Characterisation of Treg and Th17 Cells in Nasopharynx-Associated Lymphoid Tissue and their Association with Pneumococcal Carriage in Children and Adults

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By

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Abstract

Streptococcus pneumoniae (pneumococcus) is a gram-positive bacterium that can cause significant morbidity and mortality in humans especially in children and elderly. T regulatory cells (Treg) have an important role in modulation of immune responses to microbial infection. Although Th17 cells are involved in autoimmune diseases, these cells may play a protective role against pathogens. In this PhD project, Th17 and Treg cells in nasal-associated lymphoid tissue were characterised and their relationship with nasopharyngeal carriage of pneumococcus studied in children and adults.

Frequencies of Th17 and Treg in tonsillar tissue and peripheral blood samples obtained from children and adults were analysed for intracellular expression of IL17A and Foxp3 by flow cytometry. Also, tonsillar MNC and PBMC were stimulated by pneumococcal culture supernatant (CCS) derived from wild type stain D39. The ratio of Th17/Treg cells in NALT was studied in both children and adults together with their association with pneumococcal carriage.

Numbers of Th17 and Treg cells in in tonsillar tissues were shown to be significantly higher than in peripheral blood in both children and adults. The ratio of tonsillar Th17/Treg was shown to increase with age and tended to be higher in pneumococcal culture-negative children than in culture-positive. It is suggested that the balance of Th17/Treg is a crucial determinant of pneumococcal clearance or persistence/carriage in human nasopharynx. A significant increase in numbers of Th17 and Treg cells were shown following pneumococcal CCS stimulation. CCS derived from isogenic mutant strains (i.e., Ply- and CbpA-) elicited lower numbers of Th17 and Treg cells. It is suggested that pneumococcal proteins including Ply and CbpA may activate Th17 and Treg cells in human NALT, and therefore may contribute to the regulation of pneumococcal carriage or clearance in human nasopharynx. Induction of Th17 and Treg from tonsillar MNC were studied using tonsillar MNC depleted of activated and memory T cells. Stimulation with pneumococcal CCS induced Th17 from naïve T cells in tonsillar MNC in the presence of exogenous cytokines (i.e., TGF-β/IL21/IL1-β). TGFβ was shown to be crucial in Treg induction. Thus, the induction of both Th17 and Treg in human tonsillar tissue may be common in humans especially in children during natural infection/carriage, and the balance of the two may determine the clearance or carriage of pneumococcus in nasopharynx. Pneumococcal proteins including pneumolysin (Ply), its toxoid (PdB) and choline binding proteins (CBP) were shown to activate and promote Treg and Th17 cells in tonsillar MNC, thus they may play an important part in modulation of pneumococcal carriage in human nasopharynx.

Understanding the development of natural immunity to pneumococcus and to pneumococcal proteins in particular may provide important information in the development of protein-based vaccines against pneumococcal infection in humans.

Key words: Pneumococcus, Tonsillar MNC, Treg cells and Th17 cells.

Declaration

The original work of the author is presented in this thesis unless stated otherwise. All laboratory experiments described here have been performed by the author in the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

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AMP	Adenosine mono-phosphate
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
APC	Allophycocyanin
ATP	Adenosine triphosphate
BA	Blood agar
BALT	bronchial-associated lymphoid tissue
BFA	Brefeldin A
BSA	Bovine serum albumin
CBD	Choline binding domain
CBP	Choline binding protein
CbpA	Choline binding protein A
CCS	Concentrated culture supernatants
CD	Cluster of differentiation
CDC	Cholesterol dependent cytolytic
cfu	Colony forming units
ChoP	Phospho choline
CO_2	Carbon dioxide
CPCV23	23-valent capsular polysaccharide vaccine
CPS	Capsular polysaccharide
CRP	C-reactive protein
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T lymphocytes associated antigen-4
CWP	Cell wall polysaccharide
DC	Dendritic cell
dH_2O	Distilled water
ELISA	Enzyme linked immunosorbent assay
Eno	Enolase
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead-box P3
FSC	Forward scatter
g	Centrifugal force
g	Gram
GALT	gastrointestinal-associated lymphoid tissue
GC	Germinal centre
G-CSF	Granulocyte colony-stimulating factor
GITR	Glucocorticoid-induced tumour necrosis factor receptor

GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
hr	Hour
HRP	Horse radish peroxidase
Hyl	Hyaluronate lyase
IDO	Indoleamine 2, 3 dioxygenase
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
iLT	Innate-like T cells
Ion	Ionomycin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy-X-linked
syndrome	
iTreg	inducible Treg cells
LAG-3	Lymphocyte activation gene-3
LBP	LPS-binding protein
LP	Lamina propria
LPS	Lipopolysaccharide
LTA	lipoteichoic acid
LytA	Autolysin A
M	Molar
mA	Milliampere
MALT	Mucosal-associated lymphoid tissue
MAC	Membrane attack complex
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minute
ml	Millilitre
MNC	Mononuclear cell
NALT	Nasopharynx-associated lymphoid tissue
Nan A	Neuraminidase A
NaOH	Sodium hydroxide
NF-**κ**B	Nuclear factor-kappa B
ng	Nanogram
NK cell	Natural killer cell
NLR	Nod-like receptor
NLRP	Nod-like receptor Leucine rich repeat Pyrin domain containing protein
NP	Nasopharyngeal
nTreg	Natural Treg cell
OD	Optical density

PAFr	Platelet activating factor receptor
PAMP	Pathogen associated molecular pattern
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCV	Pneumococcal conjugate vaccine
PdB	Genetically detoxified pneumolysin
PE	Phycoerythrin
pg	Picogram
pH	Power of hydrogen
PiaA	Pneumococcal iron acquisition A
PIgR	Polymeric of immunoglobulin receptor
PiuA	Pneumococcal iron uptake A
Ply	Pneumolysin
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leucocytes
PNPP	P-Nitrophenyl Phosphate
PP	Peyer patch
PRR	Pathogen recognition receptor
PsaA	Pneumococcal surface adhesion A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
ROR-γt	Retinoic acid-related orphan receptor
rPly	Recombinant pneumolysin
RT	Room temperature
SC	Secretory component
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
S-IgA	Secretory Immunoglobulin A
SSC	Side scatter
STGG	Skim milk, tryptone, glucose, glycerol transport medium
TCR	T-cell receptor
TGF-β	Transforming growth factor
Th	T helper
Th1	T helper 1 cell
Th17	T helper 17 cell
Th2	T helper 2 cell
Th3	TGF-β-secreting T helper 3 cell
THB	Todd-Hewitt-broth
TLR	Toll like receptor
TMB	Tetramethyl benzidine
TNF-α	Tumour necrosis factor alpha
Tr1	IL-10- secreting T regulatory 1 cell
Treg	Regulatory T cell

TSB	Tryptone soya broth
U	Units
URT	Upper respiratory tract
v	Volts
WCA	Whole cell antigen
WHO	World Health Organization
wt	wild type
yr	year
µg	Microgram
µl	Microlitre
XLAAD	X-linked autoimmunity allergic disregulation syndrome

Chapter 1: General Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae (pneumococcus) is a gram positive and encapsulated bacterium. This bacterium was first identified in 1880 and was described independently by Sternberg and Pasteur through incubation of injected rabbits with human saliva; Pasteur used saliva from a child who had died from rabies, whereas Sternberg used his own saliva. After that instant development of septicaemic disease was observed (Watson et al. 1993). Globally, this pathogen is a major cause of morbidity and mortality among risk groups, including young children, elderly people and immunodeficient patients. It can cause a range of diseases such as acquired meningitis, septicaemia, and pneumonia. Also, it can cause acute otitis media in children (O'Brien et al. 2009).

Pneumococcus is a common cause of respiratory tract infections in both developing and developed countries, especially the former. Annually, pneumococcal diseases are responsible for around 1 million deaths of young children under 5 years (Obaro & Adegbola 2002). Healthy individuals are colonized the upper respiratory tract (URT) by asymptomatic pneumococcus. Approximately 40-50% of infants (0-2 years) in the United Kingdom are colonized by pneumococcus (Hussain et al. 2005), whereas the prevalence of nasopharyngeal carriage in African infants with the same age group, ranging from 48-90% (Abdullahi et al. 2008; Hill et al. 2008; Obaro et al. 1996). In humans, the main reservoir for *S. pneumoniea* is nasopharynx. Colonisation with pneumococcus in the nasopharnyx is very common in infants, reaching 90% of carriage rates (Bogaert, De Groot & Hermans 2004). However, the rates of carriage decrease

thereafter to 10% in late childhood and adults (Granat et al. 2009). Because of the high rates of pneumococcal colonisation in children, they may play a vital role in the transmission of the community between different individuals. Pneumococcal colonisation in some individuals can lead to infections. It might be due to acquirement of a recent serotype rather than the existing serotype in nasopharyngeal compartment (Obaro & Adegbola 2002). There was a gradual decrease of the duration of the pneumococcal carriage for specific serotypes (6, 9, 14, 15, 19, and 23) with age. Between 1995 and 2003 in Sweden the mean duration of carriage in younger children under age of 5 was 43 days which is longer than older children (25 days). Also it was shown that in children aged <1years (74 days), 1-2 years (47 days), 3-4 years (34 days) (Hogberg et al. 2007).

1.2 Virulence factors of pneumococcus

S. pneumoniae has several ways to invade and modulate the host defence by using their virulence factors. These factors range form the polysaccharide capsule to cell surface proteins and all have different roles in protecting pneumococcus from the immune system (Figure1.1.).

The definite etiological agent of pneumococcus is polysaccharide capsule. Although there are at least 93 distinct capsule serotypes which have been identified, just several of these account for the majority of pneumococcal infection. The most common serotypes in younger children are 6B, 9V, 14, 19F and 23F (Henriques-Normark & Tuomanen 2013).

Polysaccharide capsules have many functions. They enable the bacteria easily to evade the host immune system. Pneumococcus can remain protected from polymorphonuclear (PMN) leukocyte attack thanks to their polysaccharide capsule which protect them against phagocytosis and enable them to induce inflammation and disease (Kadioglu et al. 2008). This capsule delay complements attachment and immunoglobulins (Ig) with bacterial cell wall (Mitchell & Mitchell 2010). Also, pneumococcus can be protected from mucus and easily reach the epithelial surface due to the negative charges of capsules (Henriques-Normark & Tuomanen 2013). Capsulated and unencapsulated pneumococus can bind to mucus of human upper airway. A study has demonstrated that unencapsulated pneumococci were trapped into the cell surface mucus and incapable to be free. However, encapsulated pneumococci were capable to escape from mucus binding and therefore attach to the epithelial cells to initiate nasopharyngeal colonisation (Nelson et al. 2007).

There are several virulence factors beside capsule types including cell wall, pneumolysin, surface proteins, autolysin, neuraminidase, peptide permeases, hydrogen peroxide (H_2O_2) and IgA protease ('Should We Develop an Inhaled Anti-pneumococcal Vaccine for Adults?' 2005). Several proteins of pneumococci including pneumolysin, pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), and choline-binding protein A (CbpA) have been studied as vaccine candidates (Kadioglu et al. 2008) (see Table 1.1).

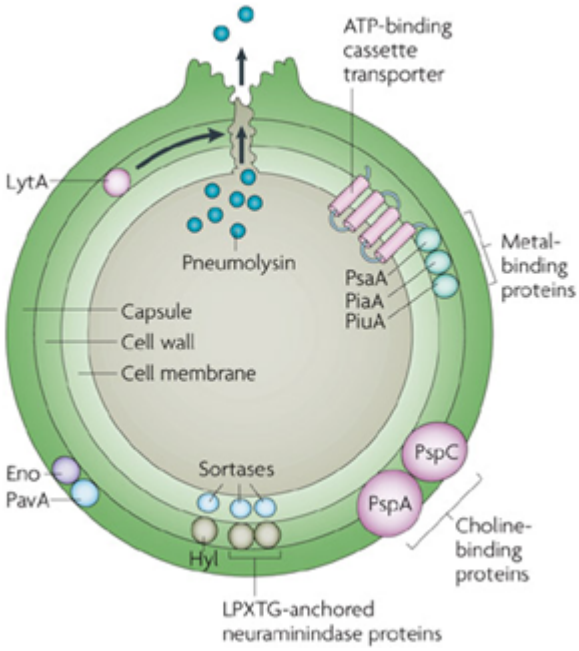

Figure 1.1 *Streptococcus pneumoniae* virulence factors.

The capsule; the cell wall; choline-binding proteins; pneumococcal surface proteins A and C (PspA and PspC); the LPXTG-anchored neuraminidase proteins; hyaluronate lyase (Hyl); pneumococcal adhesion and virulence A (PavA); enolase (Eno); pneumolysin; autolysin A (LytA); and the metal-binding proteins pneumococcal surface antigen A (PsaA), pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA;). Adapted from (Kadioglu et al. 2008).

Pneumolysin is the most widely studied pneumococcal protein virulence factor. It is a member of the cholesterol-dependent cytolytic (CDC) pore-forming toxins family (~53-kDa). It has many activities, such as classical complement activation (Kadioglu et al. 2008), stimulation of host cell apoptosis, binds to cholesterol molecules in the host cells plasma membrane and then enters into membrane to form a pore 350 to 450 Å in diameter (Price & Camilli 2009). In term of the activation of complement, the deposition of C3b on cells surface was more observed on the Ply and PspA-mutant strains and on the unencapsulated

pneumococci. In strains deficient for Ply, PspA and PspC, the clearance of pneumococci from the blood stream has been shown (Quin, Moore & McDaniel 2007). It has been shown that pneumococcal strains can release the Ply into the culture supernatant despite the absence of cells autolysis. This phenomenon is occurred especially at the beginning of the stationary phase. Thus, the release of Ply can be autolysis-independent (Price & Camilli 2009).

Additionally, it was shown that Ply could activate the nucleotide-binding oligomerization domain (Onoda et al.) receptors, Leucine rich repeat and Pyrin domain containing proteins (NLRP) inflammasome which are members of NLR (Nod-like receptors) (McNeela et al. 2010; Tian, Pascal & Monget 2009) and in turn activates caspase-1. The activation of caspase-1 is very important to generate the active form of cytokines such as IL-1 and IL-18. Both cytokines have role in an inflammatory response and recruitment the immune cells to the site of infection (Dinarello 2009).

Choline Binding Proteins (CBP) bind to the cell wall of pneumococci non-covalently. Generally, they comprise three different segments; leader peptide, N-terminal domains and conserved choline binding domain (CBD). Thirteen to sixteen CBPs can be generated from pneumococci (Bergmann & Hammerschmidt 2006). One of CBPs is the pneumopcoccal surface protein C (PspC) also known as choline binding protein A (CbpA) (Quin, Moore & McDaniel 2007). The molecular weight of PspC protein is ~75Kda and this virulence factor has many functions (Jedrzejas 2001). The main role of this protein is to promote the pneumococcal adherence either to nasopharynx causing colonisation or lung

epithelia cells. This interaction facilitates translocation of the pathogen across the layer of epithelial cells. Thus it might facilitate the host cell invasion by pneumococci (Ogunniyi et al. 2007).

PspC is capable of binding to the secretory component of human secretory immunoglobulin A, human factor H and complement component C3 (Ogunniyi et al. 2007). The adherence of PspC to that niche is through the interaction with the secretory component (SC) which known as the ectodomain of polymeric Immunoglobulin receptors (PIgR). A study demonstrated that PIgR knockout mice had a reduced colonisation if PspC lost its function (Bergmann & Hammerschmidt 2006). This protein seems to be expressed by most isolates and contributes to pneumococcal colonisation. Some studies of mice revealed that using this protein and other pneumococcal proteins such as pneumolysin, PspA, and PsaA in intranasal immunisation can protect against multiple serotypes of pneumococci and/or prevent pneumococcal carriage (Briles et al. 2000a; Briles et al. 2000b; Ogunniyi et al. 2001) therefore, CbpA is likely a candidate vaccine against nasopharyngeal carriage of pneumococcus. The ability of PspC to adhere to nasopharynx epithelial cells may contribute to the prolonged colonisation of the nasopharynx (Zhang, Choo & Finn 2002).

PspA has been demonstrated in mice to be important in invasive pneumococcal infections. PspA immunization has been shown in mice to induce protection against pneumococcal infection and nasal carriage (Shaper et al. 2004). Pneumococcus has the ability to bind lactoferrin which is source of iron and is considered bactericidal (Bergmann & Hammerschmidt 2006). There are many

sources of lactoferrin such as tears and saliva and it might protect against nasal ccolonisation. According to Shaper et al., pneumococci can be protected from the killing effect by PspA expression (Shaper et al. 2004). It has been showed by Baril and collogues (Baril et al. 2006) that PspA has an efficient ability to trigger CD4+ T cells and antibody responses. Thus it suggested that this pneumococcal protein antigen is effective vaccine against pneumococcal carriage (Baril et al. 2006).

A number of pneumococcal proteins have been studied in individual immunizations to induce protection against infection of pneumococci and nasopharyngeal carriage (Briles et al. 2000a; Briles et al. 2000b). PsaA, pnuemolysin, and PspA are expressed by nearly all of pneumococcal isolates (Morrison et al. 2000; Paton 1998; Zhang, Choo & Finn 2002). Because Ply and CbpA are important virulence factors and have been shown to induce protective immunity (Mureithi et al. 2009; Zhang et al. 2007), both proteins as well as the PdB derivative of pneumolysin are used as antigens in this study.

Table 1.1 The main role of pneumococcal virulence factors in ccolonisation and disease.

Adapted from (Kadioglu et al. 2008).

Pneumococcal virulence factors	Main function
Upper-airway colonisation:	
Capsule	1- Prevents entrapment in the nasal mucus, thereby allowing access to epithelial surfaces. 2- Suppresses effective opsonophagocytosis
ChoP	Binding of rPAF on the epithelial surface of the nasopharynx.
CbpA (also known as PspC)	Binds to human SC on a PIgR during the first step of translocation across the epithelium.
Hyaluronidase	Cleavage of extracellular matrix components that has hyaluronan
Respiratory-tract infection and pneumonia	
Ply	1- Complement activation. 2- Wide range of effects on host immune components at sub-lytic concentrations.
PspA	1- Lactoferrin binding. 2- Avoid binding of C3 onto pneumococcal surface.
LytA	Facilitate the release of pneumolysin.
PsaA	Component of the ABC transport system, which is involved in resistance to oxidative stress
PiaA and PiuA	Component of the ABC transport system.
IgA	Breakdown IgA1.

1.3 Pneumococcal colonisation

Pneumococcus is opportunistic microorganism capable of colonising the upper respiratory tract and it can cause invasive disease, especially in the high-risk groups. Since pneumococci can colonize in the nasopharynx, mucosal vaccination may be an effective way to protect against mucosal infection. Some studies in mice models have revealed that the immunization with pneumococcal antigens, including PspA, pneumolysin, PsaA, and CbpA by intranasal route, are more efficient against invasive disease as well as nasopharyngeal carriage (Alexander et al. 1994; Briles et al. 2000a; Briles et al. 2000b; Paton 1998), especially if there is a combination of virulence proteins factors. It has been recommended that non-capsular-based vaccine can probably protect both human and mice from pneumococcal proteins (Richards et al. 2010).

1.3.1 Mechanisms of pneumococcal colonisation

Pneumococcal outer surface is a highly heterogeneous and immunogenic polysaccharide capsule, which is considered as a significant virulence factor of pneumococci as a shield protecting them from phagocytosis, and crucial for colonisation. The next layer which comprises polysaccarides and teichoic acid is named the cell wall. This surface is crucial for stimulating immune response especially the inflammatory cells and triggering the complement cascade and cytokine production. The dynamic inflammatory reaction, which is caused by the pneumococcal cell wall, is the primary trigger to ccolonisation (Bogaert, De Groot & Hermans 2004).

The establishment of ccolonisation is by adhering *S. pneumoniae* with epithelial cells of the respiratory tract. Cell wall associated surface proteins such as PsaA binds to cell surface receptor of epithelial cell termed N-acetylglucosamine that is exposed after the cleavage of mucus layer by neuraminidase. Pneumococcal colonisation is generally not followed by symptomatic disease (Bogaert, De Groot & Hermans 2004) unless the local production of inflammatory factors such as IL-1 and TNF are triggered (Tuomanen 1997).

Subsequently, the receptors on the epithelial and endothelial cells are subjected to change by the cascade of inflammatory response. One of those receptors named platelet activating factor receptor (PAFr) which is up-regulated by the inflammatory factors shows a high affinity to the pneumococcal cell wall choline. When the binding occurs, pneumococci migrate through the epithelial cells of the respiratory tract leading to the invasion (Baril et al. 2006). Moreover, there is an increased affinity of choline binding protein A (CbpA) toward sialic acid and lacto-N-neotetreose on human cells that are activated by cytokines, and binds directly to the polymeric Ig receptor (pIgR), and thus the migration through the mucosal barrier (Balachandran et al. 2002). It has been recently revealed that IgA proteases of pneumococcus strongly adhere to epithelial cells of the lung in the presence of human IgA (Weiser et al. 2003). Therefore, the cleavage of opsonising IgA by pneumococcal IgA1 protease lead to in a modification (neutralisation) of surface charge (Weiser et al. 2003). In addition, CbpA is capable of bind secretory component of Immunoglobulin A (S-IgA) and thus associate with the complement pathway which compose the immune response (Balachandran et al. 2002;

Hammerschmidt et al. 1997). The activity of virus (influenza) protein such as neuraminidase may contribute to the improvement of pneumococcal adherence, especially in the presence of viral infection (Bogaert, De Groot & Hermans 2004).

Both IgA and IgG antibodies specific to pneumococcal strains have been shown to contribute to killing pneumococcus and in the reduction of pneumococcal carriage in the presence of complement and phagocytes (Weiser et al. 2003). But IgA1 protease enables the pneumococcus to interact and persist on the mucosal surface of the host tissue (Weiser et al. 2003).

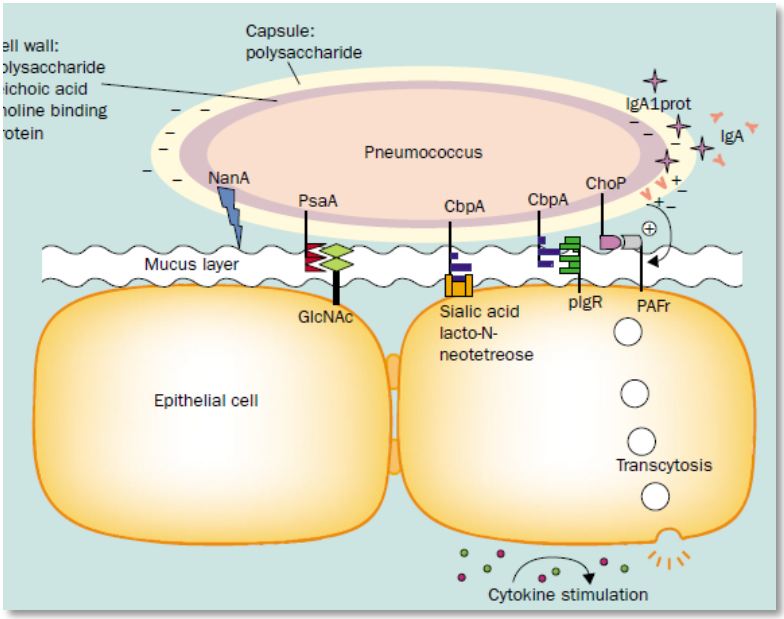

Figure 1.2 Interaction between pneumococcal surface proteins and epithelial cell components in the mucosal cavity.

The thickness of the layer of mucus is reduced by neuraminidase (NanA) which in turn exposes the N-acetylglycosamine (GlcNAc) receptors on the epithelial cells. Then after, that receptor interacts with pneumococcal PsaA. The platelet-activating-factor receptors (PAFr) is upregulated by epithelial cells in response to cytokine trigger. There was a strong affinity between cell-wall phosphocholine (ChoP) and PAFr. CbpA binds to the polymeric Ig receptor (pIgR) directly which results in microbial passage via the mucosal barrier (transcytosis). The cleavage of opsonising IgA is occurred by IgA1 protease. Consequently, this action changes the charge of pneumococcal surface and in turn enhances the attachment ChoP to the PAFr. Adapted from (Bogaert, De Groot & Hermans 2004).

1.4 Mucosal immune system

The mucosal immune system is the first line of physical and immunological defence against invaders. Mucosal-associated lymphoid tissue (MALT) is a general term for mucosal immune system in humans which includes 1)

gastrointestinal-associated lymphoid tissue (GALT), 2) bronchial/nasopharynx-associated lymphoid tissue (B/NALT). Mucosal tissue covers large area of human body and is unique in its structure and function. It provides protection to humans through induction of mucosal immunity, which depends on lymphocytes activation within MALT (Lee, Lee & Gu 2005).

Mucosal immune system consists of induction and effector sites. Induction site includes the Peyers patches (PP) in the gut and NALT in the oropharyngeal cavity, which are two major compartments of the MALT. Effecter site includes mucosal lamina propria (LP) and glandular tissue. Both inductive and effector sites are under the term of mucosal immune system working together to generate antigen-specific immune responses at mucosal surfaces (Kiyono & Fukuyama 2004).

Waldeyer's ring in humans consists of the main inductive sites in NALT. The nasal route has been considered an effective way of vaccine administration for both mucosal and systemic antibody production (Brandtzaeg 2011). Nasopharyngeal tonsil (adenoids), and paired plataine tonsils are major components in Waldeyer's ring in addition to lingual tonsils, tubal tonsils and lateral pharyngeal bands (Dolen, Spofford & Selner 1990). Human tonsils are composed of several different specific tissue sections all compartments of which take part functionally in the immune response (Brandtzaeg 2011).

1.5 Mucosal immunity against pneumococcal ccolonisation

There are various immune cells in nasopharynx involved in preventing bacterial

colonisation. The host immune responses against pneumococcus include phagocytes (neutrophils and macrophages), T cells and B cells that produce antibodies to different targets such as proteins and polysaccharides of *S. pneumoniae*. Both arms of host defence mechanisms (innate and adaptive immunity) are required against *S. pneumoniae* (Jambo et al. 2010).

1.5.1 Innate immune response to *S. pneumoniae*

During colonisation, innate immune response is considered the first and early non-specific defence, which has the ability to act fast against diverse array of pathogens. Also, it is very important to initiate adaptive immune response including T cell and B cell responses. Many immune cells participate to protect the host from any intruder, including macrophages neturophils, natural killer (NK) cells and innate-like T (iLT) cells.

1.5.1.1 Role of complement against *S. pneumoniae*

Complement is considered a bridge between innate and adaptive immune response. The classical pathway of complement activation is predominant major pathway for innate immunity against pneumococcal infections in mediating a clearance of pneumococcus (Brown et al. 2002).

The complement system incorporates three different pathways: classical pathway, alternative pathway and lectin pathway. All three pathways require the main component of complement (C3) which when activated leads to the clearance of

microorganism (Kadioglu & Andrew 2004). Opsonophagocytosis by macrophages and neutrophils and inflammation induction both result from deposition of complement components on the surface of foreign antigens and followed by microbial degradation by forming membrane attack complex (MAC). A contribution of acute phase proteins, e.g. serum amuloid protein and C-reactive protein (CRP) are found to be crucial for the activation of classical pathway during pneumococcal infections. Moreover, direct binding of C1q to the surface of pneumococcus is involved (Brown et al. 2002; Kadioglu & Andrew 2004; Mold & Du Clos 2006). In contrast to other complement pathway (alternative and lectin), the classical pathway is much contributed to protective innate response. The elimination of a microorganism begins when the components of complement deposit on its surfaces. Pneumococcus use strategies such as capsules, cell wall proteins and other antigens to inhibit the complement pathway (Paterson & Orihuela 2010).

Pneumococcus has polysaccharide capsule that plays a key role in pathogenesis. It prevents pathogens from being trapped by mucus and to be opsonophagocytosized through mechanism that involves a decrease activation of the classical pathway. Complement is key elements in protection against pneumococcal infection via opsonophagocytosis ((Brown et al. 2002; Roy et al. 2002). Co-colonisation of mice with *S. pneumoniae* and *H. influenzae* induces complement and neutrophil-like cells which help to clear pneumococci from nasopharynx through phagocytosis by neutrophils (Lysenko et al. 2005).

