.33201796	0.003911828	D39	Unt
P62204	CALM_MOUSE	1.576142894	0.004281379	Unt	D39
P27659	RL3_MOUSE	2.673556373	0.004673541	Unt	D39
P63001	RAC1_MOUSE	2.818479063	0.004782323	Unt	D39
P57759	ERP29_MOUSE	1.761878777	0.005749513	D39	Unt
Q8BK67	RCC2_MOUSE	30.65803068	0.006198819	Unt	D39
P70670	NACAM_MOUSE	2.746584767	0.006493338	Unt	D39
Q8BGQ7	SYAC_MOUSE	2.219722324	0.006736873	Unt	D39
P99026	PSB4_MOUSE	2.126885269	0.007075396	Unt	D39
Q61937	NPM_MOUSE	1.937943071	0.007282286	Unt	D39

P46664	PURA2_MOUSE	6.303266999	0.00749462	Unt	D39
Q8K297	GT251_MOUSE	1.462538981	0.007562828	D39	Unt
Q8BL97	SRSF7_MOUSE	4.705153817	0.007585064	Unt	D39
Q05144	RAC2_MOUSE	2.196653035	0.007664766	Unt	D39
P17918	PCNA_MOUSE	2.969341186	0.007700279	Unt	D39
Q9DBP5	KCY_MOUSE	2.887145768	0.008368744	Unt	D39
P24527	LKHA4_MOUSE	2.230816487	0.009274534	Unt	D39
P50427	STS_MOUSE	4.794686763	0.009421782	D39	Unt
Q05769	PGH2_MOUSE	8.607296457	0.009896371	D39	Unt
Q8BMS1	ECHA_MOUSE	2.118807241	0.009924724	D39	Unt
P29788	VTNC_MOUSE	4.949903934	0.009977579	D39	Unt
Q9D0E1	HNRPM_MOUSE	1.870445656	0.010146052	D39	Unt
P63276	RS17_MOUSE	3.676879834	0.010526276	Unt	D39
Q8R1F1	NIBL1_MOUSE	2.645122762	0.010756155	D39	Unt
P80317	TCPZ_MOUSE	1.453195545	0.01082077	Unt	D39
Q9D8U8	SNX5_MOUSE	2.644468347	0.011729807	Unt	D39
P05063	ALDOC_MOUSE	3.796021795	0.011905855	Unt	D39
P06797	CATL1_MOUSE	2.648530744	0.012002412	Unt	D39
P21981	TGM2_MOUSE	1.902548378	0.012129597	Unt	D39
Q9QUM9	PSA6_MOUSE	2.121096208	0.012334511	Unt	D39
Q3THS6	METK2_MOUSE	4.545943534	0.012350299	Unt	D39
Q9R1P3	PSB2_MOUSE	3.512178734	0.012478139	Unt	D39

Q8K124	PKHO2_MOUSE	2.382353243	0.013311689	Unt	D39
O70251	EF1B_MOUSE	1.984973589	0.013585568	Unt	D39
P97315	CSRP1_MOUSE	3.499073276	0.01366934	Unt	D39
Q9D1A2	CNDP2_MOUSE	1.726085861	0.014025886	Unt	D39
Q64337	SQSTM_MOUSE	5.42823774	0.014497411	D39	Unt
P60843	IF4A1_MOUSE	1.70517327	0.014498899	Unt	D39
Q8R1F1	NIBL1_MOUSE	2.431231881	0.01532357	Unt	D39
Q9D8Y0	EFHD2_MOUSE	3.997444422	0.015462736	Unt	D39
P98078	DAB2_MOUSE	10.61956686	0.015543638	Unt	D39
Q60932	VDAC1_MOUSE	1.426413714	0.01555357	D39	Unt
P63276	ACADS_MOUSE	7.538040735	0.015578573	Unt	D39
Q60668	HNRPD_MOUSE	2.268038403	0.01570923	Unt	D39
Q8VDJ3	VIGLN_MOUSE	8.12976964	0.015884722	Unt	D39
Q9EQH3	VPS35_MOUSE	2.491817843	0.015943459	Unt	D39
Q9DB77	QCR2_MOUSE	1.493454965	0.01605293	D39	Unt
P63242	IF5A1_MOUSE	2.33513008	0.01608664	Unt	D39
Q07417	ACADS_MOUSE	1.431227647	0.016419739	D39	Unt
Q9D662	SC23B_MOUSE	2.052219054	0.016460305	Unt	D39
Q9R1P0	PSA4_MOUSE	7.352451036	0.01663564	Unt	D39
P05555	ITAM_MOUSE	1.231636183	0.016728992	D39	Unt
Q9R0Q3	TMED2_MOUSE	2.722912842	0.016746322	D39	Unt
P08003	PDIA4_MOUSE	1.524511856	0.017599413	D39	Unt

P51569	AGAL_MOUSE	2.323925998	0.017682056	Unt	D39
O08807	PRDX4_MOUSE	1.555577253	0.018022643	D39	Unt
P28352	APEX1_MOUSE	1.970487615	0.018147219	Unt	D39
P39054	DYN2_MOUSE	1.838192772	0.018162229	Unt	D39
Q8BKC5	IPO5_MOUSE	2.28646132	0.018173817	Unt	D39
O35841	API5_MOUSE	1.689981915	0.018363087	D39	Unt
Q6P069	SORCN_MOUSE	4.880076693	0.018505487	Unt	D39
Q9R0Q7	TEBP_MOUSE	2.498295539	0.018533458	Unt	D39
Q6ZWN5	RS9_MOUSE	2.557465453	0.01884961	Unt	D39
P57780	ACTN4_MOUSE	3.238182515	0.018881343	Unt	D39
P80314	TCPB_MOUSE	2.316149378	0.019046373	Unt	D39
Q91YT0	NDUV1_MOUSE	2.148716125	0.020075349	D39	Unt
P35441	TSP1_MOUSE	1.803642887	0.020230756	D39	Unt
O08553	DPYL2_MOUSE	3.165592997	0.02026667	Unt	D39
O55131	SEPT7_MOUSE	2.828916586	0.020419327	Unt	D39
Q9EQK5	MVP_MOUSE	1.255000804	0.020816426	D39	Unt
P70372	ELAV1_MOUSE	2.821721727	0.020898504	Unt	D39
Q91V12	BACH_MOUSE	5.512172254	0.021312701	Unt	D39
P17751	TPIS_MOUSE	3.610980239	0.021801044	Unt	D39
P05201	AATC_MOUSE	2.937011655	0.0219596	Unt	D39
P29699	FETUA_MOUSE	4.163350326	0.022169526	D39	Unt
Q6PDM2	SRSF1_MOUSE	3.754354879	0.022219539	Unt	D39

P97807	FUMH_MOUSE	2.020270258	0.022483025	D39	Unt
Q91VW3	SH3L3_MOUSE	5.072936473	0.022613001	Unt	D39
Q9CY64	BIEA_MOUSE	3.23148992	0.022834834	Unt	D39
Q8K2Q7	BROX_MOUSE	6.151405839	0.02289493	Unt	D39
P51174	ACADL_MOUSE	1.639310022	0.022950091	D39	Unt
P68040	GBLP_MOUSE	3.274323429	0.023208512	Unt	D39
Q6P1B1	XPP1_MOUSE	2.033293357	0.023231434	Unt	D39
Q9JJU8	SH3L1_MOUSE	4.864985195	0.02369873	Unt	D39
P20060	HEXB_MOUSE	1.382079497	0.024442854	Unt	D39
Q9D2G2	ODO2_MOUSE	2.420475253	0.02485812	D39	Unt
Q9Z1Z2	STRAP_MOUSE	8.064938086	0.025195607	Unt	D39
Q922R8	PDIA6_MOUSE	1.801758848	0.025491396	D39	Unt
P26638	SYSC_MOUSE	3.412251234	0.025956644	Unt	D39
Q9CQQ7	AT5F1_MOUSE	2.075787207	0.026446349	D39	Unt
Q9D0K2	SCOT1_MOUSE	1.992360989	0.026560989	D39	Unt
P26443	DHE3_MOUSE	1.736682113	0.02667564	D39	Unt
Q99KV1	DJB11_MOUSE	1.743206723	0.026764301	D39	Unt
Q9CPU0	LGUL_MOUSE	6.461886858	0.026866289	Unt	D39
O70492	SNX3_MOUSE	1.504987277	0.026866483	Unt	D39
P47738	ALDH2_MOUSE	1.719026789	0.027107058	D39	Unt
P62827	RAN_MOUSE	3.153217673	0.027119196	Unt	D39
Q9JIY5	HTRA2_MOUSE	1.473621924	0.02715153	Unt	D39