1.5.1.2 Toll-like receptor (TLR)

TLRs are part of a family called pathogen recognition receptor (PRRs). These receptors can recognize conserved microbial components, known as pathogen-associated molecular patterns (PAMPs) and trigger inflammatory responses. There are more than 10 TLRs discovered in humans, some have been found to contribute to pneumococcal infection (Paterson & Orihuela 2010). Once TLRs are stimulated by PAMP, the activation of signaling pathways will lead the production of inflammatory cytokines. Maturation of dendritic cells is often triggered after TLRs are activated, and as a consequence, co-stimulatory molecules and capacity of antigen presentation will be induced and enhanced (Janeway & Medzhitov 2002).

1.5.1.2.1 TLR2

TLR2 receptor is capable of recognising a large selection of microbial ligands, including peptidoglycan, lipoproteins, zymosan (Janeway & Medzhitov 2002), and pneumococcal lipoteichoic acid (LTA) and cell wall peptidoglycan (Paterson & Mitchell 2006). TLR2 normally works by formation of heterodimers with either TLR1 or TLR6 (Janeway & Medzhitov 2002; Ozinsky et al. 2000; Takeuchi et al. 2001). In case of pneumococcal carriage, the role of TLR2 is very essential to pneumococcal clearance (van Rossum, Lysenko & Weiser 2005).

It has been investigated that TLR2 knockout mice ($TLR2^{-/-}$) following intranasal infection with pneumococcus revealed a moderate contribution in host response, compared with wild type mice. That is due to compensatory and effective role of

TLR4 recognition of pneumolysin (Knapp et al. 2004). Moreover, CD14 is a membrane-bound and soluble receptor which act as co-receptor along with TLR2 in order to augment the LPS response (Abeyta, Hardy & Yother 2003), also it can contribute to pneumococcal recognition (Schroder et al. 2003).

1.5.1.2.2 TLR4

TLR4 has been shown to activate the innate immune response to gram-negative infections mainly. This receptor has been studied widely inclduing its role to LPS response and pneumolysin. TLR4 is involved in the recognition of LPS (Paterson & Orihuela 2010; Srivastava et al. 2005), in combination with CD14 (Hoshino et al. 1999) and LPS-binding protein (LBP) (Hoshino et al. 1999).

Ply was shown to activate TLR4 on macrophages and enhances the pro-inflammatory response (figure 1.3) which may contribute to invasive disease (Malley et al. 2003). To determine the interaction between pneumolysin and TLR4 during the pneumococcal colonisation, Malley et al has compared between wild type and TLR4-deficient mice in a nasopharyngeal carriage model. The findings revealed that mice lacking TLR4 were more subjected to colonisation and to develop invasive disease (Malley et al. 2003).

However, another study by Rossum and colleagues (van Rossum, Lysenko & Weiser 2005) revealed that no difference between wild-type and $TLR4^{-/-}$ mice. The use of different bacterial strains might be one of the explanations to the apparent

difference. In case of pneumococcal pneumonia, TLR4 has been suggested to play a defensive role (Branger et al. 2004) and it has also been suggested that the important of TLR4 pneumococcal infections may be restricted to the airway surface (Paterson & Orihuela 2010).

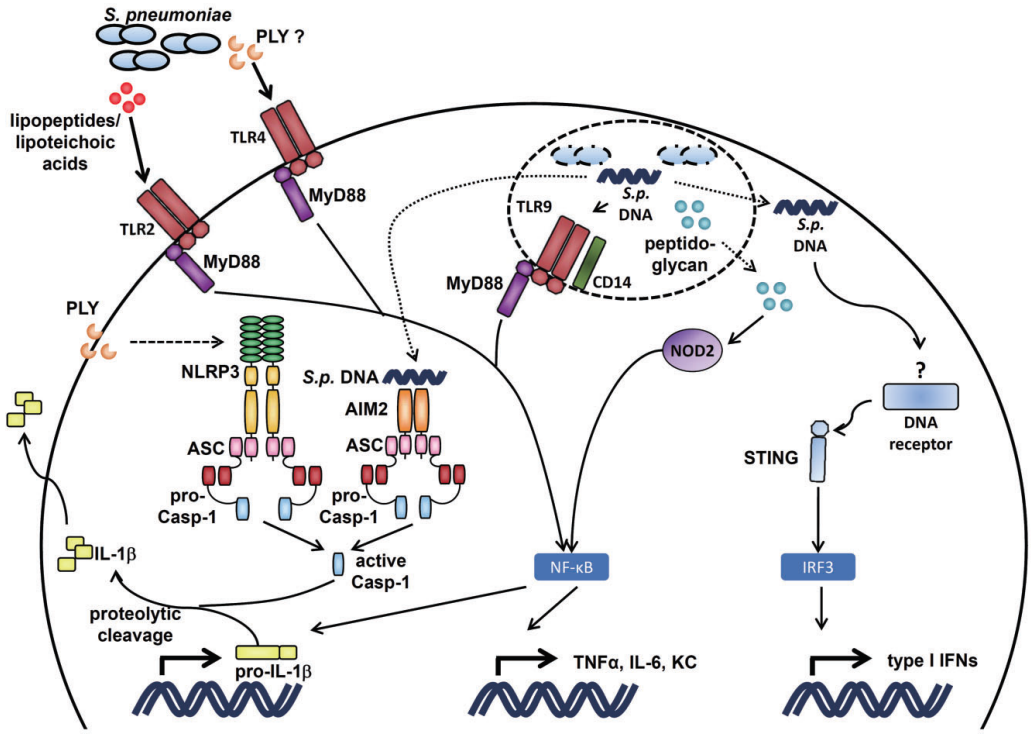

Figure 1.3 The recognition and initiation of the immune response against pneumococcal components by different PPRs.

TLR2 and TLR4 can recognise cell wall components and Ply respectively. Phagocytic cells internalize S. *pneumoniae* and then a degration in phagosomes. Therefore, bacterial peptidoglycan and nucleic acids are released. Unmethylated CpG-containing DNA activates TLR9 within the endosomes. The secrestion of NF-**k**B-dependent cytokines is elicited by TLRs as well as NOD2. Adapted from (Koppe, Suttorp & Opitz 2012).

1.5.2 Adaptive response to *S. pneumoniae*

1.5.2.1 Antibody-mediated response

It has been long assumed that immunity to extracellular encapsulated bacteria was humoral-dependent response. The immune response to pneumococcal colonisation and disease has been attributed to antibodies against capsular polysaccharide of pneumococcus (Malley et al. 2005). Antibodies to pneumococcal

capsular polysaccharide produced by vaccination can provide protection to humans (Dagan et al. 2002) and animals (Malley et al. 1998) against colonisation. Mice were protected from colonisation when specific antigens administrated systemically (Ogunniyi et al. 2000) or intranasally (Briles et al. 2000a).

However, recent studies have suggested that antibodies to capsular antigens are not required for naturally-developed immune protection against carriage following exposure to live pneumococci (Trzcinski et al. 2008), and this protection requires the presence of CD4+ cells (Malley et al. 2005). A study by McCool et al. also showed that pneumococcal carriage clearance was antibody-independent despite the presence of antibodies. (McCool et al. 2002).

Nevertheless, antibodies to capsular polysaccharide of pneumococcus induced by vaacination are known to prevent invasive disease and pneumococcal colonisation. Both secretory IgA antibodies in mucosal site and IgG in serum may mediate protection (Jambo et al. 2010). It is likely that antibodies may contribute to protection against pneumococcal ccolonisation/carriage, but recent evidences suggest CD4 T cell response, T helper 17 (Th17) in particular, may also be important, and perhaps more crucial in promoting clearance of pneumococcal carriage (Malley et al. 2005).

1.5.2.2 Cellular-mediated response

In addition to antibodies, the generation of specific memory T cells against pneumococcal antigens can be elicited by pneumococcal colonisation (Zhang,

Clarke & Weiser 2009). T cell response is triggered by antigen presenting cells (APCs) via PRR signals and induction of cytokine production (Gerosa et al. 2008). Protection against colonisation has been suggested to be mediated by CD4+ T cells, particularly IL17–producing CD4+ T cells which have the potential to recruit neutrophils through the production of chemokines (Malley et al. 2005). On the other hand, T regulatory cells have been suggested to be important in mediating pneumococcal carriage (Zhang et al. 2011).

1.6 Regulatory T cells

1.6.1 Classification and function

Regulatory T cells (Treg) have a crucial role in modulation of different immune responses, in microbial infection, allergy, tumours, autoimmunity, and transplantation (Lim, Hillsamer & Kim 2004). Natural regulatory T cells (nTregs) and inducible or adaptive regulatory T cells (iTregs) are two main subsets of Tregs that differ in term of action and generation (Fig.1.4.). The subset of CD4 T cells that expresses constitutively the interleukin (IL)-2 receptor alpha-chain (CD25) possesses potent immunosuppressive functions toward auto-reactive peripheral T cells (Huibregtse, van Lent & van Deventer 2007; Mills 2004). Thymus-derived Treg cells ($CD4^+CD25^+$Foxp3+) which are known as natural Treg (nTreg) were first defined in 1995 by Sakaguchi and colleagues. The mean proportion of these cells is approximately 5-10 % of the total CD4+ T cells in thymus, peripheral blood (Campbell et al.) and lymphoid tissues (Mills 2004). Once these cells are produced

and developed in the thymus, they migrate to the peripheral blood, remain and survive in there for normal surveillance of self antigens and avoid autoimmune responses (Piccirillo 2008).

In addition to nTreg cells, adaptive or inducible Tregs are derived from conventional $CD4^+$ T cells or naïve T cells (non-Treg cells) after exposure to antigens and they include IL-10-secreting T regulatory 1 (Tr1) cells and transforming growth factor (TGF)-beta-secreting T helper (Th) 3 cells (Miyara & Sakaguchi 2007). Table 1.2. showing the difference between these subsets in terms of markers expression. Their regulatory activity is mediated by the expression of IL10 and/or TGF-β although the specificity of antigens is still elusive in terms of inducible Treg cells (Vernal & Garcia-Sanz 2008).

The interactions between T cell receptors (TCR) of $CD4^+CD25^+$ Treg cells and self peptide-Major Histocompatibility Complexes (MHC), which are expressed by thymic stromal cells, is a requirement for $CD4^+CD25^+$Treg cell development. In contrast to T cell selection in the thymus, avidity interactions between TCR of $CD4^+CD25^+$Treg cells and self-peptide/MHC, which is presented on the cortical epithelial cells, is another necessity to Treg cell development. However, if there is a high avidity, the deletion of Treg cells will occur (Fehervari & Sakaguchi 2004). It has been stated that in mouse models, the adaptive Treg cells can be induced *ex vivo* in the presence of TGF-β (dependent manner) (Chatila 2005).

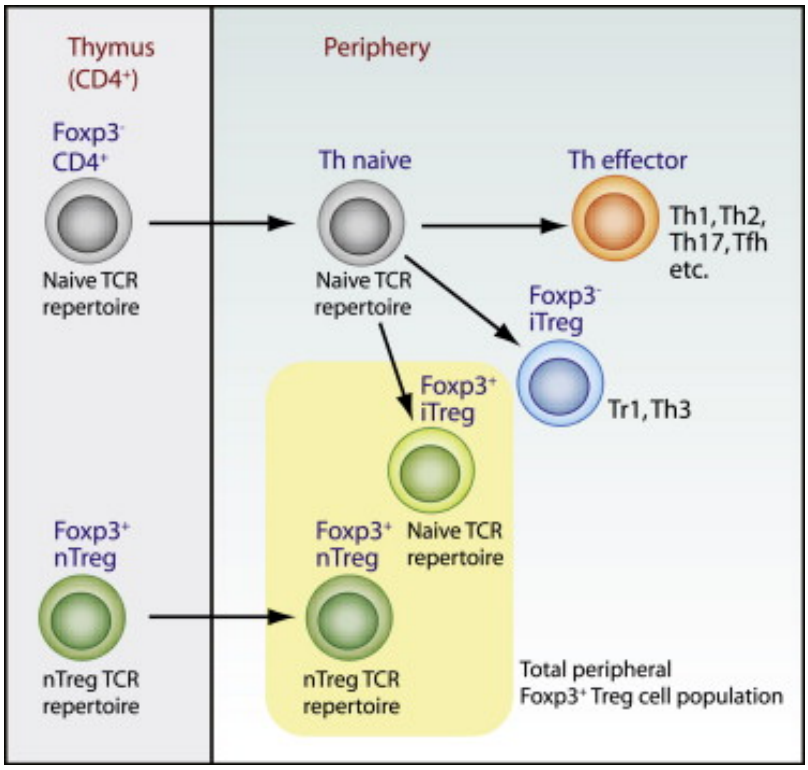

Figure 1.4 Generation of T regulatory cells.

Thymus derived regulatory T cells which are termed naturally occurring regulatory T cells (nTreg) differentiate in the thymus and migrate to peripheral tissues. Adaptive peripheral inducible regulatory T cells (iTreg) differentiate in secondary lymphoid organs and tissues and incorporate three different types of cells Tr1, Th3 and CD8 (CD4+CD25+Foxp3-). (Curotto de Lafaille & Lafaille 2009).

Table 1.2 Comparison between natural and inducible Treg cells.

Adapted from (Abdulahad, Boots & Kallenberg 2010).

Phenotype/feature	nTreg	Tr1	Tr3
Place of origin	Thymus	Periphery	Periphery
L-2Ra (CD25)	++	+/-	+
Foxp3 (activated/resting)	+High/+Low	_	_
CD45RA (activated/resting)	-/+	?	?
LAG-3	+	?	?
GITR	+	-	?
CTLA-4	+	+	+
IL-10	-	++	+/-
TGF-β	+/-	+/-	++
FR4	++	?	?
CD39	+	?	?
LAP	+	?	?
IL-1R I/II	+	?	?

(? = not known yet)

1.6.2 Expression of Foxp3 in T regulatory cells

The transcription regulator factor forkhead box P3 or winged helix (Foxp3) is a chief controller in nTregs development and function (Gavin et al. 2006). The Foxp3 is a member of the fork-head family of transcription factor. It is characterized by the existence of a winged helix DNA-binding domain. Adjacent to forkhead homology domain of C-terminal in Foxp3 gene, there are zinc finger motif and a leucine

zipper domain. Another N-terminal domain in this gene which has the capability to inhibit the transcription from nuclear factor of activated T cells and nuclear factor-$_k$B (NF-**κ**B) response elements is identified by its high proline content (Chatila 2005). Transformation of Foxp3 gene can be generated in human subjects from any deletion of a single amino acid of these domains. Thus, the function and the development of nTregs will be affected. Therefore, the spontaneous development of various autoimmune or inflammatory diseases will occur including the human genetic disease termed immune dysregulation, polyendocrinopathy, enteropathy-X-linked syndrome (IPEX) (Miyara & Sakaguchi 2007) and X-linked autoimmunity–allergic dysregulation syndrome (XLAAD) (Chatila 2005). The loss of nTregs could be compensated by infusion of $CD4^+CD25^+$Treg cells to prevent those disorders (Piccirillo 2008). The expression of Foxp3 in natural $CD4^+CD25^+$ Tregs either in humans or animal facilitates the control of their development and function. The expression of Foxp3 arises at the late stage of double positive T cell development in the thymus. The proportion of Treg cells represents approximately 6% of human CD4+ subsets. Natural Treg cells (CD4+$CD25^{high}$) can permanently express Foxp3 (Gavin et al. 2006).

It has been investigated that nTreg cells can be identified by a low expression of CD127 (IL-7-Ra) with a high expression of CD25 (Liu et al. 2006; Seddiki et al. 2006). By using these surface markers, the percentage of circulating nTreg cells in human was around 6-10% of the total of CD4+ cells. Irrespective of CD25 surface expression, Foxp3 is considered currently the most specific and reliable molecular

marker either for thymic or peripheral nTreg cells in rodents and humans. It is known that the over-expression of Foxp3 in mice has an increased development of nTreg cells, and also can suppress the T cell proliferation and cytokine production of effector T cells (Piccirillo 2008).

1.6.3 The characteristic markers of Treg cells

There are a number of constitutive expressions reported on Treg cells in addition to CD25 and Foxp3 (Table 1.2.) They include cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene-3 (LAG-3) L-selectin (CD62L) integrin $\alpha_E\beta7$ (CD103), C-C chemokine receptor 7 (CCR7), CCR4, CCR8, and CD39 (Abdulahad, Boots & Kallenberg 2010) and neuropilin-1 (Bruder et al. 2004; Fehervari & Sakaguchi 2004).

It has been shown that CD25 is crucial for nTreg cells ($CD4^+$ $CD25^{high}$) function. Mice that lack CD25 (IL-2Rα) or IL-2 can develop a fatal disease termed IL-2 deficiency syndrome. According to Fehérvari et al., number of $CD4^+CD25^{high}$ Treg cells was reduced in healthy mice after the neutralization of IL-2. As a result, organ-specific autoimmune diseases may occur. It is suggested that IL-2 is indispensable in the development, preservation and the function of $CD25^{high}CD4^+$Treg cells (Fehervari & Sakaguchi 2004).

GITR molecule is a constitutively expressed marker for nTreg cells and its role in $CD4^{+}CD25^{+}$Treg cells is considered important. The natural ligand for GITR which is expressed on APCs (DCs, macrophages, and B cells) is GITRL. Treg cells express a low level of IL-7R receptor alpha chain (CD127). This marker has been used as a marker to distinguish Tregs from other activated effector T cells. The expression of CD127 is correlated with the expression and suppressive function of Foxp3, and thus highly purified populations of Treg cells can be sorted by those surface markers described previously (Abdulahad, Boots & Kallenberg 2010). Nevertheless, activated T cells, activated T follicular helper cells (Tfh) might lose the expression of CD127 (Eddahri et al. 2006; Seddiki et al. 2006; Zhou et al. 2010). So this marker is not specific and needs to be used in combination of others.

There are also a variety of adhesion molecules and ckemokine receptors expressed by Foxp3 Treg cells that allow homing to the site of inflammation (Allakhverdi et al. 2006; Kleinewietfeld et al. 2005; Schneider et al. 2007). Foxp3+Treg cells express chemokine receptor 4 (CCR4), CCR5, CCR6, CCR7 and CCR8 relatively higher than effector T cells. For example, CCR7, which is expressed by the vast majority of Foxp3+Treg cells, enables these cells to migrate towards chemokines CCL19 and CCL21. It has been demonstrated that IL-10-producing nTreg cells can be recruited into the site of infection when they express CCR5; and inhibit the development of effector T cells and the production of IFN-γ. Therefore, the survival of pathogens for long period will be promoted in the site of

inflammation (Piccirillo 2008). Generally, when Treg cells express a range of homing receptors, these cells can migrate to the inflammation site and inhibit the immune response (Bruder et al. 2004; Fehervari & Sakaguchi 2004).

1.6.4 The immune suppression mechanisms by Treg cells

The suppression of $CD4^+$ and $CD8^+$ T effector cell proliferation occurs upon the activation of nTregs through TCR (Bacchetta, Gambineri & Roncarolo 2007). Also, antibody production by B cells, Natural killer (NK) cells, and the maturation of dendritic cells are suppressed by nTregs (Miyara & Sakaguchi 2007). Recently, it was revealed that CD39 is an ectoenzyme and is considered as activated surface marker of human Tregs. It has the ability to degrade adenosine tri-phosphate (ATP) to adenosine mono-phosphate (AMP) (Bacchetta, Gambineri & Roncarolo 2007). The generation of adenosine can inhibit the effector T cells function via the adenosine receptor 2A activation ($A_{2A}R$) (Fig.1.5.c) (Deaglio et al. 2007). Amazingly, although binding of adenosine to its receptor $A_{2A}R$ suppresses the function of effector T cells, it can augment induced Treg generation by inhibiting the expression of IL-6, whereas it encourages the secretion of TGF-β (Vignali, Collison & Workman 2008). The expression of Foxp3 and differentiation of Treg is promoted by TGF-β. However, the generation of Treg cells can be suppressed by IL-6. As a consequence, suppressing IL-6 has a great implication in Treg cells preservation (Vignali, Collison & Workman 2008).

In addition to direct effect of Tregs on T cells function, there is another suppression mechanism of Treg cells which is by targeting dendritic cells (DCs). There are direct interactions between Tregs and DCs in vivo through CTLA-4 which is constitutively expressed by Tregs, and CD80 and/or CD86 (Read, Malmstrom & Powrie 2000). It is known that the activation and maturation of DCs is crucial for the activation of T cells, but it has been suggested that the function might be orchestrated by Tregs. Indoleamine 2, 3-dioxygenase (IDO) expression by APC (i.e. DC) could be modulated by Tregs (Fig.1.5.d) and has the ability to catalyse the conversion of tryptophan to kynurenine and other metabolites and thus inhibits the effector T cells (Sakaguchi 2005). Treg cells may reduce the capability of DCs to activate T cells (Fallarino et al. 2003; Mellor & Munn 2004). It has been shown that CTLA-4 triggering can stimulate the production of IDO by human DCs (Boasso et al. 2005). $CD4^+$ $CD25^-$ cells can be converted to $CD4^+$ $CD25^+$ cells by IDO (Curti et al. 2007).

Another mechanism of Treg-mediated suppression may be through killing the effector cells (cytotoxicity). Treg cells may have the ability to release perforin and granzyme A (Fig.1.5.b), therefore promoting death of T cells, monocytes and DCs. However, through perforin-independent and perforin-dependent pathways, the T cells and B cells could be damaged by the release of granzyme B (Miyara & Sakaguchi 2007).

$CD4^+CD25^+$ Treg cells may act through cytokine production such as interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) (Fig.1.5.a). These cells may suppress the proliferation of T cells and interferon gamma (IFN-γ) production via cell-cell contact, which could be mediated by CTLA-4 (Ozeki et al. 2010). It is important to control the level of Treg cells suppression so that host protection against pathogens won't be compromised. For instance, cytokines co-stimulation molecules have a key role in host protection, and suppression of these will make the host susceptible to infection. On the other hand, autoimmunity and allergy may occur in the deficiency of suppression. When the host encounters microbes, the requirement of Treg immune regulation is crucial for preventing excessive tissue damage and possible autoimmune response during the inflammatory response (Miyara & Sakaguchi 2007).

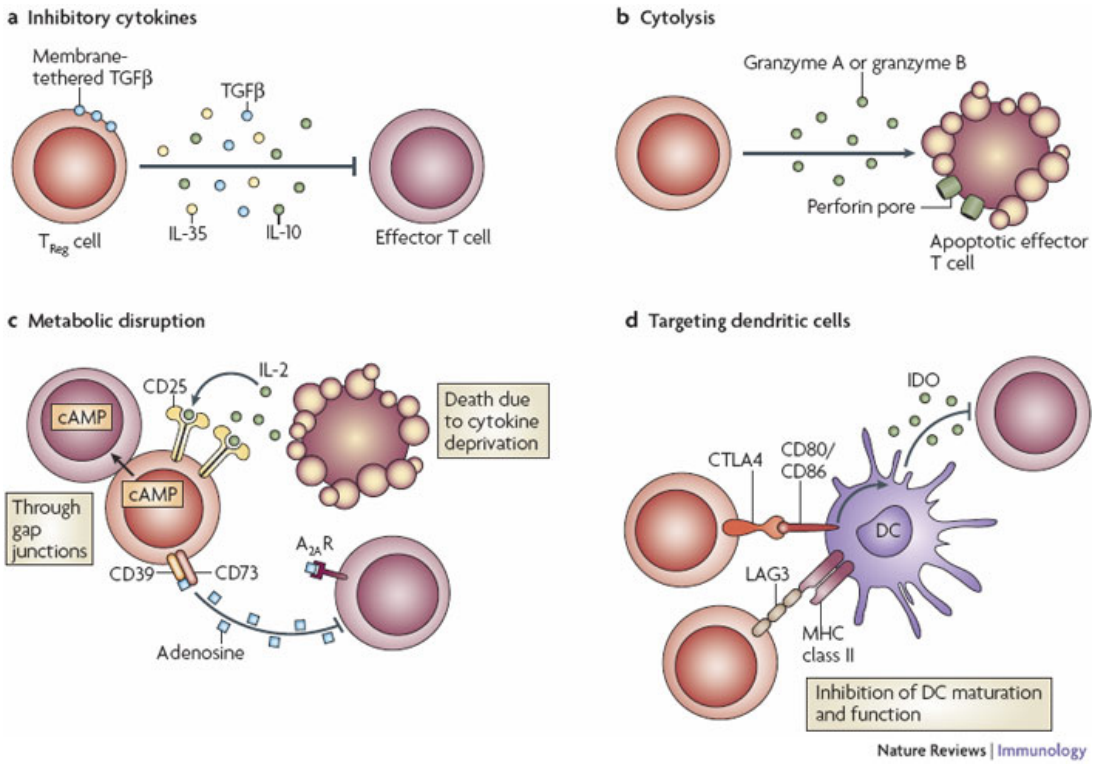

Figure 1.5 Mechanisms of suppression by Treg cells.

(a) Regulatory T cells secrete interleukin-10 (IL-10), IL-35, and transforming growth factor (TGF-β), and directly inhibit T-cell activation. (b) Activated human T_{Reg} cells directly destroy target cells such as T cells and APCs through perforin - or granzyme B-dependent pathways. (c) CD39 as activated surface marker of human Tregs degrade adenosine triphosphate (ATP) to adenosine monophosphate (AMP) and the generation of adenosine can inhibit the effector T cells function through the adenosine receptor 2A activation ($A_{2A}R$). (d) The expression of indoleamine 2, 3-dioxygenase (IDO) by APCs is provoked by cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) T_{Reg} cells, and IDO-expressing APCs in turn suppress T-cell activation by reducing tryptophan. Also, regulatory T cells can suppress APC function, by inhibiting expression of MHC molecules, CD80, CD86 and IL-12 through CTLA-4. Adapted from (Vignali, Collison & Workman 2008).

1.7 The protective role of Treg cells in infectious diseases

CD4+CD25+ Regulatory T cells have been shown to be crucial in protection against several conditions such as autoimmune disease, allograft rejection and allergy. The effector immune cells have a protective role against microbial infection, but pathogenic effects can occur during the immune-inflammatory response to the pathogens (Mills 2004). In this case, the induction of Treg cells is crucial to tackle immunopathology via TGF-β and IL-10 dependent pathway. It has been shown that memory Treg cells, which were obtained from mice infected with bacterial infection such as *Helicobacter hepaticus*, were capable of preventing intestinal inflammation progress. Also, the production of IFN-γ can be inhibited after exposing to antigens of *H. hepaticus in vitro* by the CD4+CD45RB^{low} Treg cells which were obtained from the same infected mice lack of IL-10 generation (Maloy et al. 2003).

Those generated Treg cells may be memory Treg due to the previous exposure to similar antigens (Mills 2004). It has been demonstrated that lack of IL-10 in mice has a negative impact on host tissue and can lead to increased disease severity, and pro-inflammatory cytokine production (Deckert et al. 2001). Additionally, IL-10 deficient mice has a reduced ccolonisation of *Helicobacter pylori* in gastric mucosa, although the severity of gastric inflammation was considerably greater than in wild-type mice (Chen, Shu & Chadwick 2001) and this severe inflammation was associated with IL-12 release and Th1 cells response (Mills 2004).

1.8 The role of Regulatory T cells in pathogen persistence

There are several studies demonstrating the role of Treg cells in bacterial and viral persistence, through their inhibition of T cell proliferation and cytokine production. For example, the cytotoxicity of effector CD8+ T cells is suppressed by Treg cell during viral infection such as cytomegalovirus, HIV (Aandahl et al. 2004) , and hepatitis C virus (HCV) (Cabrera et al. 2004). Once Treg cells are activated, they are expanded and suppress CD4+ and CD8+ T cell responses (Keynan et al. 2008). Additionally, a suppression of immune response to non-self antigens can be seen also from the non-specific activation, suggesting that Treg cells are not only specific for self antigens but also can be triggered by cross-reactive antigens (Keynan et al. 2008). As stated by Macleod and Wetzler, regulatory T cells can be activated by TLR activation and that leads to a reversal effect on their suppressive activity, and if the agonist of TLRs is removed, Treg cells can regain suppression (MacLeod & Wetzler 2007).

Specific Treg cells to particular viruses showed a potent inhibitory function in the presence of relevant viral antigens (i.e. human papillomavirus 16 and 18 proteins). Studies in rabbits, mice and humans on controlling T cell immune response against herpes simplex virus type 1 (HSV-1) indicated that Treg cells concentrate at the site of inflammation, but they are effective at manipulating the response of T cells. It was suggested that Treg cells act during the development of memory cells as well as during the infection especially during the acute phase. In terms of HCV, after depleting CD4+CD25+ cells from infected individual, the proliferation and the

production of cytokines by memory T cells to specific HCV antigens have been recovered (Keynan et al. 2008).