P35278	RAB5C_MOUSE	1.784884073	0.027625381	Unt	D39
P50516	VATA_MOUSE	1.54471257	0.02779199	D39	Unt
P09671	SODM_MOUSE	2.461180271	0.027884137	D39	Unt
P60335	PCBP1_MOUSE	1.6337891	0.027935143	Unt	D39
P19973	LSP1_MOUSE	3.289875291	0.027949902	Unt	D39
Q8JZV7	NAGA_MOUSE	5.691986805	0.028467251	Unt	D39
Q60597	ODO1_MOUSE	2.519215726	0.028685462	D39	Unt
Q5SSL4	ABR_MOUSE	3.983768834	0.028800419	Unt	D39
P09405	NUCL_MOUSE	2.67107106	0.028850245	Unt	D39
Q03265	ATPA_MOUSE	2.018756482	0.028973696	D39	Unt
Q3TW96	UAP1L_MOUSE	2.241007807	0.02903345	Unt	D39
Q99KK7	DPP3_MOUSE	2.32305289	0.029573875	Unt	D39
P46638	RB11B_MOUSE	3.316836292	0.029668602	Unt	D39
P14211	CALR_MOUSE	2.019503093	0.029673316	D39	Unt
Q9CQ60	6PGL_MOUSE	4.731329217	0.029950752	Unt	D39
P11835	ITB2_MOUSE	1.304994613	0.030107991	D39	Unt
P28474	ADHX_MOUSE	2.016834839	0.03019735	Unt	D39
Q9R1T2	SAE1_MOUSE	5.168798715	0.030271953	Unt	D39
P62331	ARF6_MOUSE	3.58355747	0.031172405	Unt	D39
O88456	CPNS1_MOUSE	1.828285884	0.031459787	Unt	D39
P14869	RLA0_MOUSE	3.201691579	0.031548301	Unt	D39
O09131	GSTO1_MOUSE	3.714377156	0.031800978	Unt	D39

P56399	UBP5_MOUSE	1.918789615	0.032125423	Unt	D39
P37040	NCPR_MOUSE	2.347487165	0.03228013	D39	Unt
P47757	CAPZB_MOUSE	3.004612868	0.032303897	Unt	D39
Q8BWT1	THIM_MOUSE	1.552746628	0.032562171	D39	Unt
Q00519	XDH_MOUSE	1.788364115	0.033019745	Unt	D39
Q8BHN3	GANAB_MOUSE	1.49620051	0.033101258	D39	Unt
Q9WUA3	K6PP_MOUSE	1.884056903	0.033361732	Unt	D39
O08539	BIN1_MOUSE	3.519075672	0.033866834	Unt	D39
P28656	NP1L1_MOUSE	3.470203602	0.033904363	Unt	D39
Q8K157	GALM_MOUSE	5.018381816	0.033981346	Unt	D39
Q7TQI3	OTUB1_MOUSE	2.55482455	0.034199928	Unt	D39
P68181	KAPCB_MOUSE	2.198801891	0.03424006	Unt	D39
P12970	RL7A_MOUSE	1.282329065	0.034429882	Unt	D39
P61750	ARF4_MOUSE	1.512871418	0.034724877	Unt	D39
Q78PY7	SND1_MOUSE	1.881399008	0.035174232	Unt	D39
P27773	PDIA3_MOUSE	2.01463333	0.035314008	D39	Unt
P48678	LMNA_MOUSE	1.367576811	0.035353149	Unt	D39
P54071	IDHP_MOUSE	1.552802848	0.035802048	D39	Unt
Q9EST5	AN32B_MOUSE	2.535361691	0.036108395	Unt	D39
P63038	CH60_MOUSE	1.949366729	0.036209871	D39	Unt
Q64152	BTF3_MOUSE	3.020205004	0.03626428	Unt	D39
P56480	ATPB_MOUSE	2.293072353	0.036306369	D39	Unt

Q99LP6	GRPE1_MOUSE	2.754269379	0.036402523	D39	Unt
P19783	COX41_MOUSE	2.437314609	0.03645526	D39	Unt
P70195	PSB7_MOUSE	2.770721871	0.036498119	Unt	D39
O09061	PSB1_MOUSE	2.824434857	0.037355456	Unt	D39
Q9Z0N1	IF2G_MOUSE	3.314348198	0.037414865	Unt	D39
P25444	RS2_MOUSE	2.343217194	0.037541084	Unt	D39
Q6WVG3	KCD12_MOUSE	2.543648214	0.03766201	Unt	D39
P68510	1433F_MOUSE	4.801240245	0.037731403	Unt	D39
Q8BMF4	ODP2_MOUSE	2.149403312	0.0378437	D39	Unt
Q9JHR7	IDE_MOUSE	2.547923571	0.037898051	Unt	D39
Q9DCH4	EIF3F_MOUSE	3.14650385	0.038057387	Unt	D39
O88844	IDHC_MOUSE	3.024283887	0.038287514	Unt	D39
P70296	PEBP1_MOUSE	4.031652328	0.038563626	Unt	D39
O54984	ASNA_MOUSE	2.296059918	0.038637302	Unt	D39
P97379	G3BP2_MOUSE	2.354372109	0.038912466	Unt	D39
Q3U0V1	FUBP2_MOUSE	3.152835456	0.039204583	Unt	D39
Q9ER72	SYCC_MOUSE	2.35614808	0.039210118	Unt	D39
Q00623	APOA1_MOUSE	3.212736695	0.039360214	D39	Unt
P42125	ECI1_MOUSE	1.499813332	0.039522051	D39	Unt
P16858	G3P_MOUSE	3.127622464	0.039573124	Unt	D39
O89023	TPP1_MOUSE	3.199549856	0.039622313	Unt	D39
P18242	CATD_MOUSE	1.509222434	0.039951335	Unt	D39

P50544	ACADV_MOUSE	2.946350579	0.040046807	D39	Unt
Q9Z1G3	VATC1_MOUSE	1.47861264	0.04029386	Unt	D39
Q9CQF9	PCYOX_MOUSE	2.180315167	0.040882388	D39	Unt
Q8R180	ERO1A_MOUSE	3.312927093	0.041050063	D39	Unt
P62918	RL8_MOUSE	3.954950365	0.041302551	Unt	D39
Q9DBG5	PLIN3_MOUSE	6.829569208	0.0414462	Unt	D39
Q05816	FABP5_MOUSE	3.966757688	0.041804534	Unt	D39
P97855	G3BP1_MOUSE	3.78813305	0.041936646	Unt	D39
P04117	FABP4_MOUSE	3.241224706	0.042289541	Unt	D39
P62259	1433E_MOUSE	4.035116962	0.042701011	Unt	D39
P97351	RS3A_MOUSE	5.472464539	0.043043129	Unt	D39
P62192	PRS4_MOUSE	1.66422613	0.043205063	Unt	D39
Q9D819	IPYR_MOUSE	3.800389898	0.043238725	Unt	D39
P97311	MCM6_MOUSE	3.363087727	0.04334755	Unt	D39
Q9CY58	PAIRB_MOUSE	7.477494732	0.043545079	Unt	D39
Q62422	OSTF1_MOUSE	2.780114377	0.043661059	Unt	D39
P62082	RS7_MOUSE	3.65289741	0.043902306	Unt	D39
Q00612	G6PD1_MOUSE	2.568667346	0.044238943	Unt	D39
O35855	BCAT2_MOUSE	1.32813538	0.044589075	D39	Unt
P10107	ANXA1_MOUSE	1.817930199	0.044693039	Unt	D39
Q9CZX8	RS19_MOUSE	17.56962393	0.044797288	Unt	D39
Q9CQ65	MTAP_MOUSE	4.419225333	0.044868494	Unt	D39