In comparison to uninfected people, infected individuals with chronic HCV were shown to have substantial number of Treg cells in their circulation. The higher ratio of Foxp3+ Treg cells to other lymphocytes in chronic HCV infection helps to explain how T cell responses against HCV infection were attenuated by the elevated numbers of Treg cells (Keynan et al. 2008; Ward et al. 2007). TGF-β and IL-10 may be involved in the suppressive action (Cabrera et al. 2004), although IL-10 and TGF-β independent pathway may also be involved (Keynan et al. 2008; Rushbrook et al. 2005).

1.9 T helper-17 cells

Mosmann and Coffman have discovered two different subsets of T helper (Th) cells, including Th1 and Th2 cells based on specific cytokines and effector functions (Mosmann & Coffman 1989). A new subset of T helper cells named Th17 cells which produce Interleukine-17 (IL-17) were identified more recently (Park et al. 2005). The expression of transcriptional factor retinoic-acid-related orphan receptor γ t (ROR-γ t) is the main characterization of human Th17 cells. During microbial infection, Th17 have been shown to accelerate the clearance of extracellular pathogens (van de Veerdonk et al. 2009). However, Th17 cells can be involved in chronic inflammation and in the pathogenesis of autoimmune disease such as rheumatoid arthritis, multiple sclerosis (Oukka 2008; Ouyang, Kolls & Zheng 2008). IL-17 is a family of cytokines (including IL-17A, B, C, D, F) which are

structurally homologous to each other (Afzali et al. 2007). IL-17A and IL-17F are considered the major types part of this family. These cytokines participate in the recruitment, activation and migration of neutrophils, which are important in microbial removal (Matsuzaki & Umemura 2007).

Moreover, IL17 promotes cells such as macrophages, fibroblasts, endothelial cells and epithelial cells to produce IL-1 β, IL-6, tumour necrosis factor alpha (TNF-α) and chemokines (CXCL9, CXCL10 and CXCL11) which attract interferon-producing CD4+Th1 cells (Oukka 2008). They act as multiple pro-inflammatory mediators (Bacellar et al. 2009). It have been demonstrated that these mediators have an effect on pathogenicity especially in autoimmune diseases (Lu et al. 2009; Sutton et al. 2006). It has been reported that human memory Th17 cells are categorized as CCR2+CCR5- and produce high level of IL17, whereas the cells expressing both CCR2 and CCR5 produce only a low level of IL17 (Sato, Aranami & Yamamura 2007). It was suggested that the discrimination between Th17 and Th1 could be helped by the expression of CCR5 (Sato, Aranami & Yamamura 2007). It has been reported that ROR-γ t is important for generation of Th17 cells *in vitro* and *in vivo* (Aujla, Dubin & Kolls 2007a; Lim et al. 2008; Ortega et al. 2009). Not only IL-17, but also TNF-α, IL-6, IL-21 and IL-22 can be produced from Th17 (Ortega et al. 2009; Wilson et al. 2007). Despite large quantities of IL-17 produced by Th17, this particular cytokine is also generated by other cells of the innate immune system (Oukka 2008). There are several other cells which have the capability to express IL-17 including Natural Killer T (NKT) cells, γδ T cells,

neutrophils, monocytes and NK cells (Bhan, Cornicelli & Standiford 2008). It is suggested that the immune cells of innate response are also very important for IL17 production and these IL17-producing T cells provide efficient first line defence during microbial invasion .

1.9.1 The role of IL-17 against infections

Despite IL17 is involved in autoimmune diseases, It has may play a protective role against pathogens. It has been shown that IL17 provides protection against intracellular pathogens such as mycobacterium (Gaffen 2009; Matsuzaki & Umemura 2007).

Experiments have shown that mice without IL17R had a lower survival rateafter *Klebsiella pneumonia* infection (Matsuzaki & Umemura 2007). This bacterium is associated with an increase of bacterial numbers and the decrease of neutrophil emigration into the lung (Aujla, Dubin & Kolls 2007a; Matsuzaki & Umemura 2007). This is due to reduced levels of chemokines such as CXCL1 and CXCL2 in the lung (Aujla, Dubin & Kolls 2007a). These findings support the importance of IL17 in protective response against infections. Notably, the lack of IL17R led to reduced defence in the first phase of infection (Matsuzaki & Umemura 2007). It has been reported that lack of IL-17A in mice resulted in reduction of granulocyte colony-stimulating factor (G-CSF) and CXCL1 in the lung; therefore it may lead to infection (Ouyang, Kolls & Zheng 2008). Huter et al. suggested that the differentiation of Th17 cells cannot be controlled by CD4+CD25+ natural Treg cells

in autoimmune disease (Huter et al. 2008). However, another study by Zhou et al reported that purified Foxp3 Treg cells inhibited Th17 cells and may suppressed Th17 activity *in vivo* or *ex vivo* (Zhou et al. 2010).

There are multiple steps in the pathway of IL-17-mediated protection against infections as illustrated in figure1.6. First of all, transforming growth factor-beta (TGF-β) from Treg cells and IL-6 from activated APCs helps to induce Th17 differentiation. In next stage, after the recognition of antigens, Th17 cells will produce IL-17 in the presence of IL-23 from activated APCs. Finally, $IL17R^+$ cells are activated by IL-17 and these cells; i.e. endothelial cells and bronchio-epithelial cells induce the production of G-CSF (Aujla, Dubin & Kolls 2007a), granulocyte macrophage colony-stimulating factor (GM-CSF) (Vernal & Garcia-Sanz 2008) , which activate the neutrophils. Additionally, $IL17R^+$ cells produce β-defensin to enhance bactericidal activity, so the pathogens can be killed (Matsuzaki & Umemura 2007).

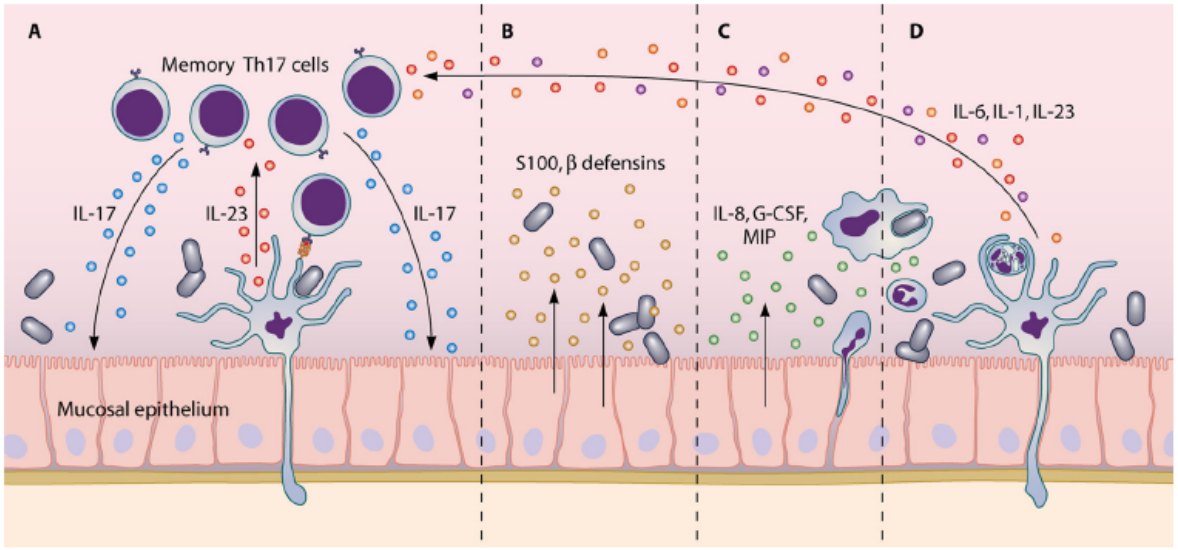

Figure 1.6 Th17 engagement in mucosal infection.

Mucosal DCs activated by pathogens secrete IL23 (A), leading the memory Th17 cells to produce IL17 and IL22 (B). These proinflammatory cytokines promote the secretion of antimicrobial peptides from epithelial cells (C). The expression of granulopoietic and chemotactic factors is driven by IL17. DCs are accumulated at the mucosal surface and phagocytosed the infected and apoptotic neurophils (D). Adapted from (Peck & Mellins 2010) .

1.9.2 The role of IL-17 against *streptococcus pneumoniae*

IL17 response can be induced by pneumococcal antigens such as cell wall polysaccharide (CWP) or whole cell antigens (WCA) (Bogaert et al. 2009; Lin, Slight & Khader 2010; Malley et al. 2006), especially with adjuvant cholera toxin and when given via natural route; i.e. mucosal administration (Malley et al. 2006). Neutralization of IL17 or IL17 receptor leads to increase in pneumococcal ccolonisation. Mice deficient of Th1 and Th2 cytokines, IFN-gamma and IL4 respectively were still capable of protection (Lu et al. 2008). Following secondary challenge with pneumococcus, recruitment of protective immune cells such as

monocytes, macrophages and neutrophils was shown to be mediated by IL-17-producing cells leading to clearance of pneumococcus. Upon antigen exposure, IL17 can be produced from CD4+Th17 memory cells in tonsillar tissues in both adults and children (Lin, Slight & Khader 2010; Lu et al. 2008). It has been reported that pneumococcal clearance in animal models is correlated with the cytokine IL-17A expression (Richards et al. 2010). Both IL-17A and neutrophils are likely to accelerate the clearance of ccolonisation of pneumococci. With the cooperation of antibody response, it may lead to effective immunity against this bacterial infection (Richards et al. 2010).

In HIV/AIDs patients, it was shown that with defects in the expansion of Th17cells, they were more susceptible to infections by *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (Crum-Cianflone, Weekes & Bavaro 2009; Hirschtick et al. 1995).

1.10 Development of pneumococcal protein vaccines

Currently, there are two types of vaccines, including 23-valent capsular polysaccharide vaccine (CPCV23) and pneumococcal conjugate vaccine (PCV). CPCV23 is immunogenic in adults and children older than 5 years. It induces a T-cell independent immune response. However, it is ineffective for children under 2 years (Bogaert, De Groot & Hermans 2004; Lu et al. 2009), because it does not induce activation of T cells (Bogaert, De Groot & Hermans 2004).

PCV, on the other hand, is more effective and immunogenic in children younger than 2 years due to the induction of a T-cell dependent immune response. Incidence of pneumococcal disease has been dramatically reduced by the introduction of PCV vaccines. However, pneumococcal infections due to non-vaccine serotypes (eg. 3, 7F, 19A) have been shown to increase following conjugate vaccination (CDC 2010; Lu et al. 2009).

Recent efforts have been made in the development of pneumococcal protein vaccines with well-preserved protein antigens among all pneumococcal strains. Protein antigens induce T cell dependent antibody response in all age groups and likely to induce immunological memory. Also, recombinant proteins are also cheap to produce. The best combination of proteins to be selected for vaccination purposes remains to be examined (Bogaert, De Groot & Hermans 2004). Ply and CbpA have been shown to be well conserved among pneumococcal strains, and are considered as promising vaccine candidates against carriage (Zhang et al. 2007; Zhang et al. 2006b).

1.11 Aims of study

As *S. pneumoniae* is a mucosal pathogen colonising human nasopharynx, local mucosal immunity in NALT is likely to be crucial in mediating protection against pneumococcal ccolonisation or carriage. It is hypothesised that mucosal Treg and Th17 in NALT are critical determinants in pneumococcal persistence/carriage or clearance. Therefore, this PhD project was designed to investigate:

1. The frequency of Treg and Th17 cells, and the ratio of Th17/Treg cells in NALT and PBMC in both children and adults, and their association with pneumococcal carriage.
2. What components of pneumococcal culture supernanant (CCS) activate/induce Foxp3+ Treg and Th17 cells?
3. Whether pneumococcal protein antigens activate/induce Th17 and Treg cells from tonsillar MNC or CD45RO-depleted MNC respectively.

Chapter 2: Materials and Methods

2.1 Human subjects and samples

Tonsillar tissues, peripheral blood and nasal swabs were obtained from subjects aged between 2 and 36 years undergoing adenoidectomy and/or tonsillectomy, Alder Hey Hospital for Children and Royal Liverpool and Broadgreen University hospitals Trusts due to upper airway abstruction or tonsillitis. Tonsillar tissues were collected into 25ml universal tube, containing 10ml Hank's balanced salt solution (HBSS) (Sigma Aldrich, UK) supplemented with 10µg/ml gentamycin and 1% L-glutamine (Sigma). Peripheral blood sample (2-5ml) was collected into a 25ml universal tube containing anticoagulant (heparin 100µl, LEO Pharma, UK). The patients' samples were then transported to the laboratory for processing. Individuals in this study who were received antibiotics and had serious infection or immunodeficiency were excluded.

2.2 Study ethics

This study was approved by the local research ethics committee, and informed consent was obtained from the volunteers and children's parents or custodian in each case. The project was performed in the laboratories of Clinical infection, Microbiology and Immunology department at the University of Liverpool.

2.3 Nasopharyngeal swab culture for detection of *Streptococcus pneumoniae*

2.3.1 Nasopharyngeal swab medium

On the day of that operation was performed, a nasopharyngeal (NP) swab was collected using a sterile Dryswab™ (Medical Wire & Equipment, UK), and reserved in screw-capped 1.5ml sterile vials containing 1ml of a special medium, contains skim milk (oxoid, UK), tryptone soya broth (TSB, from oxoid, UK), glucose (Sigma Aldrich, UK) and glycerol (Sigma Aldrich, UK). This medium is known as Skim-milk Tryptone Glucose Glycerol (STGG) transport medium (O'Brien et al. 2001) (appendix 1). Then the vial was transported into the laboratory to be stored at -80˚C and then cultured later for pneumococcus.

2.3.2 Detection and identification of pneumococcal isolates

The NP swab was thawed out at room temperature (25°C) and vortexed for approximately 10-20 second. Following a standard procedure as described previously (O'Brien & Nohynek 2003), 50µl of the NP swab was subcultured on a blood agar (BA) medium plate (Oxoid, UK), containing 5% sheep blood. Then it was streaked in 4-quadrant fashion for colony isolation by a sterile 5µl plastic loop (Technical Service Consultants Ltd, UK) using standard inoculating procedure. Then it was incubated for 18-24 hours (hr) at 37°C in CO_2 incubator. The growth on blood agar was carefully examined for the typical pneumococcal morphology, i.e. small, grayish, moist, watery surrounded by a greenish zone of alpha-

hemolysis (Fig.2.1.). Each suspected α-haemolytic growth was subcultured and confirmed by standard optochin disc inhibition test.

To perform the optochin susceptibility test, streaking the suspect alpha-hemolytic colony onto blood agar plate using 5µl plastic loops. An optochin disc (5ug) with 6mm diameters (Oxoid, UK) was placed onto the streaked area and then incubated in 5% CO_2 incubator at 37°C for 18-24 hr. If isolates sensitive to optochin (halo diameter >14mm), it is identified as *S. pneumonia (pneumococcus)* (O'Brien & Nohynek 2003).

(a) **(b)**

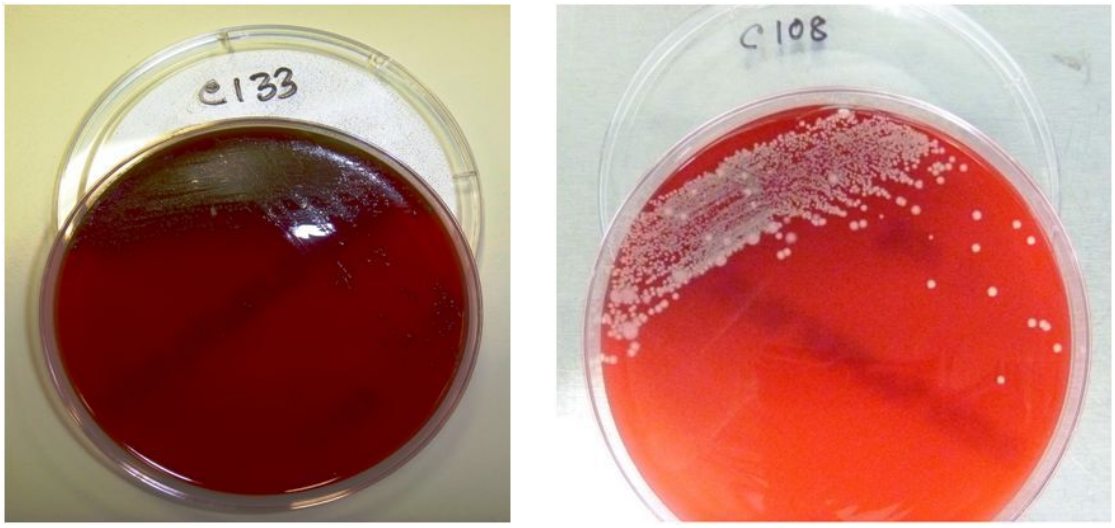

Figure 2.1 Identification of *Streptococcus pneumoniae* on blood agar plates.

Typical colonies of pneumococci with a small greenish α-haemolytic (a) compared with non-α-haemolytic non-pneumococcal colonies (b) of nasopharyngeal swabs.

2.4 Pneumococcal culture supernatant

A standard laboratory strain known as *Streptococcus pneumoniae* encapsulated type 2 strain D39 (NCTC7466) (Ogunniyi et al. 2000) was used in this project for cells stimulation beside other strains such as an isogenic choline binding protein A–deficient mutant (CbpA-), and an isogenic pneumolysin-deficient mutant (Ply-) (Berry et al. 1999). Pneumococcal concentrated culture supernatants (Pneumococcal CCS) were prepared from these three strains mentioned above. The CCS was prepared and concentrated using a specific method as described in a previous paper (Zhang, Choo & Finn 2002) and it will be described later in this chapter. During this stage of the project, different types of pneumococcal CCS were used at a protein concentration of 2µg/ml after determining the optimal concentration and dose response for cell stimulation.

2.4.1 Preparation of pneumococcal CCS

Todd-Hewitt Broth (THB) (Oxoid, UK) with yeast extract (0.5%) was used following manufacturer's instructions. Different pneumococcus strains such as wild type (D39 type 2) and mutant stains (CbpA- and Ply-) were taken out from -80°C freezer and defrosted. After that 5-10µl of each different strain was subcultured on BA medium for overnight in 5% CO_2 at 37°C. Following 18 hr approximately, typical observation of α-hemolytic colonies of streptococcus was grown on the BA. Subsequently, several colonies were picked up by sterile loop and cultured them overnight in the earlier prepared 3ml of THB in 5% CO_2 at 37°C. The turbidity of media indicates the bacterial growth. All 3ml of bacteria culture were added into

large cell culture bottles containing 100ml of THB media. The turbidity and optical density (O.D) of bacterial growth was read at 620nm using spectrophotometer. The growth was observed about 2–4 hr and depends on the how the quickness of bacterial growth was, then half hourly observation until O.D. value reach about 0.4-0.5 (approximately 10^8 cfu/ml). Culture tubes were centrifuged at 3000×g for 30 minutes (min) in order to remove the bacteria and the supernatant was filtered first through 0.4µm pore size and then 0.2µm sterile filter to remove the remaining bacteria. Finally, the filtered supernatants were concentrated to reach 10-fold at 3000×g for 30 min using Vivaspin15 concentrators (Sartorius Stedim Biotech, Germany). Numerous aliquots of CCS (1.5ml) were prepared and stored in freezer -80°C.

2.4.2 Biorad protein assay for measuring CCS protein concentration

The protein concentrations in the CCS were determined by the Bio-Rad protein assay through using Bradford protein dye reagent (Sigma) according to the manufacturer's instructions. Briefly, first, 2-fold of 8 serial dilutions of standard control bovine serum albumin (BSA) (starting with 1mg/ml) must be prepared in eppendorf tubes in order to perform the standard curve (Fig. 2.1. and table 2.1.). Furthermore, a preparation of CCS was assayed in duplicate and three different concentrations; started from the net and other two serial dilutions (1:10 and 1:100) in eppendorf tubes. A volume of 5µl of the prepared serial dilutions from standard and samples were dispensed into each well of a 96-well Costar plate in a duplicate. Then, 250µl of Bradford solution was added to the control and samples

then mixed thoroughly. The plate was incubated at room temperature in dark for 5 min. This solution is working as substrate. Samples and control were developed to navy colour. Finally, the plate was read at 595nm wavelength using a microtiter plate reader (Opsys MR, Thermo labsystems, UK). After determining the O.D of the control (BSA standard) and each sample, a calculation of protein concentration (mg/ml) was done against the standard curve produced from BSA standard. Deltasoft software (Biometallics Inc., USA) was used for analysis. Then the mean of 3 serial dilutions of CCS was compared with the control and interpolated to give appropriate concentration of BSA. The concentration of CCS that been measured by this assay was shown in table 2.2.

Table 2. 1 Mean OD of the BSA standard control measured by Biorad protein assay.

BSA (Standard)	Con. (mg/ml)	Mean OD
1	0.008	0.399
2	0.016	0.406
3	0.031	0.409
4	0.063	0.427
5	0.125	0.449
6	0.25	0.491
7	0.5	0.578
8	1	0.708

Table 2.2 Concentration of the pneumococcal CCS measured by Biorad protein assay.

Pneumococcal CCS	Conc. (mg/ml)	Concentration used for stimulation
D39wt	0.19	2µg/ml
Ply-	0.340	2µg/ml
CbpA-	0.24	2µg/ml

2.4.3 Western blotting for detection of Ply and CbpA in CCS

Western blotting was used to confirm the presence or absence of CbpA and Ply proteins in pneumococcal CCS derived from D39 strain and that there was no expression of CbpA or Ply in the CCS derived from the CbpA- and Ply- mutant strains (data shown in figure 2.2 below). The protein bands were separated on sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels dependent on the molecular weights of proteins.

2.4.3.1 Preparation of SDS-PAGE Gel:

Glass plate sandwich, casting stand and resolving (separating) gel (12%) should be prepared first. After preparing the resolving gel (appendix-1), the gel was poured quickly into the casting stand with leaving 5 mm gap from the top (up to the margin). Immediately, isopropanol was added on the top for leaving and smoothing the upper margin. The gel was left 15-20 min for setting. Isopropanol was washed from the top with d H_2O to allow drying by using filter paper for water absorbance. Then the stacking gel (appendix-1) was poured quickly into the casting stand. The

comb was applied carefully from an angle to avoid trapping of air bubbles. Finally, the gel was left 15-20 min for setting.

2.4.3.2 Sample Preparation

Samples (pneumococcal CCS) were diluted 1:3 with the prepared Laemmli buffer act as reducing buffer (appendix-1). The mixture was heated on a heat block at 100°C for 5 minutes to denature proteins. For recombinant CbpA and Ply proteins 1:60 dilutions were used. Then, 30µl of CCSwt, CbpA- and Ply- strains as well as 5µl of the ladder (Bio-Rad, UK) were loaded into the prepared gel or a 12% mini protean precast TGX™ gels (BioRad).

2.4.3.3 Gel Electrophoresis

The gel was loaded on the tank and the running buffer (appendix-1) was poured in the middle and side of gel chamber. The comb was removed gently then samples were loaded to the wells with long sample loading tips. The lid was placed on the top. Electrodes were connected with power-pack and run for 1 hr at constant volts (50m.Amp with a maximum 250V). Bromophenol blue was allowed to run through the gel until reached the bottom of the gel. At this point, the gel was taken out and put into transfer buffer. The small gel was removed carefully and the stacking gel was cut from the resolving gel using a scalpel.

2.4.3.4 Protein Transfer

A Transblot Turbo™ transfer system (Biorad) was used to perform Western blotting protein transfer into a 0.2µm nitrocellulose membrane (Transblot turbo transfer pack). The gel was placed onto a nitrocellulose membrane and then placed on to the bottom ion reservoir (anode) stack. The top ion reservoir (cathode) stack was then laid over the gel, and run for 10 min at a constant 25V and 1000Amp.

2.4.3.5 Western blotting detection of CbpA and Ply in pneumococcal CCS

Following Western blot transfer, the Nitrocellulose membrane was blocked with 5% skimmed milk in 0.05% PBS Tween-20 (appendix-1) for 90 min on the shaker with gentle shaking at room temperature (RT). The blocking solution was aspirated and 0.05% PBS Tween-20 was added for 5 min wash (repeated twice). The primary antibodies; rabbit anti-Ply and mouse anti-CbpA antiserum, were diluted 1:5000 in blocking solution then added and the membrane were incubated for 120 min at RT. The solution was poured and 0.05% PBS Tween-20 was added for 5 min wash (wash was repeated 4 times). The secondary antibody was prepared as follows: donkey anti-rabbit or anti-mouse IgG-HRP (1:10000 in blocking solution) and 3µl of StrepTactin-HRP (Bio-rad). Then it was added and incubated for 60 min. The solution was poured and 0.05% PBS Tween-20 was added for one minute wash (repeated 4 times). Finally, the substrate was prepared by adding equal volume (1:1) of Immun-star Western C chemiluminescence reagent A and B (BioRad). Before adding substrate, the membrane was washed with PBS then all PBS was removed. The prepared substrate was decanted onto the membrane and

incubated for 5 min. Excess substrate was removed with PBS and visualised and imaged using the Bio-Rad Chemi-Doc XRS system (Bio-Rad).

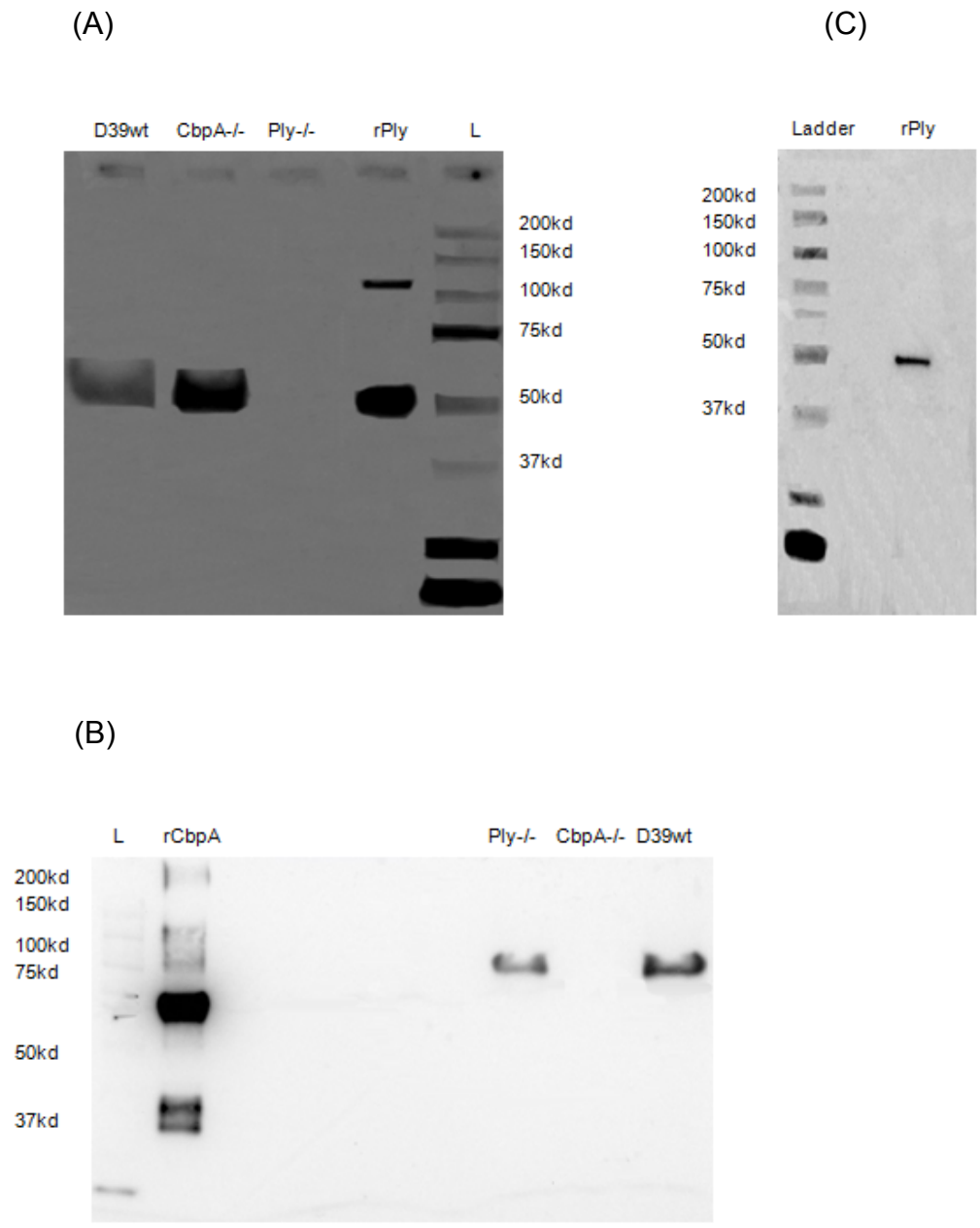

Figure 2.2 Western blot analysis of pneumococcal CCS (D39wt, CbpA- and Ply-) and recombinant Ply (rPly).