Q3U1J4	DDB1_MOUSE	4.284537499	0.044881743	Unt	D39
Q9WTP6	KAD2_MOUSE	2.037429119	0.045131097	Unt	D39
P32261	ANT3_MOUSE	3.668509429	0.045805053	D39	Unt
Q9CRB9	CHCH3_MOUSE	1.456131367	0.045842813	D39	Unt
P53810	PIPNA_MOUSE	3.798995926	0.045844414	Unt	D39
Q9D6J6	NDUV2_MOUSE	1.34943135	0.045869735	D39	Unt
P10639	THIO_MOUSE	2.635787531	0.045896606	Unt	D39
P45952	ACADM_MOUSE	1.530815955	0.046245112	D39	Unt
Q9EPL9	ACOX3_MOUSE	1.168656484	0.046334759	Unt	D39
Q63844	MK03_MOUSE	3.34063601	0.046342643	Unt	D39
A6X935	ITIH4_MOUSE	4.842399889	0.046478132	D39	Unt
P12787	COX5A_MOUSE	2.776875402	0.046532398	D39	Unt
Q8BFZ9	ERLN2_MOUSE	1.748801511	0.046590403	D39	Unt
Q7TMB8	CYFP1_MOUSE	5.105871495	0.047192717	Unt	D39
P68433	H31_MOUSE	1.584871069	0.047265942	D39	Unt
Q3UIA2	RHG17_MOUSE	1.730287729	0.047358708	Unt	D39
Q9JKR6	HYOU1_MOUSE	1.8697835	0.048007162	D39	Unt
Q9CZ13	QCR1_MOUSE	1.809981316	0.048047596	D39	Unt
P14206	RSSA_MOUSE	3.341281239	0.048155008	Unt	D39
Q91VI7	RINI_MOUSE	1.465771927	0.048208237	Unt	D39
O88544	CSN4_MOUSE	3.808386202	0.048486605	Unt	D39
Q3TRM8	HXK3_MOUSE	2.024578888	0.048707328	Unt	D39

Q9Z204	HNRPC_MOUSE	2.842328417	0.048738434	Unt	D39
Q61598	GDIB_MOUSE	3.282097131	0.04888907	Unt	D39
O70133	DHX9_MOUSE	1.912208405	0.049224289	D39	Unt
Q9QWR8	NAGAB_MOUSE	2.723477572	0.049342799	Unt	D39
Q9WV32	ARC1B_MOUSE	3.589822372	0.049361683	Unt	D39
P28063	PSB8_MOUSE	3.305475796	0.049502182	Unt	D39
P17742	PPIA_MOUSE	4.906637115	0.049504662	Unt	D39
P06745	G6PI_MOUSE	3.442409492	0.049837268	Unt	D39

Appendix 2

List of proteins showing fold changes and statistically significant differences in expression of BMDM proteins between D39 and ΔPLY.