(a) Nitrocellulose membrane blotted with pneumococcal CCS were immunostained with rabbit anti-Ply antiserum showing that, Ply band is present in D39wt, CbpA- CCS but absent in Ply- mutant CCS. (B) Blotted with mouse anti-CbpA antiserum showing that, CbpA band is present in D39wt and Ply- CCS but absent in CbpA- mutant CCS. (C) Probing with rabbit anti-Ply antiserum showing the Ply band. (L: Ladder).

2.5 Pneumococcal antigens and mitogens

Some recombinant and native proteins were used as antigen stimulants such as rPly (WT) and PdB (W433F, provided by Profs Tim Mitchell (University of Birmingham) which were purified from recombinant *Escherichia coli* that expresses the respective cloned genes. Also, a affinity purified choline binding protein preparation (CBP) from pneumococcal CCS was used; type 2 strain D39wt. Recombinant pneumolysin (Ply) protein has a strong hemolytic activity, whereas PdB antigen is a toxoid derivative form of pneumolysin with a mutation of amino acid Trp433-Phe which reduces the hemolytic activity to approximately 1% without affecting antigenicity (Palaniappan et al. 2005; Paton et al. 1991). The endotoxin levels of the recombinant proteins were <0.01ng/µg of protein, as determined by the *Limulus* assay (Zhang et al. 2006a). To determine the best concentration of those proteins for cell stimulation, a range of concentrations of rPly at 10, 50 and 100ng/ml and of PdB at 100, 250 and 500ng/ml was used for cell stimulation. The optimal-dose response of both rPly and PdB for the induction of Treg and Th17 cells was at 50ng/ml and 250ng/ml, respectively.

Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Ion) were used for stimulation of existing Th17 by co-culture with adenotonsillar cells for 5 hours. Also, these mitogens were used in induction of IL17 from naïve T cells (CD45RO-). The optimal concentration of PMA used in these experiments was at 40pg/ml and for Ion was at 1µg/ml.

2.6 Affinity purification of Choline-binding proteins (CBP)

First, Vinylsulphone-activated agarose beads (1g, Sigma, Aldrich), which were immobilized with choline matrix were prepared by washing the beads with 10ml dH_2O. Addition of 10mM choline was added in sodium carbonate buffer (pH 11.5) into column (20ml) containing the prepared agarose and then rotated overnight at RT. Agraose beads were washed with 10ml PBS to get rid of unbound choline. 5-10ml of pneumococcal CCS was added into the econo-column chromatography (Bio-Rad) containing the choline-agarose beads and incubated for 30 min. The column was washed with 0.5M NaCL. Therefore, non-specifically binding components was washed out from the column and collected in new tubes (control). After that, choline-bound materials were passed though and eluted using 10% choline in PBS (w/v) (dissolving 2g of choline in 20ml PBS). This eluate was collected into a new tube containing mixture of proteins which were designated the CBPs. Finally, this mixture was concentrated by using Vivaspin15 concentrator at 3000×g for 30 min. As this technique is similar to the method used in Rosenow et al for isolation of CBPs (Rosenow et al. 1997), but instead of using live culture of pneumococci, a prepared pneumococcal CCS was used for the purification.

2.7 Protein quantification by Nanodrop spectrophotometer

Purified CBP preparation was quantified by Nanodrop 1000 spectrophotometer (Thermo Scientific). First the Nanodrop software was opened and selected at A280. After that, 2µl of deionized water was dropped on to the detector and the machine was calibrated. Then the detector was cleaned. Two µl of the buffer which

the proteins were being reserved in, was added to the detector as a blank control. Different protein elutions were then added to the detector, separately. Between each elution measurement, the machine was blanked by the buffer. The concentration of protein was determined and displayed on the software in mg/ml. The CBP concentration of the first elution was 0.05mg/ml.

2.8 Sample processing and cell separation

Patient's samples consist of tonsils, blood and nasopharyngeal swabs were transported to the laboratory and processed within few hours.

2.8.1 Isolation of mononuclear cells from tonsils

The sample tube that contained tonsils was rinsed with 10ml of HBSS. Then, the tonsils were transferred into a sterile petri dish containing HBSS. With the help of sterile forceps and the scalpel, tonsilar tissues were cut into small pieces and then teased to release cells into the HBSS medium. The HBSS containing the cells was passed through a cell strainer with pore size 70μm (BD bioscience, USA) into a fresh centrifuge tube (Falcon 50ml tube). Fifteen ml of Ficoll-Paque (Jencons) was added to an orange falcon tube and then the cell suspension were carefully pipetted on top of the Ficoll without disturbing it by letting it slowly runs down the side of the tube. Carefully, the tube was transferred to the centrifuge and spun for 30 min at 400x gravity (g) at 10°C. The interface layer of mononuclear cells was removed into a new orange tube and top up to around 45ml with sterile PBS. The tube was centrifuged for 10 min at 400xg at 10°C. The supernatant was poured off

and 3ml of PBS or RPMI medium (Sigma) was added to the cell pellet. Any remain tissue left over from tonsils was transferred into the sample tube with virkon and disposed into appropriate bins for autoclaving.

2.8.2 Serum preparation and isolation of peripheral blood mononuclear cells (PBMCs)

Blood sample was placed in the centrifuge and centrifuged for 10 min at 400g at 10°C. After that, the top yellow layer (serum) was pipetted off carefully using a sterile pasteur and transferred to 1.5ml eppendorf tube. Tube of serum was placed in -80°C freezers in the box marked serum. The rest of the blood was diluted with an approximately equal volume of PBS. Subsequently, the blood was treated as the same manner as described for tonsils except the cell pellet was re-suspended in 1ml of PBS or RPMI medium.

2.9 Cell counting

Cells isolated were diluted [tonsillar MNC (1:100) and PBMC (1:10) into eppendorf tube (1.5ml) through adding 10μl to an eppendorf containing 990μl of PBS. Subsequently, 10μl of the cell' suspension were transferred to the haemocytometer for counting in a 4 large squares. After that, the mean of the cells was obtained and multiplied by 10^4 and dilution factor if so, to give the number of the cells per ml. Alternatively, a disposal haemocytometer was used by transferring 10ul into it and the cells were counted by automated cell counter (Bio-Rad, UK).

2.10 Cell culture and stimulation:

Mononuclear cells (MNC) from peripheral blood and tonsillar tissues were isolated by Ficoll-Paque density centrifugation. MNC were re-suspended and cultured at $4X10^6$/ml in RPMI-1640 medium (sigma) supplemented with 1% L-glutamine, 10µg/ml gentamycin and 10% FBS (Sigma). In a sterile condition, aliquot of 0.5 ml of the cells was prepared per stimulation. Cells were cultured in 96-well flat bottom plate (Corning Incorporated, Corning, USA) with medium containing cells only or the addition of different stimulants after optimal concentration was determined such as D39wt CCS (2ug/ml), Ply- (2ug/ml), CbpA- (2ug/ml), recombinant ply (0.05ug/ml, PdB (0.25ug/ml) and CBP (2ug/ml). The plate was incubated in 5% CO_2 for up to 24 hr at 37˚C. Additionally, the cells were stimulated and reactivated by PMA (40pg/ml) and Ion (1ug/ml) in the presence of 3ug/ml brefeldin A (BFA) as a protein transport inhibitor (BD GolgiStop, eBiosciences) and incubated at 37°C in 5% CO_2 for 5hr. Plate was washed with 0.02% PBS/BSA and the cells were harvested into a new eppendorf tube for both extracellular and intracellular staining.

2.11 Depletion of CD45RO+ cells or CD45RO+ CD25+ cells from tonsillar MNC

In experiments designed for the induction of Th17 cells from naïve T cells, cellular depletion of CD45RO+ (memory and effector T cell phenotype) cells was performed by using magnetic cell sorting (MACS) according to the instructions of the manufacturer (Miltenyi Biotec). For experiments on the induction of Treg cells,

both CD45RO+ cells and $CD25^+$ cells were depleted from tonsillar MNC using MACS sorting.

The cells were firstly prepared using magnetic labelling. The cell numbers were determined and the suspension of the cells centrifuged at 300xg for 10 min. After discarding the supernatant, the cell pellets were re-suspended in 80µl of buffer (0.5% BSA in PBS) per 10^7 total cells. Twenty micro-liters of anti-human CD45RO+ micro-beads per 10^7 total cells were added, mixed well, and incubated for 15 minutes in the refrigerator (2-8˚C). The cells were washed by adding 2ml of buffer per 10^7 cells and then centrifuged at 300xg for 10 min. The cells were re-suspended up to 10^8 in 500µl of the buffer. The magnetic separation with LS column (Miltenyi Biotech) was used after labeling the cells with proper micro-beads. The LS column was placed in the magnetic field of suitable MACS separator. After rinsing the column with 2ml of the buffer, the cells suspension was applied onto the column. Eventually, the unlabelled cells (CD45RO- cells fraction) that passed through were collected into a new tube and the magnetic-labeled cells with CD45RO+ were retained on the column. This cell fraction can be eluted as a positive selected cell fraction.

Likewise, similar methods were performed for depletion of CD25+ cells. However, to deplete both CD25+ and CD45RO+, the appropriate amount of beads was added together at the same time to obtain CD25- CD45RO- MNC population (containing naïve T cells). The purity of depleted cells that yielded either CD45RO-

cells or CD25- CD45RO- was > 96%.

2.12 Induction of Th17 cells

In this experiment, CD45RO+ cell depleted MNC were used and cultured for 7 days in the medium alone or with the addition of different combination of cytokines that were in the presence or without pneumococcal stimulants. A number of cytokines were tested separately for optimal induction, including IL21, IL1-β and TGF-β. The optimal concentration of IL21 and IL1-β was 50ng/ml and of TGF-β was 2.5ng/ml. This combination of cytokines (IL21/IL1-beta/TGF-β) was added in the cells with or without antigens (all recombinant cytokines were bought from R & D Systems and also optimized for the best dose). Different controls including medium alone, cytokines alone and antigen alone were included. On day 7, the supernatants were collected and stored in the freezer at -80°C for further analysis by ELISA techniques to measure the level of IL17A, IL17F and IL22. Following antigenic stimulation (with/without cytokines), PMA/Ion and brefeldin A (BFA) were added to the cells on day 7 and incubated for another 5 hours in the incubator. The cells were harvested and washed with a cold buffer (BSA/PBS 0.02%) and transferred into the new eppendorf tubes for intracellular staining.

2.13 Induction of Treg cells

CD45RO+ and CD25+ cell--depleted tonsillar MNC were co-cultured for 7 days with pneumococcal antigens in the presence or absence of 2.5ng/ml of TGF-β (R&D system). Different controls were used including medium alone, antigen

alone, or cytokine alone were used to determine the individual effect in the induction of Treg. On day 7, the cells were harvested and the plate was washed with a cold buffer (BSA/PBS 0.02 %) and transferred into the new eppendorf tubes for intracellular staining, which is described in 2.15.

2.14 Flow cytometric analysis

Adenotonsillar MNC or PBMC were incubated for 20 min at RT in the dark place with mouse monoclonal anti-human antibodies. These antibodies were labeled with different fluorochromes (i.e. phycoerythrin (PE), fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE)-Cy5), such as anti-CD4, anti-CD25 and anti-CD127 as the surface staining for Treg phenotyping (BD Biosciences). After that, cells were washed with cold PBS/BSA 0.02% to remove the non-binding antibodies. Before analysis by flow-cytometry, 300-400uL of the washing buffer (depends on the cells numbers) was added to the cells and then the suspension was transferred into the FACS tube. Flow-cytometric data were analyzed using WinMDI software (version 2.9). Lymphocytes were gated and based on FSC/SSC and Treg cell markers (CD25 and CD127) and were determined within the CD4 and CD3 gate.

2.15 Intracellular staining for IL-17A and Foxp3

The fixation/permeabilisation solution (eBioscence) was prepared by adding one part of concentrated solution to 3 parts of diluents. A volume of 300µl of the prepared fixation/permeabilisation solution has been used to fix the surface marker

for 30-60 min at 4˚C in the dark place, followed by washing with 1-2ml of permeabilisation buffer diluted 1x. Subsequently, monoclonal antibodies labelled with fluorescence such as anti-foxp3-alexa@488, anti-IL17A-PE (BD bioscience) were added and incubated for at least further 30 min in the dark place at RT. However, a 100ul of fixative solution (eBiosciences) was used instead of using fixation/permeabilisation solution for IL17 staining. The cells were washed with permeabilisation buffer 1x. Finally, the cells were re-suspended in the buffer for analysis by flow-cytometry (FACSCalibur; BD Biosciences). The frequency of Treg cells was determined as the percentage of Foxp3+ or $CD4^{+}CD25^{high}/CD127^{low}$ cells in CD4+ T cells.

2.16 Enzyme-Linked Immunosorbent Assay (ELISA)

2.16.1 Measurement of cytokine production in cell culture supernatants

There are several ELISA techniques but the one used in this study for cytokine detection was sandwich technique for obtaining a high sensitivity. ELISA (Ready-SET-Go was used and bought from e biosciences. It was for determining the level of cytokines in the supernatant after stimulating the cells with pneumococcal antigens and other related proteins. The supernatant which was stored in the freezer at -80°C was performed by ELISA kit according to the instructions of the manufacturer.

2.16.1.1 Measurement of IL-17A

ELISA 96-well plate (Corning Costar) was coated with 100µl/well of capture antibody in Coating Buffer. The plate was sealed and incubated overnight at 4°C. Next day, the wells were aspirated and washed 3 times with buffer (1x PBS, 0.05% Tween-20) using plate washer. To increase the effectiveness of the washes, the wells were soaked (~1 min) during each wash step. Plate was blotted on absorbent paper to remove any residual buffer. One part of 5X concentrated assay diluent was diluted first with 4 parts of dH_2O. Then the wells were blocked with 200µl/well of 1X assay diluent and incubated at RT for 1hr. The plate was washed once with wash buffer. To prepare the top concentration of the standard (500pg), 1X assay diluent was used. Then 100µl/well of top standard concentration was added to the appropriate wells. Two-fold serial dilutions of the top standards were performed to make the standard curve for a total of 8 points. After optimizing the samples' dilution in the block buffer, 1:10 and 1:5 were the greatest dilutions for supernatants derived from both MNCs and CD45R0- depleted MNCs (after 3 and 7 days of incubation, respectively). A hundred µl/well of the diluted samples were added to the appropriate wells. The plate was sealed and incubated at RT for 2 hr (or overnight at 4°C for maximal sensitivity). The plate was aspirated and washed as mentioned above and repeated for a total of 3-5 washes. A hundred µl/well of detection antibody diluted in 1X assay diluent was added into the wells. The plate was sealed and incubated at room temperature for 1 hr. The plate was aspirated and washed as mentioned above and repeated for a total of 3-5 washes. Addition of 100µl/well of avidin-horse radish peroxidase (HRP) diluted in 1X assay diluent

(dilution was prepared as noted in appendix 1) was added and the plate was sealed and incubated at RT for 30 min. The plate was aspirated and washed and repeated for a total of 5 washes. In this wash step, the wells were soaked in wash buffer for 1-2 min prior aspiration. To each well, 100µl of tetramethyl benzidine (TMB) substrate solution was added and the plate was incubated at RT for 15 minutes. Finally, to stop the reaction, 50µl of stop solution (2N H_2SO_4) was added to each well. The plate was read at 450nm 15 min later using a plate reader (Opsys MR, Thermo labsystems, UK). The cytokine concentration (pg/ml) was calculated against the standard curve, with the help of microplate analysis DeltasoftPC (Biometallics, Inc., USA).

2.16.1.2 Measurement of IL-17F

IL17F was measured following a stimulation of CD45RO+cell-depleted MNC. Human IL-17F ELISA Ready-Set-Go® set (eBioscience) was used following manufacturer's instructions. The top standard concentration was used at 500pg/ml. The protocol was the same as described for IL17A; except that the samples were diluted (1:10) in blocking buffer and been optimised for supernatants derived from CD45RO+ cell- depleted MNC.

2.16.1.3 Measurement of IL-22

IL22 was measured following stimulation of CD45RO+ cell-depleted MNC. Human IL-22 ELISA Ready-Set-Go® set (eBioscience), was used following manufacturer's instructions. The top standard concentration was used at 500pg/ml. The protocol

was the same as described for IL17A; except, the samples were diluted (1:40) in blocking buffer and been optimised for supernatants derived from CD45R0-depleted MNC.

2.16.1.4 Measurement of TGF-beta

Human TGF-β ELISA Ready-Set-Go® set (eBioscience) was used for this assay to detect human TGF-β in tonsillar MNC culture supernatants following 72 hr stimulation by pneumococcal CCS.This assay was conducted to recognise the mature/active form of TGF-β without the association of latency association peptide (LAP). A standard ELISA procedure was performed following manufacturer's instructions as in IL17A. Unlike IL17A, IL17F andIL22, the samples (but not the standard) were acid-activated (HCl treatment) in order to activate latent TGF-β to immunoreactive form and then neutralized. Briefly, the samples were diluted first to 1:10 (the optimised dilution). After that, 20µl of 1N HCl were added to per 100µl of samples then incubated for 10min at RT. Twentey µl of 1N NaOH was added for neutralisation. The final concentration of the samples were calculated and corrected to dilution factor (1.4.) and the final dilution become 1:14. The top standard concentration was used at 1000pg/ml, and then the procedure was the same as described for IL-17A measurement.

2.16.1.5 Measurement of CbpA-specific IgG antibody in cell culture supernatants

CbpA specific antibodies (anti-CbpA) were detected in cell culture supernatant by ELISA using the following protocol. Antigen coating solution (1µg/ml) was prepared from recombinant CbpA (1mg/ml) into 10ml of sterile PBS. A 96-well plate (Corning) was coated with 100µl of coating solution per well. The plate was then incubated overnight in 4°C. Next day, the plate was washed 5 times with PBS containing 0.05% Tween 20. The plate was then blocked (200µl/well) with blocking buffer (10% FBS in PBS) at 37°C for 1 hr. During that time, samples and standards were prepared by diluting them (1:30) in blocking buffer. Human immunoglobulin (sandoglobulin, Sandoz, UK), containing high IgG antibody titers to CbpA antigens, was used as a reference standard for measurement of IgG antibodies. Two-fold serial dilutions of the top standards were performed to make the standard curve for a total of 8 points. The plate was incubated at RT for 2 hr. The plate was washed 5 times with washing buffer. 100µl of alkaline phosphatase-conjugated mouse anti-human IgG (1:2000 dilutions) (Sigma) was added into each well. Then the plate was incubated at RT for 2 hr. Thereafter, the plate had been washed 5 times with the buffer. Addition of 50µl of freshly prepared P-Nitrophenyl phosphate (PNPP) substrate into every well (appendix-I), then the plate was incubated in the dark room at RT until the colour devolved. Finally, the absorbance (OD) was measured using a microtiter plate reader (Opsys MR, Thermo labsystems, UK) at 405nm. The concentration (units/ml) of each sample was calculated against the standard curve with serial dilution of Sandoglobulin (Sandoz, UK). DeltasoftPC microplate

analysis software (BioMetallics, Inc., USA) was used for data analysis. The optical density (OD) at 405nm was measured after 60 min of adding the substrate solutions. The anti-CbpA titre of sandoglobulin was assigned as the reciprocal dilutions at which their corresponding OD reached to 1.00 at that time point. Based on this, the stock solution of Sandoglobulin was assigned to an antibody titre of 1000units/ml for anti-CbpA.

2.17 Statistical analysis

Statistical analysis in this study was performed using GraphPad Prism software (version 5.5). Differences between two groups were analysed by student t test. The difference between un-stimulated and stimulated cells by all different pneumococcal CCS and proteins' antigens was assessed by paired t (parametric) test. Difference among groups (>2) were analysed by one-way variance (ANOVA). Two-factor correlation analysis was performed by Pearson's correlation test (parametric). A p value of < 0.05 was considered statistically significant

Chapter 3: Association of Treg and Th17 cells in Nasopharynx-Associated Lymphoid Tissue with Pneumococcal Carriage in Children and Adults

3.1. Introduction

Regulatory T cells (Treg) have been shown to be essential in immune regulation during microbial infection, allergy, autoimmunity and transplantation. There are two main types of Treg, known as natural Treg (nTreg) and inducible Treg (iTreg). Treg express a number of markers, including CD25, CTLA-4, GITR, LAG-3, CD62L, CD103 and CD39 (Abdulahad, Boots & Kallenberg 2010). $CD25^{high}$ Treg possess potent immunosuppressive function towards auto-reactive peripheral T cells observed either *in vivo* or *ex vivo* (Huibregtse, van Lent & van Deventer 2007; Mills 2004). In addition to the surface markers, Treg express an intracellular transcription factor Foxp3 which is considered to be a specific molecular marker for both thymic and peripheral Treg (Piccirillo 2008).

It has been shown that Treg play an important role in suppressing immune responses to pathogens, including T helper 1 (Th1), T helper 2 (Th2) or cytotoxic T lymphocytes (CTL) type response (Mills 2004). Treg have been suggested to contribute to the persistence of bacterial and viral infection via their ability to inhibit activation of effector immunity including the production of proinflammatory cytokines (Chen & Konkel 2010). Th1 and Th2 were first discovered by Mosmann and Coffman based on specific cytokines and effector functions (Mosmann & Coffman 1989). A new subset of T helper (Th) cells named Th17 has been identified more recently. Th17 cells have been shown to produce Interleukine (IL)-17A, IL-17F and IL-22. Th17 cells express transcriptional factor retinoic-acid-related orphan receptor γ t (ROR-γ t). During infection, Th17 have been shown to accelerate the clearance of extracellular pathogens (Miossec 2009). Th17 are

generally characterised as CD4+ IL17A-producing cells, although co-expression of chemokine receptors including CCR4 and CCR6 have also been used for the identification of memory Th17 cells (Korn et al. 2009) .

Unlike Treg, Th17 may have a detrimental role in the pathogenesis of autoimmune diseases. Th17 may play an important role in protection against microbial infections. It has been shown that Th17 provide protection against intracellular pathogens such as mycobacterium (Matsuzaki & Umemura 2007). However, Th17 may contribute to tissue damage, particularly during intracellular infection (Bacellar et al. 2009). It has been reported that clearance of pneumococcal colonisation in animal models was correlated with cytokine IL-17A expression (Lu et al. 2008; Richards et al. 2010). It has been suggested that IL-17A may promote the clearance of pneumococci through chemokine-mediated neutrophils. In combination with antigen-specific antibody responses, Th17 will likely elicit an effective immunity against this bacterial infection (Richards et al. 2010). In this study, we aimed to study the association between Th17 and Foxp3+Treg in nasopharynx-associated lymphoid tissue (NALT) and their relationship with nasopharyngeal carriage of pneumococcus in children and adults.

3.2. Aim of the study

To investigate:

1- The frequency of Treg and Th17 cells, and the ratio of Th17/Treg cells in NALT and PBMC in both children and adults, and their association with pneumococcal carriage.
2- Whether pneumococcal CCS induces/activates Th17 and Treg.
3- The effect of Treg cells in NALT on antibody production.

3.3. Experimental design

To determine the frequency of Treg and Th17, freshly isolated MNC from tonsillar tissue and peripheral blood were stained with fluorescence-labelled mouse anti-human antibodies to CD4, CD25, CD127 and Foxp3 (for Treg) or intracellular IL-17A (for Th17) and analyzed by flowcytometry on a FACSCalibur (BD Bioscience). The frequencies of Treg and Th17 were then analysed in association with pneumococcal carriage in children and adults.

To determine whether pneumococcal stimulation induces Treg or Th17, tonsillar MNC were stimulated with pneumococcal culture supernatant (CCS, prepared from wild type pneumococcus D39) for 24 hours then the intracellular expression of Foxp3 and IL17A were analysed by flow-cytometry following cell permeabilisation and fixation (eBioscience).

3.3.1. Human subjects and samples

In this study, tonsillar tissues and peripheral blood samples were taken from adults and children aged between 2-36 years who had tonsillectomy. On the day of the operation, a nasopharyngeal (NP) swab was taken and stored at -80˚C for later pneumococcus culture. Individuals who had serious infection or immunodeficiency and had received antibiotics 3 weeks prior to operation were excluded from this study. Informed consent was obtained from the children's parents or custodian in each case.

3.3.2. Detection of Th17 cells

To determine the frequencies of Th17 in NALT and peripheral blood, tonsillar MNC or PBMC were isolated and stimulated by Phorbol 12-myristate 13-acetate (PMA) and ionomycin (Ion) for 5 hr in order to activate Th17. The optimal concentration of PMA used in this experiment was at 40pg/ml and for Ion was at 0.5μg/ml. The concentrations were determined from testing a range of different concentrations and optimal concentrations were chosen.

3.3.3. Staining of the surface and intracellular markers of Treg and Th17 cells

Mononuclear cells (MNCs) were isolated either from the tonsillar tissues or peripheral blood by Ficoll gradient centrifugation, the cells were stained with anti-CD3, -CD4, -CD25 and -CD127 (BD Biosciences) as surface markers for Treg and

incubated for 20 minutes (min) at the room temperature (RT) and in the dark. Fixation/Permeabilisation buffer (eBiosciences) were used after staining the surface markers and incubated for 30 min at 4˚C. Subsequently, cells were incubated with mouse anti-Foxp3-Alexa-488 and anti-IL17-PE (BD Bioscience) for 30 min in the dark at room temperature. Cells were then washed with permeabilisation buffer. Finally, the cells were re-suspended in the buffer for analysis by flow cytometry (FACSCalibur; BD Biosciences).

Th17 cell and Treg were determined within CD3+ and CD4+ T lymphocyte gate and the frequencies of Th17 cells were expressed as the percentage of CD4+ T cells staining positive for IL17A. Frequencies of Treg were expressed as the percentage of CD4+ T cells staining positive for Foxp3 or as $CD25^{high}$, $CD127^{low}$. Flow cytometric data were analysed using WinMDI software (version 2.9).

3.3.4. Cell culture and stimulation by pneumococcal CCS

Tonsillar MNC or PBMC were incubated with wild-type pneumococcal CCS for 24 hours and followed by addition of brefeldin A (BFA) for 6 hr (details see in chapter 2, materials and methods). Briefly, tonsillar MNC or PBMC were isolated and cultured at 4 X 10^6/ml in RPMI medium (sigma) supplemented with glutamine, penicillin, streptomycin and 10% FBS. The cells were cultured in 96-well flat bottom plate (Corning Incorporated, Corning, USA) with or without the addition of pneumococcal CCS at 2µg/ml (protein concentration). The culture plate was incubated for up to 24 hr at 37˚C, in the presence of CO_2 (5%). The cells were

harvested into a new eppendorf tubes for both extracellular and intracellular staining as described in chapter 2, materials and methods.

3.3.5. ELISA assay for IL17A measurement

To assess the level of IL17A in culture supernatant of tonsillar MNC, ELISA plate (Corning Costar 9018) was coated with 100µl/well of capture antibody in Coating Buffer. The plate was incubated overnight at 4°C. Next day, the wells were aspirated and washed 3 times with washing buffer (1x PBS, 0.05% Tween-20) using plate washer. Then the wells were blocked with 200µl/well of blocking buffer for one hour. The plate was washed once with wash buffer. Then 100µl/well of top standard concentration was added to the appropriate wells. Two-fold serial dilutions of the top standards were performed to make the standard curve for a total of 8 points. 100µl/well of the diluted samples were added to the appropriate wells and incubated at RT for 2 hr. The plate was washed before 100µl/well of diluted detection antibody was added into the wells and incubated at room temperature for 1 hr. Following wash, 100µl/well of avidin-horse radish peroxidase (HRP) was added and incubated at RT for 30 min. The plate was washed for a total of 7 washes. To each well, 100µl of substrate solution was added and incubated at room temperature for 15 min. Finally, 50µl of stop solution (2N H_2SO_4) was added to each well. The plate was read at 450nm using a plate reader.

3.3.6. ELISA assay for TGF-β measurement

This assay was conducted to recognise the mature/active form of TGF-β without the association of latency association peptide (LAP). To detect human TGF-β in tonsillar MNC culture supernatants following 72 hr stimulation by pneumococcal CCS, a standard ELISA procedure was performed following manufacturer's instructions, which was similar to the above protocol except that samples were acid-treated with HCl and neutralised prior to evaluation in this assay. Also, the top standard concentration was used at 1000pg/ml (details see materials and methods).

3.3.7. Detection of CbpA-specific IgG antibodies in cell culture supernatants by ELISA

ELISA technique was performed as described in chapter 2. Briefly, recombinant CbpA antigen was used for coating ELISA plate and then incubated overnight at 4°C in the fridge. After that the plate was washed and then blocked with 10% FBS-PBS. Following incubation of cell culture supernatants, alkaline phosphatase conjugated anti-human IgG was added and incubated and followed by the addition of PNPP substrate. Finally, the plate was read at 405nm to measure the optical density. DeltasoftPC microplate analysis software (BioMetallics) was used for data analysis.

3.3.8. Statistical analysis

Differences between groups were analysed by ANOVA and student-t test. Correlation between two factors was analyzed by Pearson's correlation test. Student's paired-t test was used to compare un-stimulated and stimulated cells. A *p* value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (version 5).

3.4. Results

3.4.1. Frequencies of Treg in human NALT and peripheral blood

To determine the frequency of Treg in tonsillar tissue and peripheral blood, tonsillar MNC and PBMC were stained with a combination of CD4, CD25 and CD127 surface markers or Foxp3. The frequency measured by $CD4^{+}CD25^{high}CD127^{low}$ and that by CD4+Foxp3+ showed that same pattern.

Representative figures were shown in Fig.3.1. As can be seen in figure 3.2.a, the mean frequency of Treg cells in tonsillar MNCs (8.5%) was higher than in PBMCs (5.5%). There was a difference between children and adults in the frequency of Treg cells in both tonsillar tissues and peripheral blood. In children, the mean frequency of Treg cells in tonsillar MNC was 9.7% compared to 6.2% in PBMCs. In adults, the respective frequencies for tonsillar MNC and PBMC were 5.6% and 4.1% respectively (Fig.3.2.b). Overall, Treg frequency in tonsillar tissue of children (mean: 9.7%) was higher than in adults (5.6%).

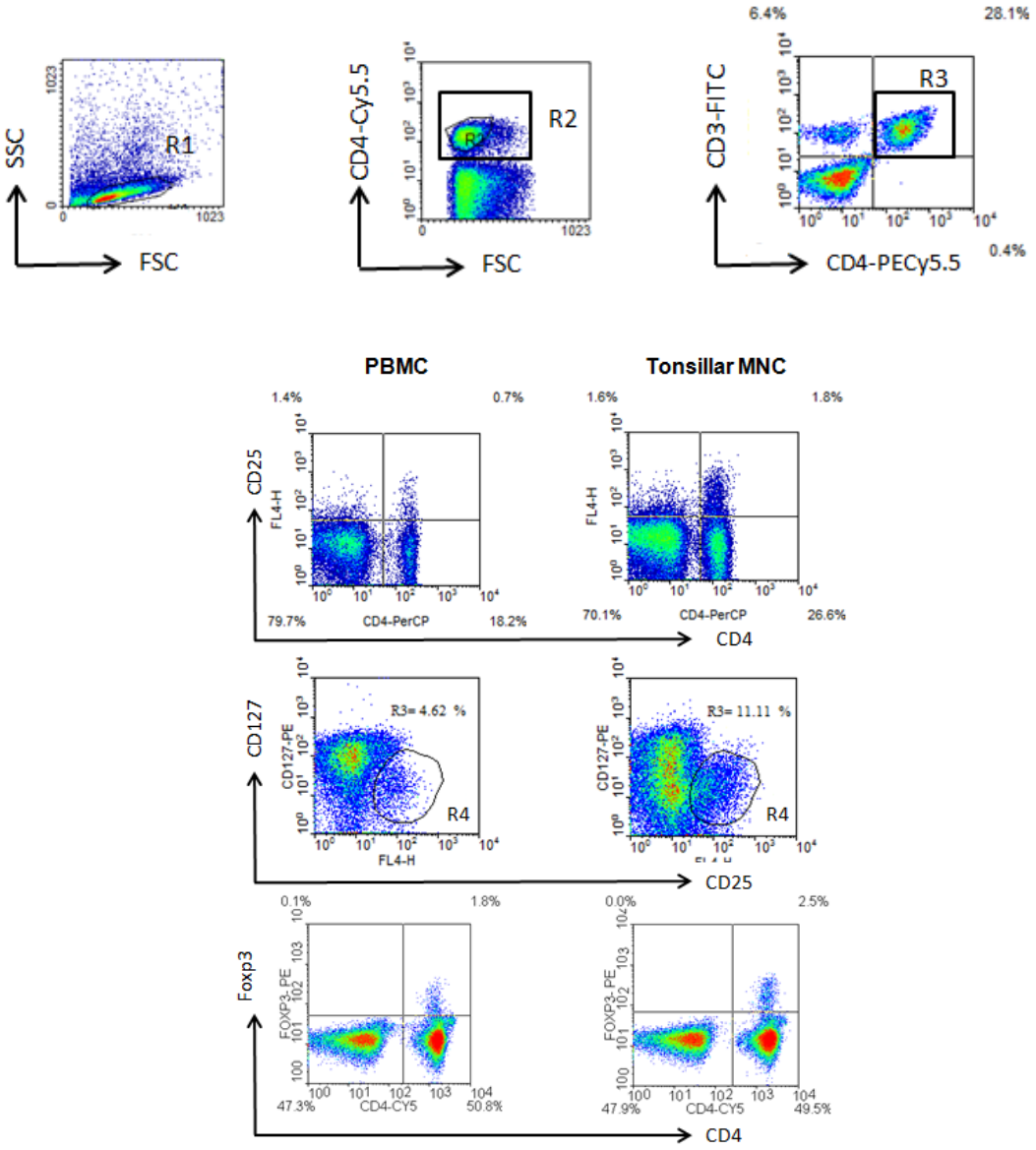

Figure 3.1 One representative sample analysis showing gating strategy for identification of Treg (CD4+CD25highCD127lowFoxp3+) in tonsillar MNC and PBMC.

Freshly isolated tonsillar MNC and PBMC were stained for CD3, CD4, CD25 and CD127. Lymphocytes were defined by typical FSC and SSC (R1) (A). CD4+ T cells were identified by positive staining of CD3+ and CD4+ cells (R2) Treg were identified by gating on CD4+ T cells (R2), and positive staining of CD25high CD127low (Treg). The percentage of Treg cells in CD4+ T cell population was defined as Treg frequency (R4).

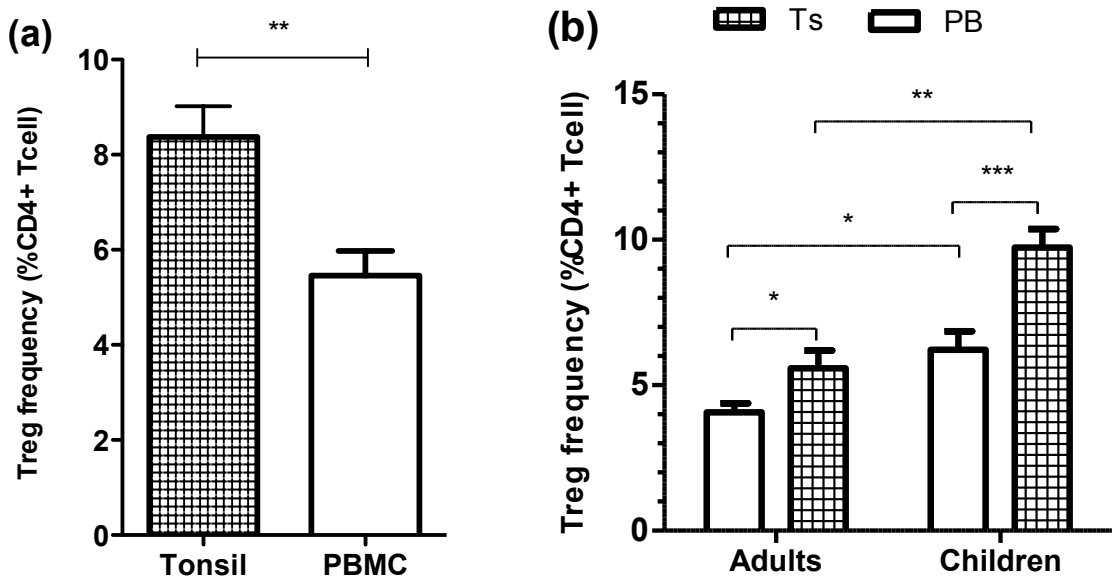

Figure 3.2 Frequencies of Treg cells in tonsillar MNC and PBMC.

(a) The frequency of Treg cells in tonsillar MNC is shown to be higher than in PBMC (**p< 0.01, n=23). (b) Frequencies of Treg cells in tonsillar MNC and PBMC in both children (n=17) and adults (n=6) are shown. Mean+SEM are shown. *p<0.05, **p< 0.01, ***p< 0.001.

3.4.2. Relationships between Treg frequencies, age and pneumococcal carriage

Frequencies of Treg in tonsillar tissue were analysed in association with ages of patients. There appeared to be an inverse relationship between the Treg frequency in tonsillar tissues and age (Fig.3.3, ***p<0.001). Overall, younger children <10 years were shown to have higher Treg numbers than those those subjects >10 years.

As we know from previous results that pneumococcal carriage rate also decreases with age, we analysed the relationship between Treg frequencies in tonsillar tissue and pneumococcal carriage in children. As can be seen from figure 3.4, children who were pneumococcal culture positive in nasopharynx had a high frequency of tonsillar Treg cells than those children who were pneumococcal culture negative (**p< 0.01). The mean frequency of tonsillar Treg cells in culture positive children was 9.2% compared to 6.8% in culture negative children.

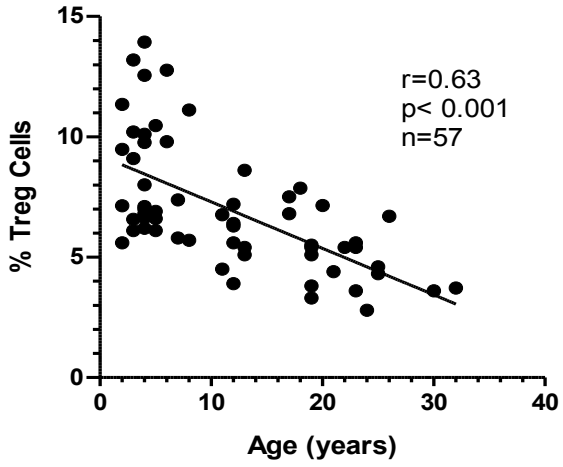

Figure 3.3 Relationship between frequencies of tonsillar Treg cells and age.

There is an inverse correlation between frequencies of tonsillar Treg cells and patients age (r=-0.63, n=57, ***p< 0.001). An age-related decrease in Treg numbers was shown between different age groups.

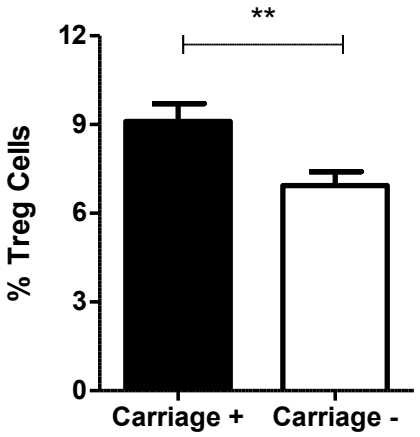

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Figure 3.4 Comparison of tonsillar Treg frequencies between culture+ and culture- children:

Frequencies of tonsillar Treg cells were compared between children who were culture positive (n=18) for pneumococcus and those who were culture negative (n=21). Mean + SEM are shown **p< 0.01 culture+ vs culture -.

3.4.3. Frequencies of Th17 cells in NALT and PBMC in children and adults

To determine the frequencies of Th17 in NALT and peripheral blood, tonsillar MNC and PBMC were analysed by intracellular cytokine staining for IL-17A and the frequency was expressed as the % of IL17A-producing cells in CD4+ T cell population. A marked difference in Th17 frequencies between tonsillar MNC and PBMC was shown (figure 3.5.a). The mean frequency of Th17 in tonsillar MNC was 1.4% compared to 0.4 % in PBMC following stimulation by PMA/Ion (figure 3.5. b). There was a significant difference between children and adults in the frequency of Th17 cells in tonsillar MNC (figure 3.6.a). The mean frequency of Th17 cells in tonsillar MNC in adults was higher than in children (figure 3.6.b, ***p< 0.001).

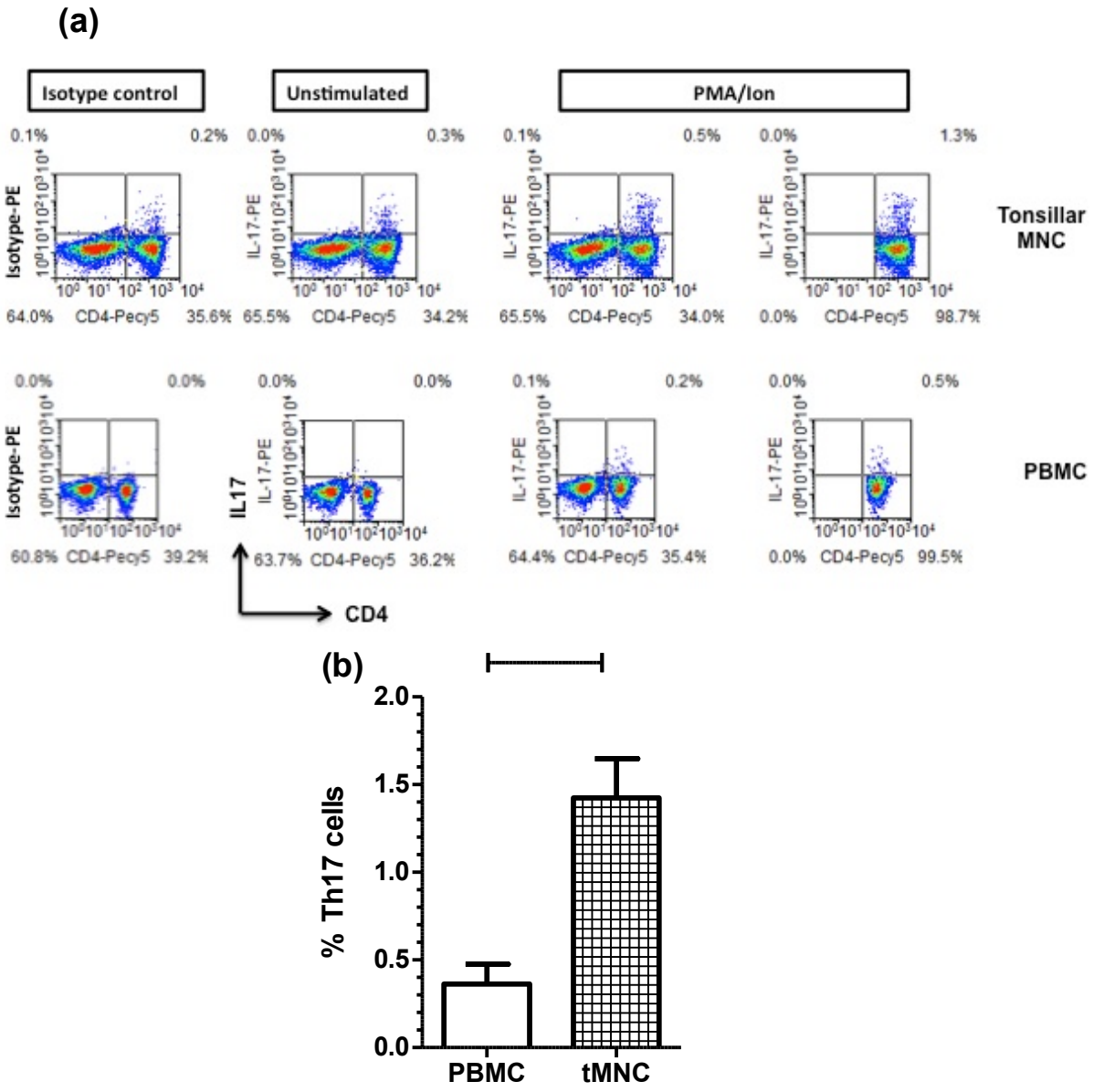


Figure 3.5 Frequencies of Th17 cells in tonsil and PBMC.

Tonsillar MNC and PBMC were stimulated with PMA/Ion for 5 hours followed by intracellular cytokine staining of IL17A. (a) A representative FACS dot plots showing intracellular staining of IL17A in tonsillar MNC and PBMC gated for lymphocytes. Frequency of Th17 is expressed as % of IL17+ cells in CD4+ T cells. Compared with unstimulated control, Th17 cells numbers between tonsillar MNC and PBMC after stimulation with PMA/Ion (**p< 0.01, PBMC vs tMNC, n=10). Mean+SEM are shown.

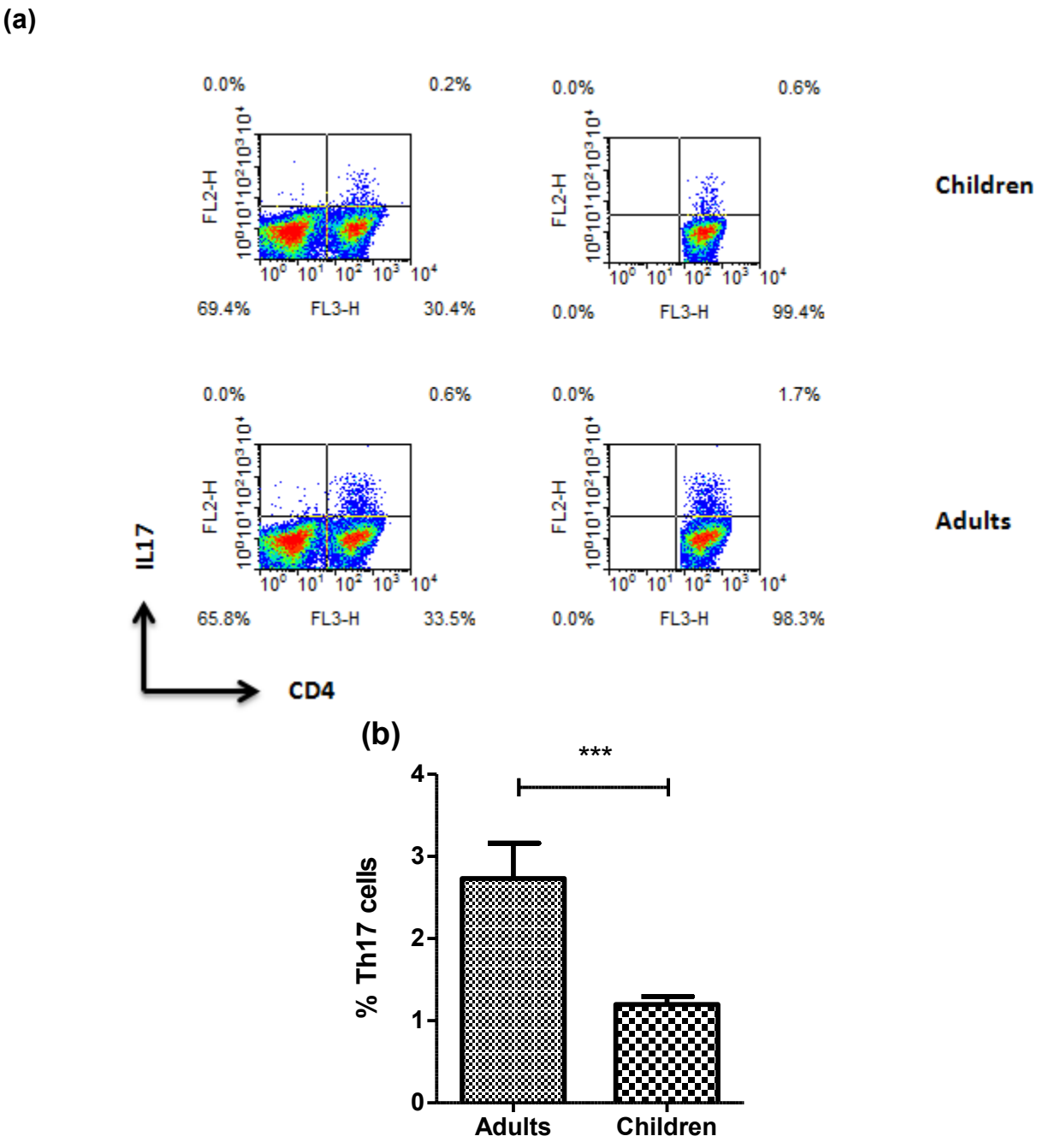


Figure 3.6 Comparison of frequencies of tonsillar Th17 between adults and children.

Frequencies of Th17 in tonsillar MNCs in adults and children following stimulation by PMA/Ion for 5 hours. (a) A representative density plot of intracellular staining of IL17A in tonsillar MNC CD4+ T cells. (b) The mean frequencies of tonsillar Th17 in adults (n= 22) and children (n= 35). Mean + SEM are shown. ***p< 0.001, children vs adults.

3.4.4. Association of Th17 frequency and age and pneumococcal carriage

The relationship between frequencies of tonsillar Th17 and ages of patients were analysed. There was a significant difference between older children younger children. The frequencies of tonsillar Th17 in younger children <10 were significantly lower than those subjects >10 years (figure 3.7.).

Relationship between frequencies of tonsillar Th17 and pneumococcal carriage in children was also analysed. Children who were culture-negative for pneumococcus tended to have a relatively higher frequency of tonsillar Th17 cells compared to in those children who were culture-positive (figure 3.8, $p>0.05$), although the difference was not statistically significant. The mean frequency of tonsillar Th17 cells in culture-positive children was 1% compared to 1.3% in culture-negative children.

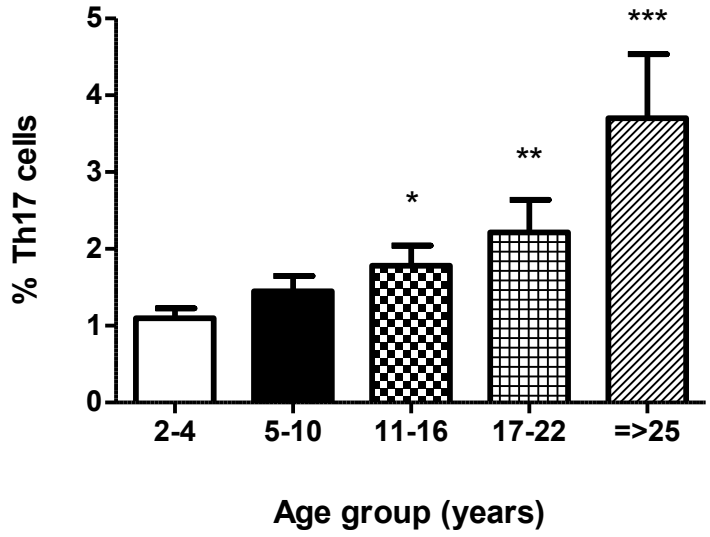


Figure 3.7 The relationship between Th17 numbers and age.

Tonsillar MNC were stimulated by PMA/Ion for 5 hours in the presence of brefeldin A. Flow cytometry of intracellular staining of IL17A in CD4+ T lymphocytes was used in this experiment. An age-related increase in Th17 numbers was shown between different age groups 2-4 years (n=22), 5-10 years (n=8), 11-16 years (n=5) 17-22 years (n=13) and =>25 years (n=9). Mean+ SEM are shown. *p< 0.05, **p<0.01, **p<0.001 compared to groups 2-4 years. ANOVA and student-t test were used.

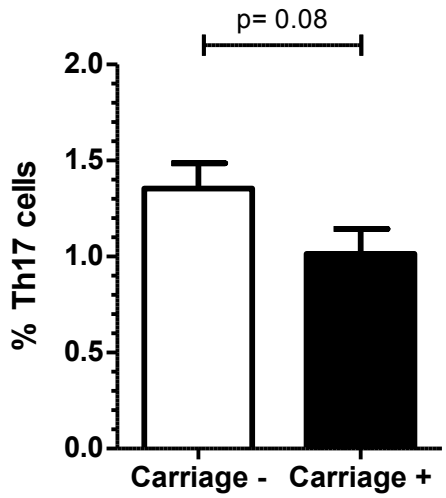


Figure 3.8 Comparison of tonsillar Th17 frequencies between culture+ and culture- children.

Comparison between children who were culture+ (black column, n=14) for pneumococcus and those who were culture- (white column, n=21) in Th17 frequency in tonsillar MNC. Mean+SEM are used. p>0.05, culture+ vs culture -.

3.4.5. Relationship between the ratio of tonsillar Th17/Treg, age and pneumococcal carriage

It has been postulated that mucosal Th17 and Treg play opposite roles in mediating immunity to an infection. We sought to find out whether the ratio of adenotonsillar Th17/Treg is important in mediating pneumococcal carriage in children and adults. There appeared to be a positive correlation between the ratio of Th17/Treg in tonsillar tisuues and age ((figure 3.9, ***$p < 0.001$). In adults, the ratio of tonsillar Th17/Treg MNC was shown to be significantly higher than in children with a mean ratio of 0.67 in adults and 0.23 in children respectively (figure 3.10, ***$p < 0.001$).

When the ratio of tonsillar Th17/Treg was analysed in association with pneumococcal carriage, children who were culture positive for pneumococcus showed a higher ratio of Th17/Treg than those children who were negative culture with a mean ratio of 0.27 and 0.14 respectively (figure 3.11, *$p< 0.05$).

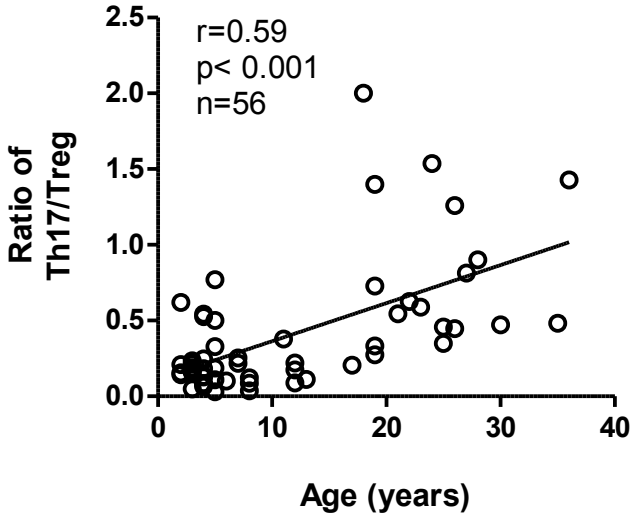


Figure 3.9 Relationship between the ratio of Th17/Treg and age.

There is a positive correlation between Th17/Treg ratio and patients age (r=0.59, n=56, ***p< 0.001). An age-related decrease in the ration is shown between different age groups.

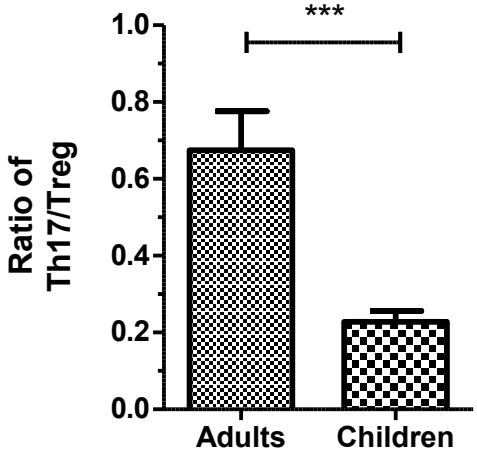


Figure 3.10 The ratio of Th17/Treg cells in adults is higher than children.

The ratio of tonsillar Th17/Treg in adults (n=21) and children (n=35). Mean+SEM are shown. ***p<0.001, adults vs children.