Protein ID	Protein Name	Fold-Change	P Value	Highest mean condition	Lowest mean condition
Q9JHU4	DYHC1_MOUSE	1.385064657	0.001661589	ΔPLY	D39
P12970	RL7A_MOUSE	2.234302212	0.001704381	ΔPLY	D39
P20108	PRDX3_MOUSE	1.58224077	0.0017172	ΔPLY	D39
P48036	ANXA5_MOUSE	1.28588439	0.002342855	D39	ΔPLY
O70492	SNX3_MOUSE	1.579150389	0.002850724	ΔPLY	D39
Q61878	PRG2_MOUSE	3.034641004	0.003621433	ΔPLY	D39
O08585	CLCA_MOUSE	1.613394599	0.004924546	ΔPLY	D39
O35744	CH3L3_MOUSE	1.364646549	0.00548276	ΔPLY	D39

P97370	AT1B3_MOUSE	1.054808158	0.005721293	D39	ΔPLY
Q9QUR6	PPCE_MOUSE	5.168609419	0.006353662	D39	ΔPLY
P56395	CYB5_MOUSE	1.537017405	0.006645976	ΔPLY	D39
P32261	ANT3_MOUSE	1.721146325	0.006923802	D39	ΔPLY
P13020	GELS_MOUSE	1.890977297	0.007786837	D39	ΔPLY
P20029	GRP78_MOUSE	1.345579989	0.00832135	ΔPLY	D39
A1L314	MPEG1_MOUSE	2.617543431	0.008686058	ΔPLY	D39
P24527	LKHA4_MOUSE	2.028573122	0.009167517	D39	ΔPLY
Q9DB20	ATPO_MOUSE	1.493838613	0.011143863	ΔPLY	D39
P56399	UBP5_MOUSE	2.298121001	0.012025566	ΔPLY	D39
P51150	RAB7A_MOUSE	1.586118376	0.01220897	ΔPLY	D39
Q62186	SSRD_MOUSE	1.277493982	0.012750592	ΔPLY	D39
Q60865	CAPR1_MOUSE	1.585991359	0.012864772	ΔPLY	D39
Q9WV80	SNX1_MOUSE	1.108313969	0.013116334	D39	ΔPLY
Q91VC3	IF4A3_MOUSE	1.177236218	0.013579942	ΔPLY	D39
P06800	PTPRC_MOUSE	1.823204021	0.013882324	ΔPLY	D39
Q99KV1	DJB11_MOUSE	1.563169748	0.014842375	ΔPLY	D39
Q9D7X3	DUS3_MOUSE	1.849182047	0.015327191	ΔPLY	D39
O70251	TRFL_HUMAN	1.555634515	0.015612869	D39	ΔPLY
P68433	H31_MOUSE	1.869215601	0.017125267	ΔPLY	D39
Q8R081	HNRPL_MOUSE	1.340350662	0.017305884	D39	ΔPLY
P05063	ALDOC_MOUSE	2.681917387	0.018957952	D39	ΔPLY