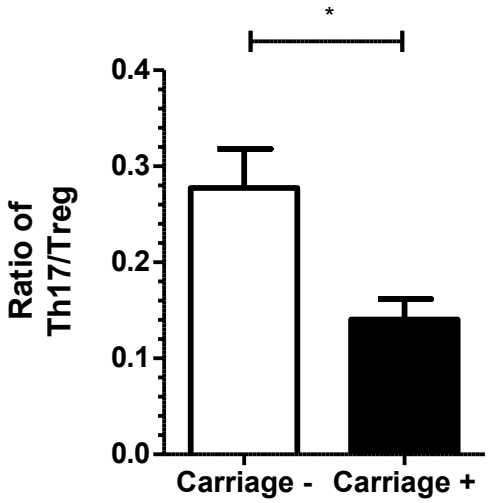


Figure 3.11 Relationship between the ratio of Th17/Treg cells and pneumococcal carriage in NALT in children.

The ratio of tonsillar Th17/Treg in children who were culture positive (n=13) with pneumococcus compared with those who were culture negative (n=22). Mean+SEM are shown. *p< 0.05 culture+ vs culture -.

3.4.6. Response of Th17 and Treg in pneumococcal carriage

3.4.6.1. Pneumococcal culture supernatant activates Treg and Th17 response

To determine whether *Streptococcus pneumoniae* activates Treg and/or Th17 cell response, Pneumococcal (concentrated) culture supernantant (CCS) was prepared and used for stimulation of tonsillar MNC. As can be seen from figure 3.12.a+b, there is a significant increase in both Th17 and Treg following pneumococcal CCS as compared to medium control (Todd-Hewitt Broth). That suggests pneumococcal CCS (which contains secreted components of pneumococcus) can activate both Th17 and Treg.

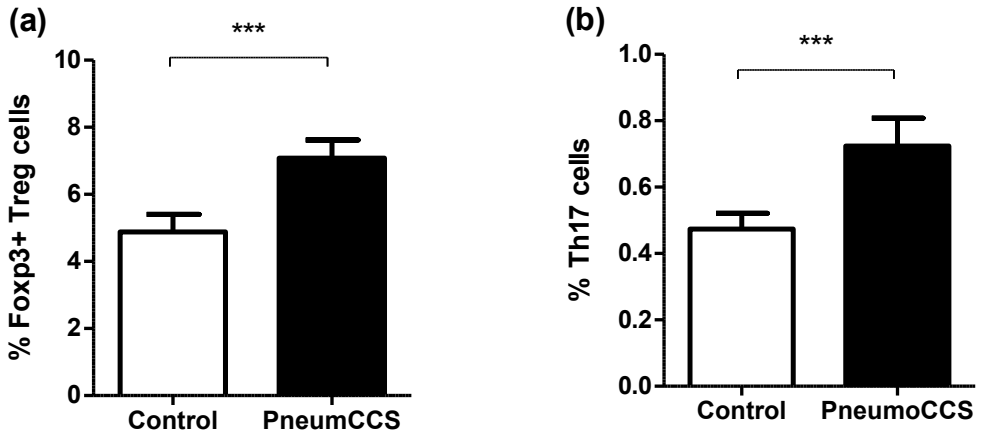


Figure 3.12 Activation of tonsillar Th17 and Treg by pneumococcal CCS.

Tonsillar MNC was co-incubated with pneumococcal CCS or with medium alone (Todd-Hewitt Broth, THB) for 24 hours (n=6). (a) The response of Treg cells and (b) Th17 response (*p< 0.05, ***p< 0.001 as compared to medium alone (control). Mean+ SEM are shown.

3.4.6.2. Association of pneumococcal carriage and frequencies of tonsillar Th17 and Treg following pneumococcal CCS stimulation

After stimulation of tonsillar MNCs with pneumococcal CCS (D39) for 24 hours, Frequencies of Foxp3+ Treg was determined (see materials and methods) as the percentage (%) of CD4+ cells. Pneumococcal CCS stimulation appeared to induce a stronger increase in Treg frequency in culture-positive children compared to culture-negative children (Fig.3.13.a, *p< 0.05). By contrast, following *in vitro* stimulation by pneumococcal CCS, the frequency of tonsillar Th17 was shown to be higher in culture-negative children (mean: 0.8%) compared to culture-positive children (mean: 0.4%) (Fig.3.13.b, **p< 0.01). Similarly, when the ratio of tonsillar Th17/Treg was analysed in association with pneumococcal carriage after pneumococcal stimulation, a higher ratio of Th17/Treg was shown in children who were culture-negative for pneumococcus than those children who were culture-positive (Fig.3.13.c, *p< 0.05).

Furthermore, data in Fig.3.14 showed that there was an inverse correlation between tonsillar Th17 and Treg frequencies in children after pneumococcal stimulation. It is possible that the pathway for the induction of one cell subset inhibits the pathway of the other.

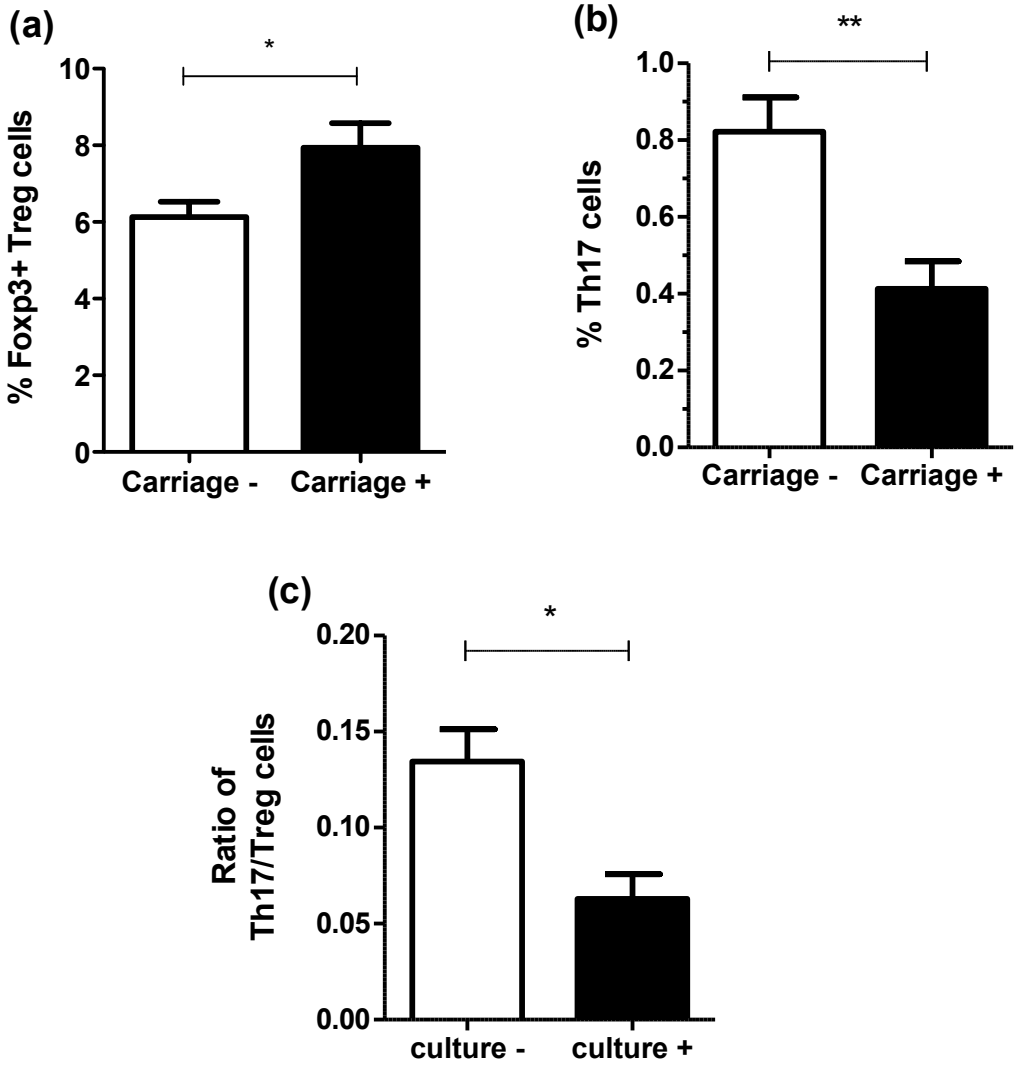


Figure 3.13 Association of pneumococcal carriage status and Th17 and Treg response following stimulation by pneumococcal CCS in children

Comparison of the frequencies of tonsillar Foxp3+ Treg (a), Th17 (b) and of the ratio of Th17/Treg (c) between culture+ and culture- children. (culture +, n=13 and culture -, n=27). Significance (*p<0.05, **p< 0.01, student-t test) between culture – and culture + children.

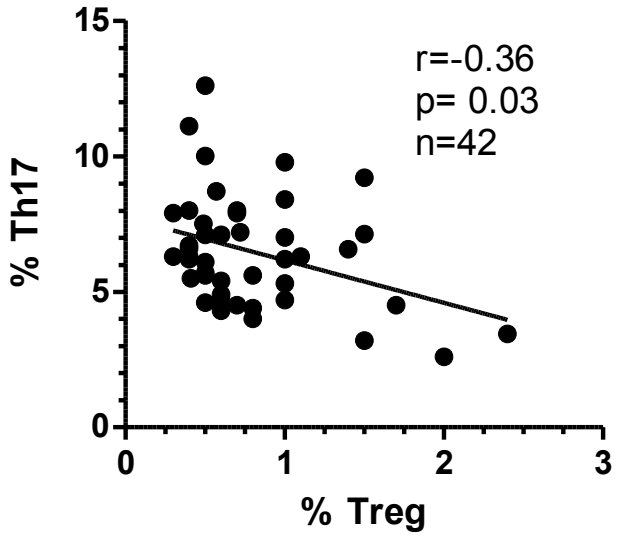


Figure 3.14 Relationship between Th17 andTreg requencies in children.

There is an inverse correlation between frequencies of tonsillar Treg cells Th17 cells in children following pneumococcal stimulation (r=-0.36, n=42, *p< 0.05).

3.4.6.3. Production of IL17A and TGF-beta following pneumococcal CCS stimulation

In vitro production of IL17A in human tonsillar MNC after stimulation with pneumococcal D39wt CCS was analysed by ELISA in cell culture supernatants. Kinetics of IL17A production in culture supernatants at different time points (0, 24, 48, and 72 hours) following pneumococcal CCS stimulation was performed. The peak concentration of IL17A cytokine was shown at 72 hours following stimulation (figure 3.15 ***$p<0.001$).

After stimulation by pneumococcal CCS, the level of IL17A in cell culture supernatant of adults was shown to be significantly higher than in children (Fig.3.16.a). The concentration of TGF-beta in cell culture supernatants of children was higher than adults although there was no significant difference between adults and children (Fig.3.16.b)

Following pneumococcal CCS stimulation, the mean IL17A concentration in MNC culture supernatant of children who were culture-negative for pneumococcus was also shown to be higher than in children who were culture-positive (figure 3.17.b, **$p<0.01$). By comparison, the mean concentration of TGF-beta in MNC culture supernatants was higher in children who were culture positive (+) than in those who were culture negative (-) (Fig.3.17.a, *$p< 0.05$).

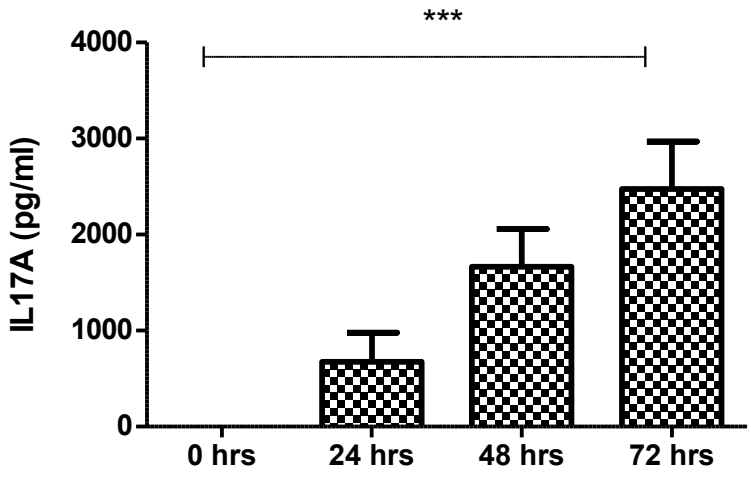


Figure 3.15 Time curve of IL17A production in tonsillar MNC following pneumococcal CCS stimulation.

The level of Il17A cytokine was measured by ELISA assay after cells stimulation with pneumococcal CCS at different time points (0hr-72hr; n=10). Mean+SEM are shown. ***p< 0.001 one-way ANOVA to compare between different time points.

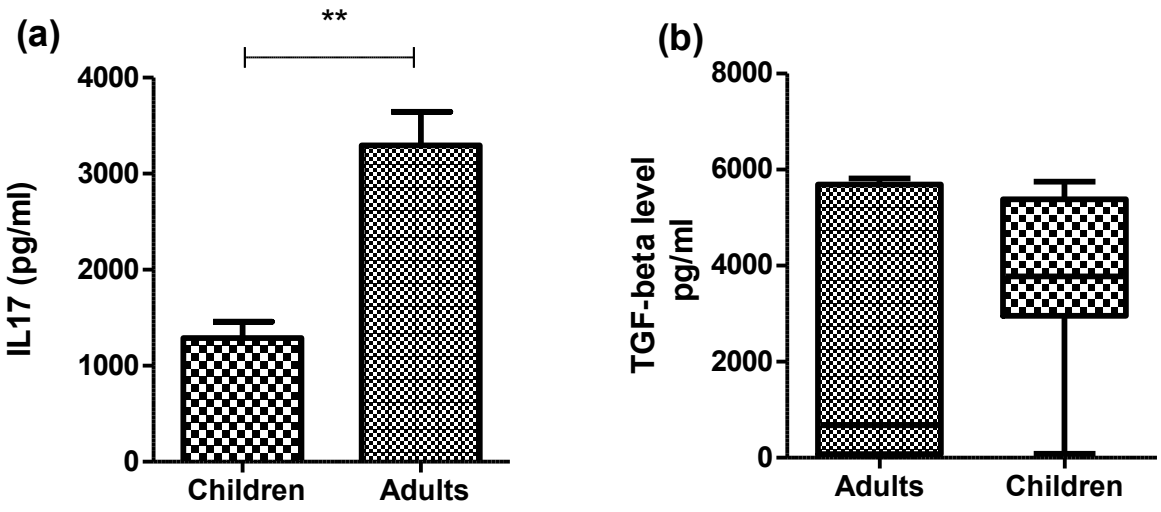


Figure 3.16 Comparison of the concentrations of IL17A and TGF-β in tonsillar MNC culture supernatants between adults and children.

Cytokine measurement of IL17A (a) TGF-beta (b) in MNC culture supernatant of adults (n=5 a) and children (n=20), after 72 hours of CCS stimulation. Data represent the median with interquartile range of cytokine levels (pg/ml) measured by ELISA. Mean+ SEM are shown and student-t test was used (a). Data represent the median with interquartile range of TGF-beta levels (pg/ml) measured by ELISA (b). **p<0.01, compared adults vs children.

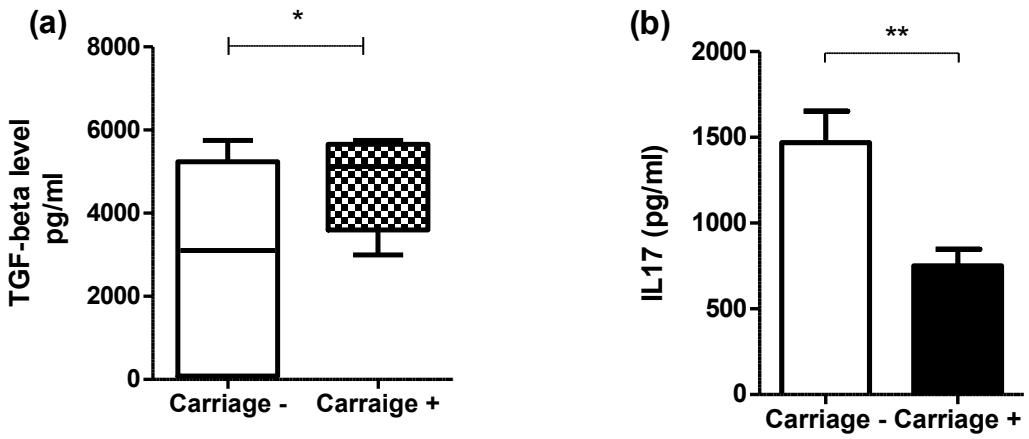


Figure 3.17 The concentrations of IL17A and TGF-β between between culture (-) and culture (+) Children.

Concentrations of TGF-beta (a) and IL17 (b) in tonsillar MNC culture supernatant following pneumococcal CCS stimulation were analysed by ELISA in culture+ (n=11) and culture- children (n=16). Data represent the median with interquartile range of TGF-beta levels (pg/ml) measured by ELISA (a). Mean+ SEM are shown and student-t test was used (b). *p<0.05, **p<0.01 comparison between culture (–) and culture (+).

3.4.7. Effect of tonsillar Treg cells on pneumococcal CbpA- specific antibody production

To assess the effect of Treg cells in NALT on antibody production, depletion of Treg cells from tonsillar MNC was performed using anti-CD25 antibody labelled magnetic beads and MACS cell separation. There was a significant increase in the level of IgG antibody to choline-binding protein (CbpA) following pneumococcal CCS stimulation in Treg-depleted MNC as compared to that in unfractionated MNC (Fig.3.18, **p< 0.01).

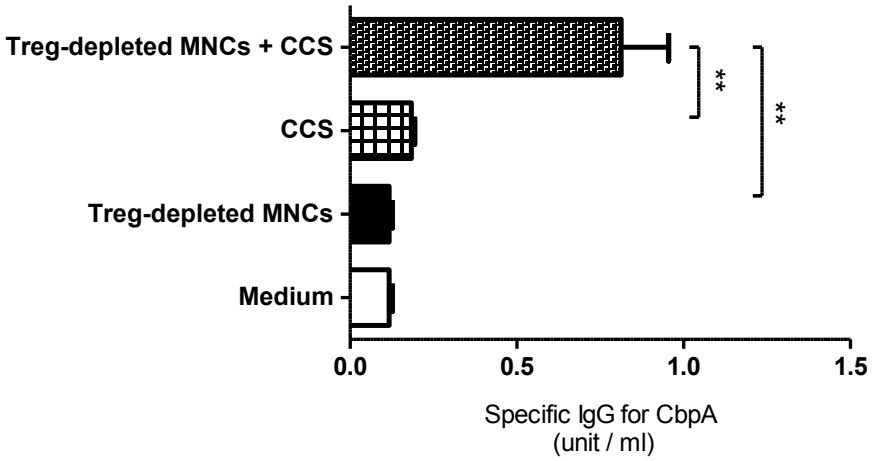


Figure 3.18 Depletion of Treg cells from MNCs increases the level of CbpA- specific IgG antibody production following pneumococcal CCS stimulation.

Culture supernatant was collected after 14 days of pneumococcal CCS stimulation of either unfractionated MNC or Treg-depleted MNC. The level of CbpA-specific IgG antibody production was measured by ELISA. n=8. Mean+ SEM are shown. **p< 0.01, compared to Treg-depleted MNC.

3.5. Discussion

Data on the relationship between mucosal Th17 or Treg and age in humans are limited. This is the first study to show that there is a differential correlation between mucosal Th17 and Treg with age.

It has been hypothesised that mucosal Treg and Th17 cells in NALT may play a crucial role in mediating the persistence or clearance of pneumococcal carriage in human nasopharynx. To determine whether this is true, we have performed a number of experiments to analyse the frequencies of Th17 and Treg in tonsillar tissues of children and adults and their relationship to pneumococcal carriage status.

It has been shown that the frequency of Treg cells in tonsillar tissue in each subject was generally higher than in peripheral blood. This may suggest the mucosal compartment within human nasopharynx develops a higher number of Treg due to the exposure of microorganisms than in peripheral blood (representing systemic compartment which is normally sterile), and also suggest the presence of antigen-specific (inducible) Treg in human NALT. Furthermore, we showed that there was a difference between adults and children in the frequency of Treg in tonsillar tissues. The frequencies of tonsillar Treg of children were generally higher than in adults, and there appeared an age-associated decrease in tonsillar Treg numbers (Fig.3.1). That appeared to be an inverse relationship between the Treg frequency in tonsillar tissues and age (Fig.3.3). Overall, younger children <10 years were shown to have higher Treg numbers than those those subjects >10

years. This would support the hypothesis that extensive exposures to microorganisms in childhood are associated with the induction of Treg. It may be that the local colonisation with microorganisms could induce antigen-specific Treg cells thus leading to high numbers of Treg in NALT tissues, and this may contribute to the persistence/carriage of specific microbes (Zhang et al. 2011). Induction of pathogen-specific Treg has been shown during infections with HIV, TB and leishmania in the local inflammation site (i.e.; lymphoid tissue) (Andersson et al. 2005; Belkaid et al. 2002; Nilsson et al. 2006; Shafiani et al. 2010). It has been suggested that the accumulation of stimulated natural and adaptive Treg cells might be induced by microbial pathogens in peripheral tissues and that may help to maintain the host homeostasis (Andersson et al. 2005; Belkaid 2008). By the age of 2 years in this study, the frequency of tonsillar Treg already reached a high level. Considering that earlier childhood (<2 years) is a period of intense exposure to multiple microorganisms, during which bacterial colonisations are common, the priming of antigen-specific Treg cells during this period is also likely to be intense and lead to the enrichment of Treg in the nasopharynx.

Pneumococcal carriage is common in children especially in younger children. With increasing age, pneumococcal carriage rate gradually decreases (Bogaert, De Groot & Hermans 2004; Granat et al. 2009). During carriage, antigen-specific immunity develops to protect against the subsequent colonisation (Cohen et al. 2011; Richards et al. 2010; Zhang, Clarke & Weiser 2009). There appeared to be a difference in the frequency of Treg cells which was higher in culture-positive than in culture-negative children. This suggests that the induction/promotion of Treg in

tonsillar tissues may contribute to pneumococcal colonisation in the nasopharynx. Also, the delayed clearance or pneumococcal colonisation persistence in children may occur in the existence of Treg cells (Zhang et al. 2011).

In a mouse study, it has been suggested that the successful prevention of nasopharyngeal carriage with the same serotype of pneumococcus was not necessary (Richards et al. 2010). Also, young children who had previous pneumococcal carriage may develop serotype-independent immunity and thus reduce the rate of subsequent colonisation, regardless of the serotypes. (Granat et al. 2009). This was consistent with a study conducted on African children. It has showed that the pneumococcal response at mucosal surface was highly regulated by the presence of Treg cells. Thus, it prolonged the nasopharyngeal colonisation (Glennie et al. 2012). Regulation of the mucosal immune system is common in other bacterial colonisation including *Neisseria meningitidis* (Davenport et al. 2007). MenB antigens enhanced the acquisition of mucosal immunity which is accompanied by the induction of regulatory activity. So, the activity of Treg cells is capable of limiting cellular response to the bacteria (Davenport et al. 2007).

Then, the frequency of Th17 cells was analysed in tonsillar MNC following stimulation by PMA and ionomycin. It has been shown that Th17 frequency in tonsillar MNC was significantly higher than peripheral blood (Fig.3.5). It has been previously shown that the Th17 detected in NALT tissues were primarily memory Th17 cells, as depletion of CD45RO+ T cells abrogate the Th17 cells in tonsillar MNC (Gray et al. 2014). It is likely that the mucosal Th17 in human NALT were

primed largely by previous microbial infection/colonisations in the nasopharynx. The relationship between frequencies of tonsillar Th17 and ages of patients was analysed. It has been demonstrated that the frequency of tonsillar Th17 in adults was higher than in children and further analysis showed there was an age-associated increase in the Th17 frequency. Numbers of tonsillar Th17 in younger children <10 were significantly lower than those in subjects >10 years (Fig 3.7.).

It is known pneumococcal carriage rate decreases with age. It has been hypothesised that mucosal Th17 in the nasopharynx may play a role in the age associated decrease in pneumococcal carriage because the human nasopharynx is a site of colonisation of many microorganisms that may prime for Th17 in NALT.

The relationship between frequencies of tonsillar Th17 and pneumococcal carriage in children was analysed. There was a trend to show culture-negative children had higher frequency of tonsillar Th17 cells compared to in culture-positive children, although this difference was not statistically significant (Fig 3.8). This is consistent with the hypothesis that Th17 may play a role in mucosal defence against microbial infection (Wu et al. 2007). There is growing evidence of Th17 cells playing an important role in mediating host mucosal immunity to a number of pathogens including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans* (Acosta-Rodriguez et al. 2007; Curtis & Way 2009; Huang et al. 2004).

It has been sought to determine the relationship between the ratio of Th17/Treg in NALT and different ages. There appeared to be a positive correlation between the ratio of Th17/Treg in tonsillar tissues and different ages. The mean ratio of tonsillar Th17/Treg MNC was shown to be significantly higher in adults than children (Fig 3.9). As we showed earlier that mucosal Treg frequency also appeared to be associated with pneumococcal carriage rate, we sought to determine the relationship between the ratio of Th17/Treg in NALT and carriage status of pneumococcus. Indeed, the mean ratio of Th17/Treg was shown to be higher in culture-negative children than culture-positive children. This suggests the ratio of Th17/Treg may be a crucial determinant of pnemococcal clearance. It may be argued that as the ratio of tonsillar Th17/Treg increases with age and both are inversely correlated with carriage rate, some other age-associated yet unknown immunological factor(s) may be important in this aspect.

To ascertain the importance of the ratio of tonsillar Th17/Treg in pneumococcal carriage, children less than 10 years were further analysed, as within the age group, the mean ratio of Th17/Treg changes little. It was shown that in this age group, culture-negative children had higher ratio of tonsillar Th17/Treg than culture-positive children. This finding would support that a higher Th17/Treg ratio in NALT contributes to clearance of mucosal carriage of pneumococcus.

In this study, pneumococcal culture supernatant (CCS, containing secreted pneumococcal components including proteins) derived from a wild type strain was used to stimulate tonsillar MNC. It was shown to elicit a significant increase in

Th17 number which was higher in culture-negative children than culture positive (Fig.3.13.b). This would support the presence of memory Th17 in tonsillar tissue, presumably primed by previous pneumococcal colonisation in children, as pneumococcus is one of the most common colonisers in early childhood. Similarly, stimulation by pneumococcal CCS also elicited an increase in Foxp3+ Treg number (Fig.3.12.a). Our previous work suggested that within the adenotonsillar tissue, there were memory/effector Th17 and Treg (Gray et al. 2014; Zhang et al. 2011), as specific stimulation by pneumococcal antigens could activate these cells. Moreover, the increase of Treg was shown to be higher in culture-positive children than culture negative children (Fig.3.13.a) whilst the ratio of Th17/Treg cells was much higher in culture negative than culture positive (Fig.3.13.c). Again, this suggests the presence of pre-existing specific-Treg in NALT presumably primed by previous pneumococcal colonisation. Additionally, it may support the hypothesis that induction of antigen-specific Treg *in vivo* can be promoted by local colonisation with pneumococcus at mucosal site, which leads to the proliferation of Treg cells following stimulation with pneumococcal WCA (Zhang et al. 2011).

It has been shown in this study that there was an inverse correlation between tonsillar Th17 and Treg frequencies (Fig.3.14). So it is possible that the pathway for the induction of one cell subset inhibits the pathway of the other. The induction of tonsillar Th17 cells in NALT may be inhibited by the early induction of inducible/ Treg cells in NALT in early childhood due to the enhanced exposure to microorganisms in the nasopharynx (Gray et al. 2014). Therefore, the increased

priming for IL17A secretion may consider as an important mechanism to clear pneumococcus from nasopharynx (Malley & Anderson 2012). Repeated exposure to pneumococcus in childhood may contribute to increase the inducible Treg cells in early childhood, and that may delay the induction of Th17 and contribute to the persistence of pneumococcal carriage. Priming for Th17 cells in early life, that may inhibit induction of Treg in NALT may be an attractive vaccination strategy against pneumococcal colonisation (Gray et al. 2014).