P51174	ACADL_MOUSE	1.307318935	0.019490872	ΔPLY	D39
P68181	KAPCB_MOUSE	2.80386122	0.020674311	D39	ΔPLY
Q3TCN2	PLBL2_MOUSE	1.142010233	0.020945676	ΔPLY	D39
Q8BK67	RCC2_MOUSE	4.636331775	0.022368437	ΔPLY	D39
P28798	GRN_MOUSE	1.537182639	0.022661515	ΔPLY	D39
Q6GQT1	A2MP_MOUSE	1.925724344	0.02271057	D39	ΔPLY
Q60931	VDAC3_MOUSE	1.258249325	0.024180801	ΔPLY	D39
P17225	PTBP1_MOUSE	1.217778025	0.024528721	D39	ΔPLY
O08529	CAN2_MOUSE	1.349956566	0.024998362	ΔPLY	D39
P62204	CALM_MOUSE	2.314139439	0.025116719	D39	ΔPLY
Q91YQ5	RPN1_MOUSE	1.628166023	0.025807444	ΔPLY	D39
Q9JHI5	IVD_MOUSE	2.062611836	0.026707272	ΔPLY	D39
Q9D0E1	HNRPM_MOUSE	1.257388349	0.028044564	ΔPLY	D39
Q9Z0K8	VNN1_MOUSE	1.65279028	0.028972344	D39	ΔPLY
P26443	DHE3_MOUSE	1.227011793	0.029134145	ΔPLY	D39
Q921M7	FA49B_MOUSE	1.650489907	0.031734081	D39	ΔPLY
P08113	ENPL_MOUSE	1.321342115	0.03195283	ΔPLY	D39
P70441	NHRF1_MOUSE	3.923073511	0.032552962	D39	ΔPLY
Q9JHJ0	TMOD3_MOUSE	1.30625782	0.033088606	D39	ΔPLY
Q9D0F9	PGM1_MOUSE	1.419436304	0.033954173	ΔPLY	D39
P68037	UB2L3_MOUSE	2.832193126	0.034192126	D39	ΔPLY
Q9WTP6	EFTU_MOUSE	2.199033709	0.03493641	D39	ΔPLY

P57759	ERP29_MOUSE	1.379524965	0.035236405	ΔPLY	D39
P63328	PP2BA_MOUSE	1.769081599	0.035439692	ΔPLY	D39
O70251	ODPA_MOUSE	1.572032327	0.035586525	D39	ΔPLY
Q8BWT1	THIM_MOUSE	1.410962611	0.036097463	ΔPLY	D39
Q07417	ACADS_MOUSE	1.320823073	0.036361943	ΔPLY	D39
Q91W90	TXND5_MOUSE	1.166905832	0.036789294	ΔPLY	D39
P97315	CSRP1_MOUSE	2.313454053	0.038402542	D39	ΔPLY
Q9DCX2	ATP5H_MOUSE	2.134074444	0.039190222	ΔPLY	D39
P35486	ODPA_MOUSE	1.498517037	0.040244057	ΔPLY	D39
P61161	ARP2_MOUSE	1.446831412	0.040620191	D39	ΔPLY
Q9EST5	AN32B_MOUSE	1.410663597	0.04190145	ΔPLY	D39
Q8BFR5	EFTU_MOUSE	1.44837975	0.041974635	ΔPLY	D39
Q9Z0J0	NPC2_MOUSE	1.549722133	0.042015485	ΔPLY	D39
P10605	CATB_MOUSE	1.099956596	0.042201885	ΔPLY	D39
P29391	FRIL1_MOUSE	1.792616783	0.042623639	ΔPLY	D39
P42125	ECI1_MOUSE	1.353475549	0.043136793	ΔPLY	D39
P63037	DNJA1_MOUSE	1.853756014	0.043655208	ΔPLY	D39
O70251	EF1B_MOUSE	1.672783555	0.043701083	D39	ΔPLY
Q9DCW4	ETFB_MOUSE	1.4144385	0.043715786	ΔPLY	D39
P35278	RAB5C_MOUSE	1.488965019	0.044043877	ΔPLY	D39
Q02105	C1QC_MOUSE	1.608389067	0.044199533	ΔPLY	D39
P51863	VA0D1_MOUSE	1.363642882	0.044302246	ΔPLY	D39

P29699	FETUA_MOUSE	1.695610269	0.044990934	D39	ΔPLY
O08795	GLU2B_MOUSE	1.739987156	0.045853753	ΔPLY	D39
P28063	PSB8_MOUSE	1.780353668	0.046235856	D39	ΔPLY
Q9QUM9	PSA6_MOUSE	1.531831846	0.046589335	D39	ΔPLY
P19157	GSTP1_MOUSE	1.96747817	0.048204918	D39	ΔPLY
Q3V3R1	C1TM_MOUSE	1.436876232	0.048655796	ΔPLY	D39
Q9WTP6	KAD2_MOUSE	1.914690505	0.048723097	ΔPLY	D39

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