Production of IL17A in human tonsillar MNC following stimulation with pneumococcal CCS was also observed, which was significantly higher in adults than children (Fig.3.16.a), which was consistent with tonsillar Th17 frequencies. IL17A produced from Th17 cells, is involved in the recruitment and activation of neutrophils that is associated with phagocytic killing (Lu et al. 2009), and likely act through the induction of pro-inflammatory mediators such as granulocyte colony-stimulating factor (G-CSF) and CXC chemokines from epithelial cells (Aujla, Dubin & Kolls 2007b; Kolls & Linden 2004). A study carried out on adult volunteers revealed that rhIL-17A exposure to alveolar macrophages significantly increased the killing of opsonised pneumococcus (Wright et al. 2013). Also, a similar study by Lu et al. (Lu et al. 2008) has shown the same pattern but they used human neutrophils instead for rhIL-17A exposure in order to increase the pneumococcal killing that is mediated by neutrophils. In addition, it has been shown that the clearance of pneumococcal colonisation by activated monocyte/macrophages could be mediated via IL17A cytokine (Zhang, Clarke & Weiser 2009). It is possible

that IL17A promotes pneumococcal clearance through recruitment of neutrophils and monocyte/macrophages to mucosal site. It is suggested that macrophages are involved in the clearance of primary and secondary bacterial colonisation, but neutrophils are very crucial for the secondary colonisation (Zhang, Clarke & Weiser 2009). Studies in mice showed that the response of IL-17A following immunisation is associated with decreased pneumococcal carriage which supports a critical role of IL17A in pneumococcal clearance (Lu et al. 2009).

TGF-β is known to be important in the induction of Foxp3+Treg and Th17. At low concentrations, TGF-β1, together with IL -6 and IL-21 induces the expression of IL-23 receptor, and promotes the differentiation of Th17 cells. At high concentrations, TGF-β1 promotes the development of Foxp3+Treg cells and suppresses the expression of IL-23 thus inhibiting the differentiation of Th17 (Zhou et al. 2008). This study has shown that the level of TGF- β in tonsillar cell culture supernatants was high, even without any stimulation, in both children and adults. There was a significant increase in TGF-beta concentration following pneumococcal CCS stimulation (Fig.3.16.b). It has been shown that many cells produce TGF-β including macrophages, antigen-presenting cells (APCs), epithelial cells as well as Treg of the mucosal tissues (Chen & Konkel 2010; Pyzik & Piccirillo 2007). It is possible that within adenotonsillar tissue, a number of cells produce TGF-β constitutively thus there is a relatively high level of TGF-β in NALT, which is considered as a favourable environment for the development of Treg cells when there are pathogen/antigens (eg. pneumococcus) in the nasopharynx. There was

no marked difference between children and adults although the median level of TGF-β in children was silightly higher than adults.

It has been shown in murine studies that nasopharyngeal carriage of pneumococcus induces Treg cells that were associated with high levels of TGF-β in the nasopharynx. The initial colonisation at a low density may activate TGF-β-signalling leading to a high TGF-β environment and persistence of pneumococcal carriage (Neill et al. 2014). It has been shown that the level of TGF-β in tonsillar MNC culture supernatants was higher in children who were culture positive (+) than in those who were culture negative (-). On the contrary, IL17 production was higher in culture (-) than culture (+) (Fig.3.17.). Together with the finding that a higher Treg frequency was observed in culture-positive than negative children, this result supports the hypothesis that TGF-β signalling plays an important role in Treg cell induction in human NALT and nasopharyngeal carriage of pneumococcus.

To determine whether the Treg in NALT are functional, we analysed their effect on antibody production by tonsillar cells. Depletion of Treg from tonsillar MNC leads to a significant increase in antibody production to pneumococcal choline binding protein following pneumococcal CCS stimulation (Fig.3.18). Treg cells may have direct or indirect effects on the antibody response through inhibition of effector T cell responses.

3.6. Summary

Our results have suggested that there are prominent numbers of Th17 and Treg cells in tonsillar tissues that were significantly higher than in peripheral blood in children and adults, which may play an important role in mediating nasopharyngeal carriage of pneumococcus. In general, the frequency of Treg in NALT decreases with age, whereas Th17 increase with age. Also the ratio of tonsillar Th17/Treg was shown to increase with age and shown to be higher in pneumococcal culture-negative children than in culture-positive children. Pneumococcal simulation of tonsillar cells elicited a significant increase in both Th17 and Foxp3+ Treg suggesting the presence of antigen-specific Th17 and Treg in human NALT, presumably primed by previous colonisation of pneumococcus. Our results suggest the ratio (or balance) of Th17/Treg in nasopharynx-associated lymphoid tissue is a critical determinant of pneumococcal clearance or persistence/carriage in human nasopharynx.

Chapter 4: Effect of Pneumococcal Proteins on Activation of Tonsillar Treg and Th17 Cells

4.1. Introduction

In the previous chapter, it has been shown that adenotonsillar tissue has a significant number of Th17 and Treg cells, and pneumococcal stimulation induces increases in numbers of Th17 and Treg. In this study, we aimed to study whether pneumococcal proteins are involved in activation of Th17 and Treg.

Pneumococcus is a major cause of morbidity and mortality amongst all ages, especially children and elderly. It can cause a wide range of diseases such as meningitis, septicaemia, and pneumonia. Also, it also causes otitis media in children.(Siber 1994; Zhang et al. 2006b). Pneumococcus has several virulence factors, including capsule, cells wall, pneumolysin and surface proteins. Pneumococcal proteins including pneumolysin, pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), and choline-binding protein A (CbpA) have been studied as candidate vaccines against pneumococci infection (Briles et al. 2000a; Briles et al. 2000b).

Pneumolysin is a cholesterol-dependent cytolytic pore-forming toxin. It is expressed in virtually all clinical isolates (Paton 1998). It has a number of actions, such as complement activation (Kadioglu et al. 2008), stimulation of host cell apoptosis, binding to cholesterol molecules in host cell membranes and then entering into the membrane to form a pore 350 to 450 Å in diameter (Price & Camilli 2009). It has been shown that Ply and its toxoids as vaccine candidates have the capability to induce a protective immunity against invasive infection and ccolonisation in animal models (Kirkham et al. 2006; Ogunniyi et al. 2001). Studies

in animal model have shown that immunization with pneumococcal antigens, including PspA, pneumolysin, PsaA, and CbpA by intranasal route, may induce immune protection against invasive disease as well as nasopharyngeal carriage (Briles et al. 2000a; Briles et al. 2000b; Ogunniyi et al. 2001).

Pneumococcus colonises the upper respiratory tract and can cause invasive disease in some individuals, especially in the high-risk groups. Since pneumococci can evade and colonize the nasopharynx, mucosal vaccination may be an effective vaccination strategy to protect against pneumococcal infection (Zhang, Choo & Finn 2002). It has been found that the pneumococcal protein antigens are probable has the efficiency to induce protection against a vast majority of pneumococcus serotypes as well as they might be effective as vaccine and they are inexpensive for manufacture. Thus, there is a strong possibility that Immunisation of mice with pneumococcal protein antigens can lead to a protection from pneumococcal infection (Paton 1998). A combination of these antigens may provide a high degree of protection to a wide range of pneumococcal strains than using a single protein antigen. These proteins provide a T-cell dependent antigen response which is likely to elicit high immunological memory response in human infants. (Paton 1998). It has been suggested that CD4 (Kadioglu et al. 2004; Malley et al. 2005) and T helper 17 (Th17) cells (Lu et al. 2008; Zhang, Clarke & Weiser 2009) in particular have important role in animal studies in mediating the clearance of nasopharyngeal ccolonisation of pneumococcus. For this reason, in this study, we studied pneumococcal protein antigens which are likely activate Treg and Th17 cells, as we have shown in previous study that pneumococcal proteins may

involved in the accumulation and activation of numbers of tonsillar Treg cells with effector/memory phenotype (Zhang et al. 2011) as well as Th17 cells (Gray et al. 2014). Pneumococcal whole cell antigen (WCA) can promote the increased numbers of Treg in adenoidal MNC (Zhang et al. 2011). It has been demonstrated in mice models that Th17 cell signature cytokines (IL17A, IL17F and IL22) have a crucial role for protection against several pathogens in the respiratory tract (O'Connor, Zenewicz & Flavell 2010). That suggested the mechanism of Th17 pathway might be very important for pathogen clearance at mucosal surfaces (Curtis & Way 2009; O'Connor, Zenewicz & Flavell 2010).

4.2. Aims of study

In this chapter it was sought to examine:

1. What components of pneumococcal culture supernanant (CCS) activate Foxp3+ Treg and Th17 cells,
2. Whether recombinant pneumolysin protein (rPly), its toxoid (PdB) and CBP activate Th17 and Treg cells.

4.3. Design of experiment

Tonsillar MNCs were stimulated by pneumococcal CCS either derived from wild type (D39wt), derived from Ply- or CbpA- mutant strains, or by purified pneumococcal proteins. Following stimulation, intracellular staining of Foxp3 and

IL17A was performed followed by flow-cytometry for analysis of frequency of Th17 and Treg in tonsillar MNC.

4.3.1. Human subjects and samples

In this study, tonsillar tissues samples were obtained from adults and children aged between 2-36 years who had tonsillectomy. Individuals who were taken antibiotics within 3 weeks prior to the operation and who had serious infection or immunodeficiency were excluded from the study. Informed consent was obtained from children's parents or custodian in each case.

4.3.2. Pneumococcal culture supernatant

A standard pneumococcal strain (D39) was used prepare pneumococcal culture supernatant (CCS). Pneumococcal CCS was also prepared from an isogenic choline binding protein A–deficient mutant (CbpA-), and an isogenic pneumolysin-deficient mutant (Ply-) strain, (Berry et al. 1999) and (Ogunniyi et al. 2000). Pneumococcal CCS was prepared from all three strains mentioned above and concentrated by a method as previously described. To determine the optimum concentration of pneumococcal CCS for cell stimulation, a range of concentrations at 1, 2, and 4μg/ml was used for cells stimulation. To assess the response of both the $CD4_+$ Th17 cells CD4+Foxp3+ Treg cells and also Th17 cytokine responses, tonsillar MNC stimulation by different types of pneumococcal CCS were used at

2μg/ml after determining the optimal concentration and dose response for cell stimulation.

4.3.3. Purified pneumococcal protein antigens

Recombinant proteins including wild type pneumolysin (rPly) and its toxoid PdB (W433F) were used as antigen stimulants. Recombinant Ply has a strong hemolytic activity, whereas PdB is a toxoid derivative form of pneumolysin with a mutation of amino acid Trp433-Phe which reduces the haemolytic activity to approximately 1% without affecting antigenicity (Palaniappan et al. 2005; Paton et al. 1991). The endotoxin level of the recombinant proteins were <0.01ng/μg of protein, as determined by the *Limulus* assay. To optimise the concentrations of those proteins for cell stimulation, a range of concentrations of rPly at 10, 50 and 100ng/ml and of PdB at 0.1, 0.25 and 0.5μg/ml was used for cell stimulation. The optimal doses of rPly and PdB for activation of Treg and Th17 cells were at 50ng/ml and 0.25μg/ml respectively. Additionally, choline-binding proteins (CPB) that contain native proteins such as CbpA and PspA were prepared from D39wt pneumococcal CCS by affinity purification using a choline column (Zhang et al. 2010). The optimal concentration of CBP was determined and used at 2μg/ml in cell stimulation.

4.3.4. Stimulation of tonsillar MNC by pneumococcal CCS and pneumococcal proteins

Tonsillar MNCs were isolated from the tonsillar tissues by Ficoll were then resuspended at $4x10^6$ /ml in RPMI-1640 culture medium and stimulated with pneumococcal CCS derived from wild type D39wt, and CbpA- and Ply- mutant strains at 2µg/ml or recombinant proteins (Ply, 50ng/ml), (PdB 0.25µg/ml) or (CBP 2µg/ml). Briefly, the cells were stimulated with CCSwt and other mutant types of CCS for 24 hr at 37˚C, in the presence of CO_2 (5%) and followed by addition of Brefeldin A (BFA) for 6 hr to immobilise IL-17A inside the cells. Cells were collected in 0.02% of BSA in PBS. To measure the Th17 cytokine production with *in vitro* CCS stimulation, cell culture supernatants were collected after 72 hr and stored at –70°C before analysed by ELISA.

4.3.5. Intracellular staining of Treg and Th17 cells

Following stimulation, tonsillar MNC were stained with T cell markers followed by intracellular staining for Foxp3 and IL17A. Fixation/Permeabilization buffer (eBiosciences), was used after staining the surface markers and incubated for 30 min at 4˚C. Subsequently, cells were incubated with mouse anti-Foxp3-Alexa-488 and anti-IL17A-PE (BD Bioscience) for 30 min in the dark place at RT. Then, cells were washed with permeablisation buffer 1x. Finally, the cells were re-suspended in the buffer for analysis by flow cytometry (FACSCalibur; BD Biosciences).

Th17 cell populations and Tregs were determined within the CD4+ and CD3+ gate and the absolute numbers of both Th17 and Treg cells were determined as the percentage of cells staining by anti-IL17A and Foxp3 respectively. Flow cytometric data were analyzed using WinMDI software (version 2.9).

4.3.6. IL17A cytokine measurement in tonsillar MNC culture supernatant by ELISA

Human IL-17A Ready-Set-Go® ELISA sets (eBioscience, UK) was used, following manufacturer's instructions and the detailed procedures are described in materials and methods' chapter. To assess the level of IL17A cytokine in culture supernatant, tonsillar MNC was stimulated with D39wt pneumoCCS, CbpA- and Ply- for 72 hr then culture supernatant was collected and stored in -80˚C for the analysis. Briefly, ELISA plate was coated with 100µl/well of capture antibody for overnight incubation at 4°C. The plate was washed and then blocked for one hour. Diluted samples (1:20) were added to the appropriate wells and incubated for 2 hr. After washing the plate, detection antibody was added into the wells and incubated for 1 hour. Avidin-horse radish peroxidase (HRP) was added and incubated for 30 min. Substrate solution was added and incubated at room temperature for 15 min. Finally, 50µl of stop solution (2N H_2SO_4) was added to each well. The plate was read immediately at 450nm using the plate reader (Opsys MR, Thermo labsystems, UK). The cytokine concentration (pg/ml) was calculated against the standard curve, with the help of microplate analysis software DeltasoftPC (Biometallics, Inc., USA).

4.3.7. Statistical analysis

Data were tested for normality and followed by analysis by student's t test non-normally distributed data were analysed by Wilcoxon signed rank test (nonparametric test), to assess the difference between un-stimulated and stimulated cells. A *p* value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (version 5).

4.4. Results

4.4.1. Optimal doses of pneumococcal CCS and recombinant proteins for Th17 and Treg cell stimulation

To optimise dose response of D39wt CCS and pneumococcal recombinant proteins (rPly and PdB), tonsillar MNCs were stimulated for 24 hours at different doses to analyse the frequencies of both Treg and Th17 cells among CD4+T populations. The optimal concentration was determined by stimulation of 4 samples (n=4) with different concentrations. Different doses were used for D39wt CCS (1.0, 2.0 and 4.0µg/ml), for rPly (10, 50 and 100ng/ml) and for PdB (0.1, 0.25 and 0.5µg/ml). The optimum doses for Treg and Th17 response for D39wt CCS, rPly, and PdB were 2µg/ml, 50ng/ml and 0.25µg/ml respectively (Fig.4.1. and Fig.4.2.). The concentration of 2µg/ml was used to stimulates tonsillar MNCs by all three different pneumococcal CCS (D39wt, Ply- and CbpA-) in the following experiments.

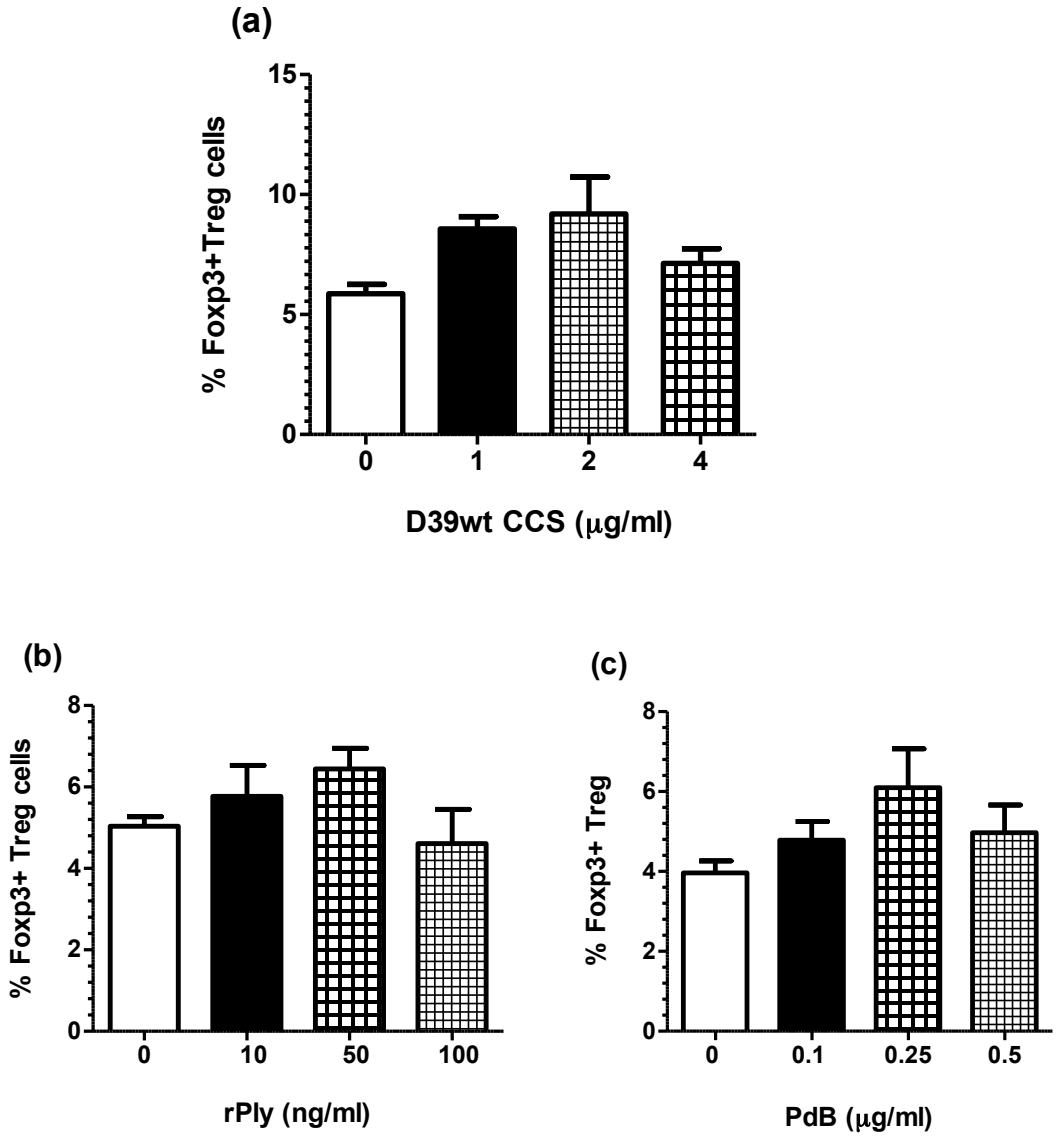


Figure 4.1 Dose-response curve for Treg cell stimulation.

Pneumococcal D39wt CCS (a), recombinant Ply (b) and PdB (c) proteins were used to stimulate tonsillar MNC followed by analysis of Treg frequency (n=4). Results are expressed as mean percentage of cells + SEM (error bars).

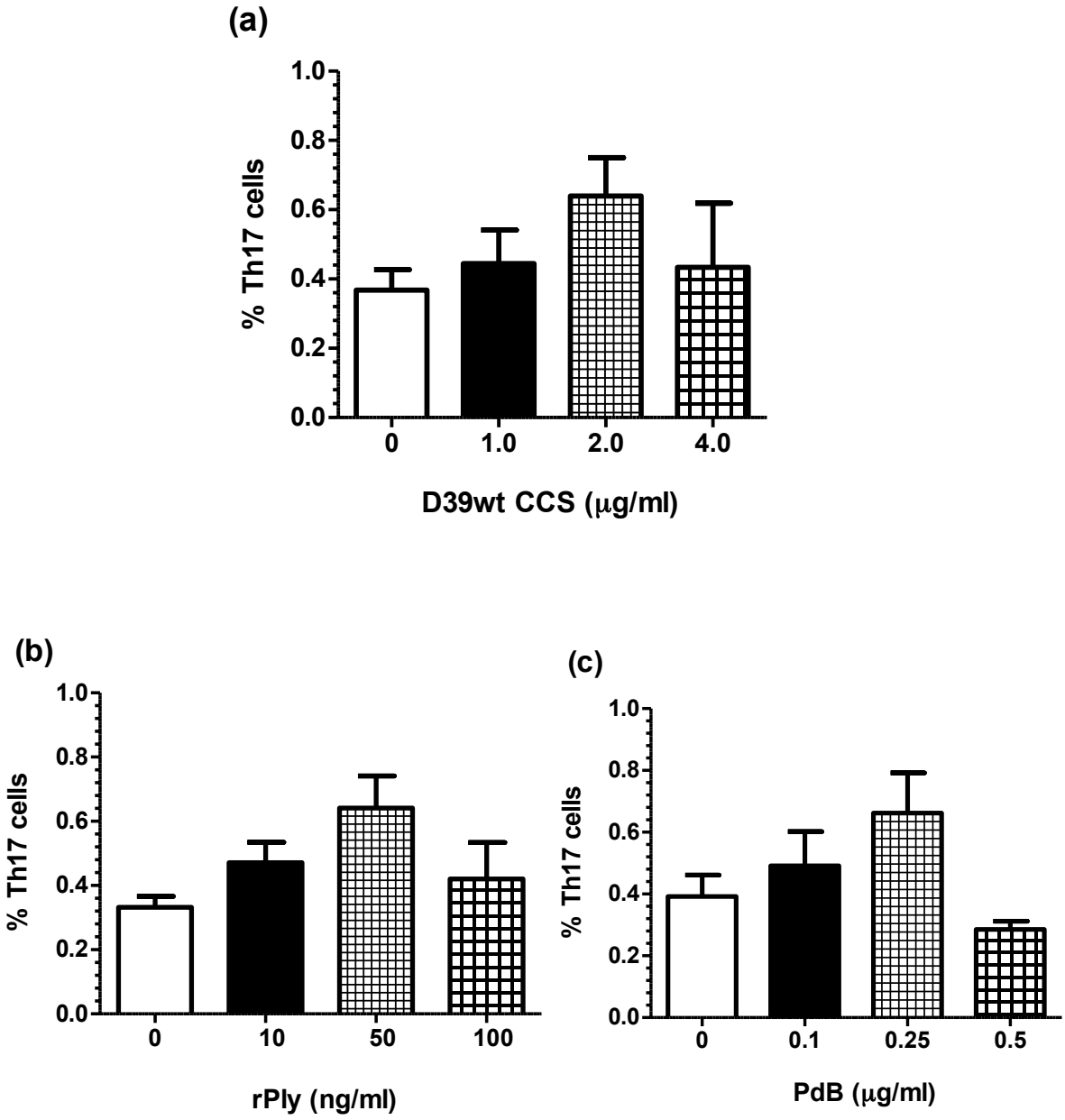


Figure 4.2 Dose-response curve for Th17 cell stimulation.

Pneumococcal D39wt CCS (a), recombinant Ply (b) and PdB (c) proteins were used to stimulate tonsillar MNC followed by analysis of Th17 frequency (n=4). Results are expressed as mean percentage of cells + SEM (error bars).

4.4.2. Pneumococcal CCS derived from CbpA- and Ply- mutant strains elicit lower Treg response than wild type CCS

To determine whether pneumococcal proteins pneumolysin or CbpA contribute to activation of Treg in NALT, tonsillar MNCs were stimulated for 24 hours by pneumococcal CCS derived from wild type, from Ply- or CbpA- mutant strains. Following stimulation, the cells were stained for Foxp3+ Treg as described in methods. Fig.4.3.a shows a representative experiment and Fig.4.3.b shows the mean frequencies of Treg cells. As can be seen from the figures, CCS from CbpA- (**$p<0.01$) and Ply- (*$p<0.05$) mutant strains induced less Foxp3+ Treg than pneumococcal CCS derived from wild type (D39wt) strain; although both Ply- (**$p<0.01$) and CbpA- (*$p<0.05$) CCS appeared to induce higher Foxp3+ Treg cells than unstimulated cells.

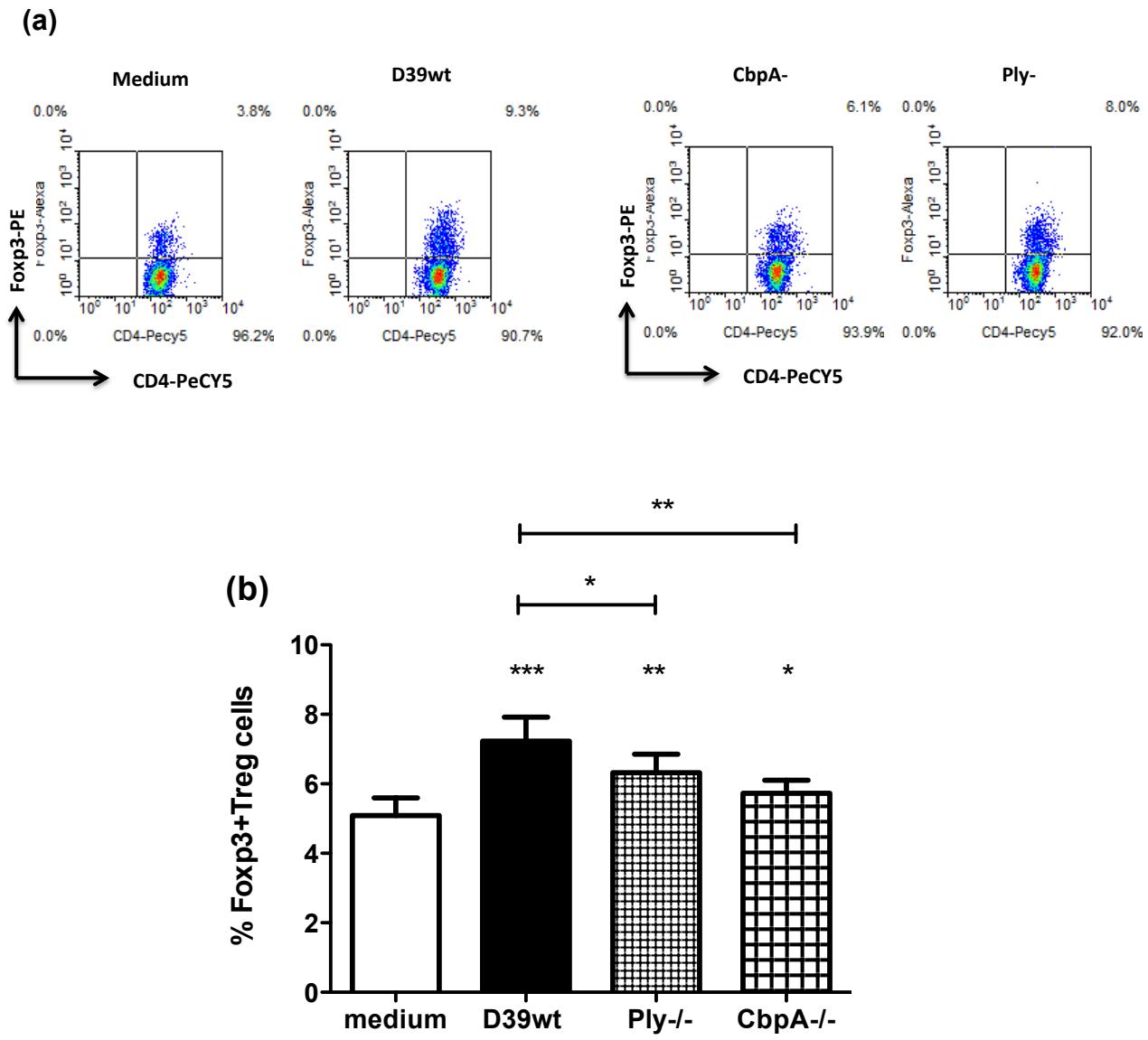


Figure 4.3 Activation of tonsillar Treg cells by D39wt CCS and CCS from CbpA- and Ply- mutants.

Density plot shows expression of Foxp3 in Treg cells gated for CD4+ T lymphocytes in one representative experiment (a). The mean frequency of Foxp3+Treg cells was measured after 24 hours of stimulation with D39wt, CbpA-, or Ply- CCS. ***p<0.001, **p<0.01, *p<0.05, compared to medium control. *p<0.05, **p<0.01, mutants compared to wild type pneumococcal CCS, n=14). Paired-t test was used. Mean+ SEM are shown.

4.4.3. Pneumococcal CCS from CbpA- and Ply- strains elicited lower Th17 than wild type CCS

To determine whether pneumococcal proteins pneumolysin or CbpA contribute to activation of Treg in NALT, tonsillar MNCs were stimulated for overnight by pneumococcal CCS derived from wild type or from Ply- and CbpA- mutant strains followed by the addition of BFA for 6 hours then cells were stained with anti-IL17A. Our results showed that CCS deficient of CbpA and Ply elicited lower numbers of CD4+ Th17 cells. Fig.4.4.a shows a representative experiment and Fig.4.4.b shows the mean frequencies of Th17 cells. As can be seen from the figures, CCS from CbpA- (*$p<0.05$) and Ply- (*$p<0.05$) mutant strains induced less Th17 cells than pneumococcal CCS derived from wild type (D39wt) strain. Also, both Ply- (*$p<0.05$) and CbpA- (*$p<0.05$) can promote/activate Th17 cells compared to unstimulated cells.

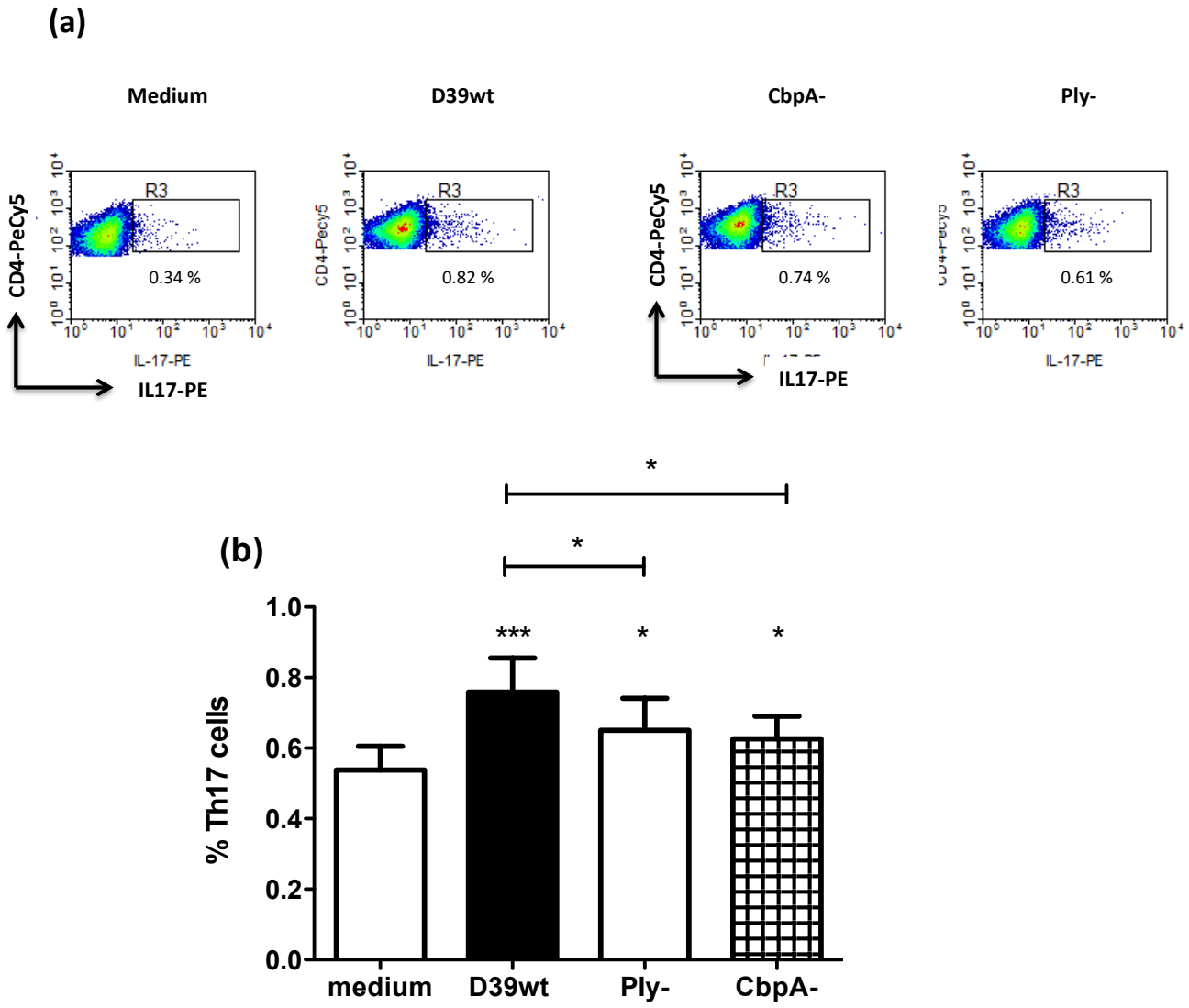


Figure 4.4 Activation of tonsillar Th17 cells by D39wt CCS and CCS from CbpA- and Ply- mutants.

Dot plots showing IL17A-producing cells gated for CD4+ T lymphocytes in one representative experiment (a). The mean frequencies of Th17 cells were shown after 24 hours of stimulation with CCS from D39wt, CbpA-, or Ply- strains. ***p<0.001, *p<0.05, compared to medium control. *p<0.05, mutants compared to wild type pneumococcal CCS, n=14). Paired-t test was used. Mean+ SEM are shown.

4.4.4. Pneumococcal CCS derived from CbpA- and Ply- mutants elicited lower IL17A production than wild type CCS:

Tonsillar MNCs were stimulated with all different CCS for 3 days then the cell culture supernatant was collected and analysed for IL17A detection. Similar results were shown as above for Th17 frequencies. The mean concentration of cytokine IL17A in culture supernatant of tonsillar MNCs was shown to be high after D39wt CCS stimulation when compared with unstimulated cells. As can be seen from the figure 4.5. CCS from wild type strain induced higher IL17A than CCS of CbpA- mutants (*$p < 0.05$) and Ply- CCS (**$p < 0.01$) ($n=18$). Also, Ply- (*$p < 0.05$) and CbpA- (**$p < 0.01$) CCS were shown to induce higher IL17A than unstimulated cells.

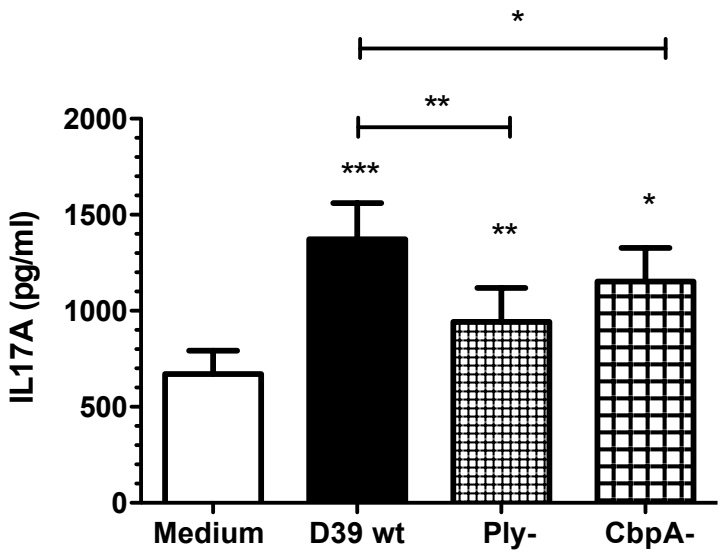


Figure 4.5 IL17A concentrations in adenotonsillar MNC culture supernatants induced by CCS from D39wt, CbpA- or Ply- mutant strains.

IL-17A was measured after 24 hours of stimulation with D39wt, CbpA-, or Ply- CCS. *p<0.05, **p<0.01, ***p<0.001 compared to medium control. *p<0.05, **p<0.01 mutants compared to wild type pneumococcal CCS, n=18. Paired-t test was used. Mean+ SEM are shown.

4.4.5. Effect of pneumococcal proteins (rPly, PdB and CBP) on Treg and Th17 frequencies:

As above results showed stimulation with CCS derived from Ply- and CbpA-mutants elicited less Treg and Th17 compared to wild type CCS that suggests Ply and CbpA may contribute to the activation of Treg and Th17, purified pneumococcal proteins were then used in this experiment to determine if individual protein activates Foxp3+Treg and Th17 cells.

As can be seen from figure 4.6, both rPly (n=13) and PdB (n=11) induced an increase in Foxp3+Treg frequency (means 6.5% and 5.5% compared to (4.5%) in unstimulated cells). CBP (n=8) stimulation also showed an increase in Foxp3+ Treg frequency (mean 6 %, compared to 4.5% in medium control. **p< 0.01, *p<0.5, paired-t test was used.

Effect of the proteins on Th17 frequency was shown in figure 4.7. There were increases in Th17 frequencies following stimulation by rPly (mean: 0.84%, n=13), PdB (mean: 0.76%, n=9) and CBP (mean: 0.82%, n=8) compared to unstimulated control (mean: 0.52%). *p<0.5, **p<0.01, paired-t test was used.

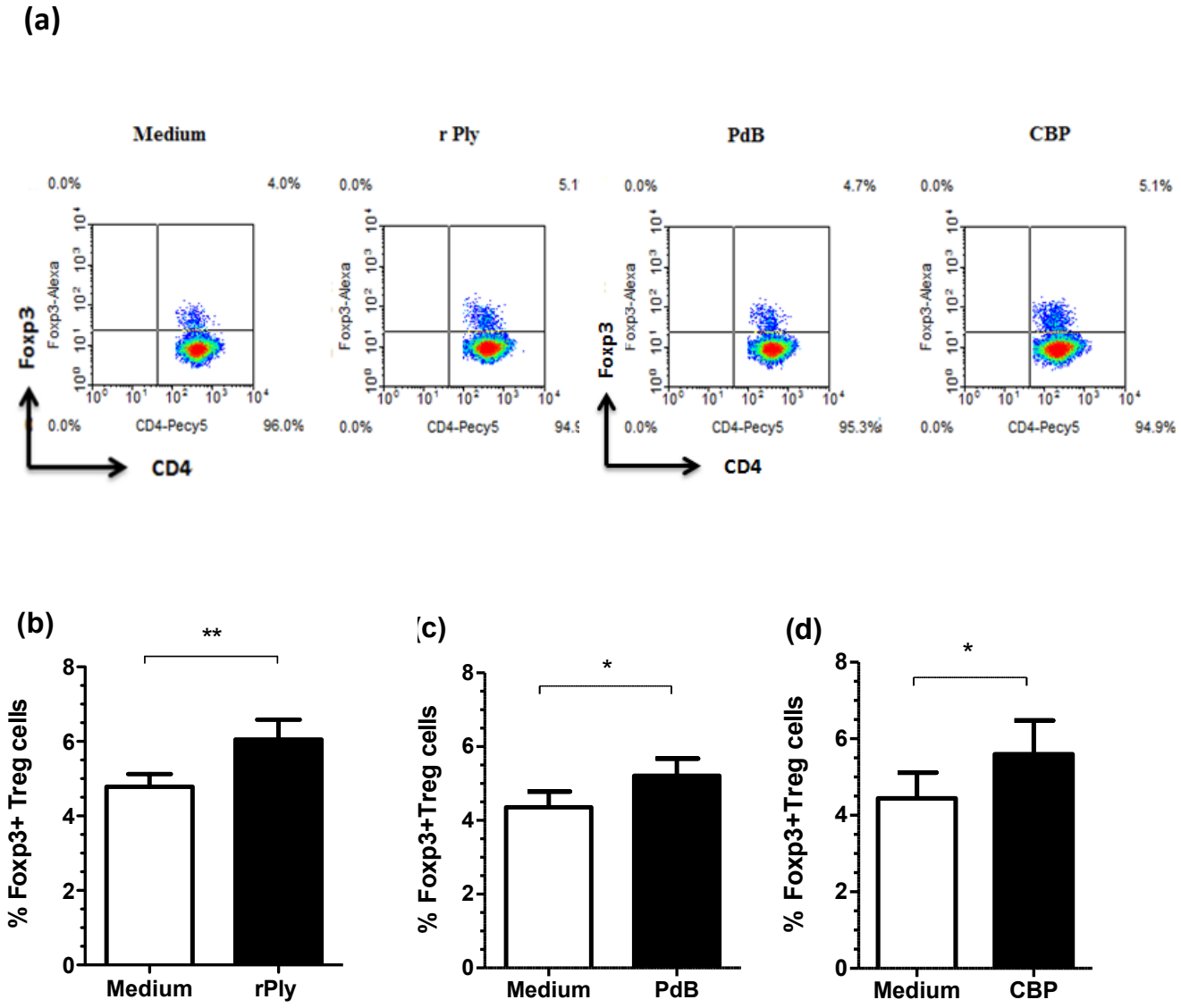


Figure 4.6 Pneumolysin, toxoid (PdB) and CBP increased Foxp3+ Treg in tonsillar cells.

Density plot shows the percentage of Foxp3+Treg cells in CD4+ T cells in one representative experiment (a). Mean frequency of Foxp3+Treg cells after tonsillar MNC were shown following stimulation by rPly (b), PdB (c) and CBP (d) for 24hr. **p< 0.01, *p< 0.05 compared with un-stimulated cells (medium control). Mean+ SEM are shown.

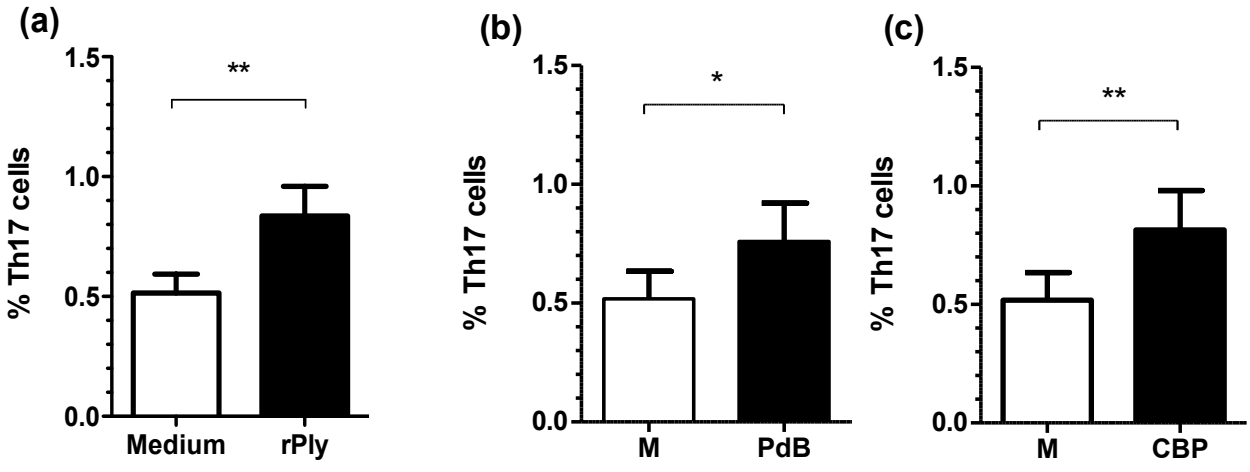


Figure 4.7 Pneumolysin, toxoid (PdB) and CBP activate tonsillar Th17 cells.

Mean frequency of Th17 cells after stimulation tonsillar MNC by rPly (a), PdB (b) and CBP (c) for 24hr. **p< 0.01, *p< 0.05 compared with un-stimulated cells (medium control). Mean+ SEM are shown.

4.5. Discussion

Data on mucosal Treg and Th17 cells in relationship with pneumococcus in humans are limited. It is unclear what components of pneumococcus contribute to the activation of Treg cells in tonsillar tissue. A previous study showed that a pneumococcal whole cell antigen (WCA) induced CD4+ T cell including Treg proliferation in tonsillar MNC and that was inhibited by treatment of proteinase, therefore suggesting that pneumococcal protein(s) contribute to Treg activation in NALT (Zhang et al. 2011). It would be useful to identify what pneumococcal components activate Treg or Th17 cells, which may add important information to novel vaccination strategies against pneumococcal infection.

In this study, it has been investigated whether pneumococcal Ply and CbpA proteins activate Foxp3+Treg and Th17 cells in NALT. The reaults showed that wild type (D39wt) pneumococcal CCS elicited a significant increase in the Treg and Th17 frequencies whereas the CCS from mutant strains (Ply- and CbpA-) were shown to elicit a lower increase in Treg and Th17 frequencies than wild type CCS. Also cytokine measurement of IL17A also showed similar results in that the mutant strains derived CCS induced less IL17A. Taken together, our findings suggest that pneumococcal Ply and CbpA proteins may contribute to the activation of Foxp3+ Treg and Th17 cells in tonsillar MNCs.

Further examination was performed whether purified individual proteins activate Treg and Th17. The results showed that rPly, PdB and CBP proteins all induced an increase in Foxp3+Treg, and also appeared to induce an increase in Th17. It

has been demonstrated recently that WCA and pneumolysin toxoid (PdB) could induce the production of Th17 in human peripheral blood mononuclear cells (PBMC) even though Th17 cell numbers detected were low (Lundgren et al. 2012). Thus our overall findings in this study support the hypothesis that pneumococcal proteins has an important role in activation of Treg and Th17 cells.

The mucosal response to pneumococcal proteins may be highly regulated by $CD25^{high}$Treg cells and this regulation may lead to persistent colonisation and delay in the clearance of pneumococcus (Glennie et al. 2012). It has been shown that Ply could be used in immunizations to induce protection against pneumococcal infection and may prevent nasopharyngeal carriage (Briles et al. 2000a; Briles et al. 2000b). A study carried out by Pido-Lopes et al also supported that the Treg cells have a negative impact on mucosal CD4 response that specific for pneumococcus. Depletion of pneumococcal-specific $CD25^{high}$Treg cells from tonsillar MNCs was associated with a significant increase in CD4+ T cell proliferation to Ply (Pido-Lopez et al. 2011).

The Treg and Th17 cells detected in adenotonsillar tissues are likely to be primed by previous pneumococcal colonisation in the nasopharynx. The findings that pneumococcal Ply and CbpA proteins may activate these cells suggest that pneumococcal proteins could prime and activate both these Treg and Th17 cells. Our previous results support that Foxp3+ Treg and Th17 are enriched in adenotonsillar tissues and the results herein further support the presence of

antigen-specific inducible Treg and Th17 in human NALT, and they may play an important role in the modulation of pneumococcal carriage or clearance.

4.6. Summary

These findings in this study provided supporting evidence that pneumococcal proteins including pneumolysin, its toxoid and choline binding proteins may activate Th17 and Treg cells in human NALT, and that they may contribute to the modulation of pneumococcal carriage or clearance in human nasopharynx. Also, these protein antigens may constitute vaccine candidates in mucosal immunization against pneumococcal infection in humans.

Chapter 5: Induction of Th17 and Treg Cells in Nasopharynx-Associated Lymphoid Tissue by Pneumococcus

5.1. Introduction

Generally, leucocytes and epithelial cells can produce cytokines that promote cell recruitment and activation of host immune response in infected tissues (Hirst et al. 2004).

T helper 17 (Th17) cells, a CD4+T cell subset, produce several cytokines including IL-17A, IL-17F and IL-22. It is thought that these are potent pro-inflammatory cytokines and have an important role to clear extracellular pathogens. IL-17A is considered to mediate the recruitment of neutrophils to inflammation tissue that is followed by a release of chemokines and metalloproteinases from several tissues (Bettelli et al. 2008; Lee et al. 2010) because of the induction of antimicrobial peptides, including lipocalin-2, Reg3γ, β-defensins, and calprotectin (Blaschitz & Raffatellu 2010; Ishigame et al. 2009; Zheng et al. 2008), IL17A, IL17F and IL22 contribute to control the dissemination of pathogens form mucosal tissues and that becomes apparent during colonic infection with *C. rodentium*, and has the capability to control the severity of gut pathology (Zheng et al. 2008). Furthermore, these cytokines can be expressed in response to mucosal infections of bacteria and fungi such as *K. pneumoniae* infection in the lung, *C. rodentium* and *S. typhimurium* infection in the gut and *Candida albicans* infection of the oral cavity (Dubin & Kolls 2008).

Upon naive T cell activation with TGF-β, they can differentiate into Treg cells with high expression of Foxp3. However, synergy of TGF-β with IL6, promotes the cells to differentiate into Th17 with high expression of R0Rγt (Bettelli et al. 2006;

Mangan et al. 2006). The expression of CD25 and Foxp3 in inducible Treg cells could be up-regulated by signalling pathways which are initiated by TCR activation, co-stimulatory molecules, IL-2R, programmed death ligand 1 (PDL1) and TGF-β receptor (Francisco et al. 2009; Zhang & Zhao 2007). Treg cells can be differentiated from naïve T cells or expanded from the pre-existing Treg cells. Inducible Treg cells are generally considered antigen specific (Chatenoud 2011). It has been suggested by Fantini that TGF-β can directly induce the expression of Foxp3 through binding to inhibitory Smad 7 promoter in order to switch off its expression. A feedback regulation of TGF-β signalling may be induced and as a consequence Foxp3 accumulates and then promotes the conversion of Treg cells from T cells (Fantini et al. 2004).

In the previous chapters, it has been shown that there were significant numbers of Th17 and Treg in adenotonsillar tissue, and pneumococcal protein antigens including pneumolysin could activate these tonsillar Th17 and Treg. It is hypothesised that pneumococcal protein antigens may induce Th17 and Treg from naïve T cells which may contribute to the accumulation of these cells in human NALT due to previous colonisation of pneumococcus.

5.2. Aims of study

In this chapter it has been sought to examine:

1- Whether pneumococcal proteins such as pneumolysin or its derivatives induce Th17 and Treg cells from naïve T cells in human NALT, and

2- What cytokines promote the induction of Th17 and Treg cells in NALT?

5.3. Design of experiment

Tonsillar MNC are depleted of activated and memory T cells by CD45RO+ cell depletion (so only naïve T and non-T cell populations remaining), and CD25+ cell depletion (Treg depletion), followed by stimulation with pneumococcal culture supernatant (CCS) derived from wild-type (wt), CbpA- or Ply- strains, or by purified recombinant pneumolysin (Ply) and its toxoid (PdB) antigens for 7 days. Various cytokines are added to test for the induction of Th17 cells. The induction of Treg cells will be evaluated as well in the presence or absence of TGF-β. The response of Th17 cells following stimulation will be assessed by measurement of IL-17A, IL-17F and IL-22 by ELISA and by intracellular cytokine staining followed by flowcytometry. Treg cell response following stimulation will be evaluated by analysis of expression of Foxp3, CD25 and CTLA-4.

5.3.1. Human subjects and samples

Tonsillar tissue samples were obtained from children aged between 2-16 years who had tonsillectomy. Individuals who were on antibiotics within 3 weeks prior to the operation and who had serious infection or immunodeficiency were excluded from the study. Informed consent was obtained from children's parents or custodian in each case.

5.3.2. Pneumococcal culture supernatant

Pneumococcal concentrated culture supernatant (pneumococcal CCS) was prepared from *Streptococcus pneumoniae* encapsulated type 2 strain D39 and was used in this project for cell stimulation. CCS from Isogenic strains including choline binding protein A deficient mutant (CbpA-), and pneumolysin-deficient mutant (Ply-) were also tested. Tonsillar MNC depleted of activated and memory T cells were stimulated by these pneumococcal CCS preparations for 7 days with or without exogenous cytokines, followed by analysis of the frequencies of Th17 and Treg and related cytokines.

5.3.3. Purified pneumococcal protein antigens

Recombinant proteins including pneumolysin (rPly) and PdB txoid (W433F). rPly has a strong haemolytic activity, whereas the PdB antigen is a toxoid and a derivative of pneumolysin with a point mutation of amino acid Trp433-Phe which reduces the haemolytic activity to approximately 1% without affecting antigenicity (Palaniappan et al. 2005; Paton et al. 1991). The endotoxin levels of the recombinant proteins were < 0.01ng/µg of protein, as determined by the *Limulus* assay (Zhang et al. 2006a). A sublytic concentration of 50ng/ml of rPly and 0.25µg/ml of PdB were used for cell stimulation. The induction of Treg and Th17 cells was determined on day 7.

5.3.4. Depletion of CD45RO+ and CD25+ cells from tonsillar MNCs

In this study, cellular depletion of CD45RO+ (activated and memory T cells) cells and CD25+ cell depletion (Treg depletion) were performed using magnetic cell sorting (MACS) according to the instructions of the manufacturer (Miltenyi Biotec). Both CD45RO+ cells and/or $CD25^{+}$ cells were depleted from tonsillar mononuclear cells (TMNCs) population in some experiments, eg. for induction of Tregs. To deplete both CD25+ and CD45RO+ for Treg induction experiments, the appropriate amount of beads was added together at the same time to obtain CD25- CD45RO- population (MNC containing naïve T cell). The efficiency of cell depletion for CD45RO or CD25 cells were >96%.

5.3.5. Induction of Th17 cells from naïve cells

CD45RO-depleted MNCs were used and cultured for 7 days in the medium alone or with the presence of different combination of cytokines, and pneumococcal stimulants, before analysis of Th17 frequency. The optimal concentrations of cytokines were determined (i.e. 50ng/ml of IL-21 and 50ng/ml of IL1-beta and 2.5ng/ml of TGF-β) and these were used in the cell culture with or without antigens (all recombinant cytokines were from R & D system and doses optimized). Also, controls including in medium alone or with cytokine alone, were used. On day 7, the cell culture supernatants were collected and stored in the freezer at -80°C for further analysis by ELISA techniques to measure the level of IL17. Also, Phorbol myristate acetate (PMA), Ionomycin (Ion) and brefeldin A (BFA) were added to the cells and incubated for another 5 hours in the incubator. The cells were harvested

and the plate was washed with a cold buffer (BSA/PBS 0.02%) and transferred into the new eppendorf tubes for intracellular staining which was described previously.

5.3.6. ELISA for IL17A, IL17F andIL22 measurement

Human IL-17A, IL17F and IL22 ELISA Ready-SET-Go (eBioscience, UK) were used following manufacturer's instructions for determination of the level of these cytokines in cell culture supernatants following stimulation. Detailed procedures were described in materials and methods' chapter (see chapter 2). Briefly, ELISA plate was coated with 100µl/well of capture antibody for overnight incubation at 4°C. The plate was washed and then blocked for one hour. Appropriate diluted samples (1:10 for IL17A and IL17F; 1:40 for IL22) were added to the appropriate wells and incubated for 2 hr. After washing the plate, detection antibody was added into the wells and incubated for 1 hour. Avidin-horse radish peroxidase (HRP) was added and incubated for 30 minutes. Substrate solution was added and incubated at RT for 15 min. Finally, 50µl of stop solution (2N H_2SO_4) was added to each well. The plate was read immediately at 450nm using the plate reader (Opsys MR, Thermo labsystems, UK). The cytokine concentration (pg/ml) was calculated against the standard curve, with the help of microplate analysis software DeltasoftPC (Biometallics, Inc., USA).

5.3.7. Induction of Treg cells

CD25+ and CD45RO+ cell depleted tonsillar MNCs were cultured for 7 days in the medium alone or with the addition of TGF-β and pneumococcal stimulants followed

by analysis of Treg frequency by flowcytometry. The optimal concentration of TGF-β was determined and used at 2.5ng/ml. On day 7, the cells were harvested and the plate was washed with a cold buffer and intracellular staining was performed which was described previously.

5.3.8. Statistical analysis

Student's paired t test was performed to evaluate the difference between un-stimulated and stimulated cells for normally distributed data or Wilcoxon matched-pairs signed rank test for non-normally distributed data was applied. Pearson's correlation test was used to analyse the correlation between two different factors. A p <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism software (version 5).

5.4. Results

5.4.1. Induction of Th17 cells in tonsillar MNC by pneumococcal CCS

To assess the induction of Th17 in naïve T cell-containing tonsillar MNC, the CD45RO+ cell depleted MNC (Figure 5.1.) were stimulated by D39wt pneumococcal CCS for 7 days without any exogenous cytokines added then followed by the addition of PMA/Ion and BFA for the last 5 hours. Intracellular staining of IL17A performed as described in the materials and methods. The mean frequency of Th17 was increased to 0.38% compared to 0.20% in medium control (Fig.5.2.a, n=6, *$p<0.05$). The concentrations of IL17A (n=8, *$p<0.05$) in the cell culture supernatants were measured by ELISA and shown to be increased following stimulation by D39wt CCS (Fig.5.2.b).

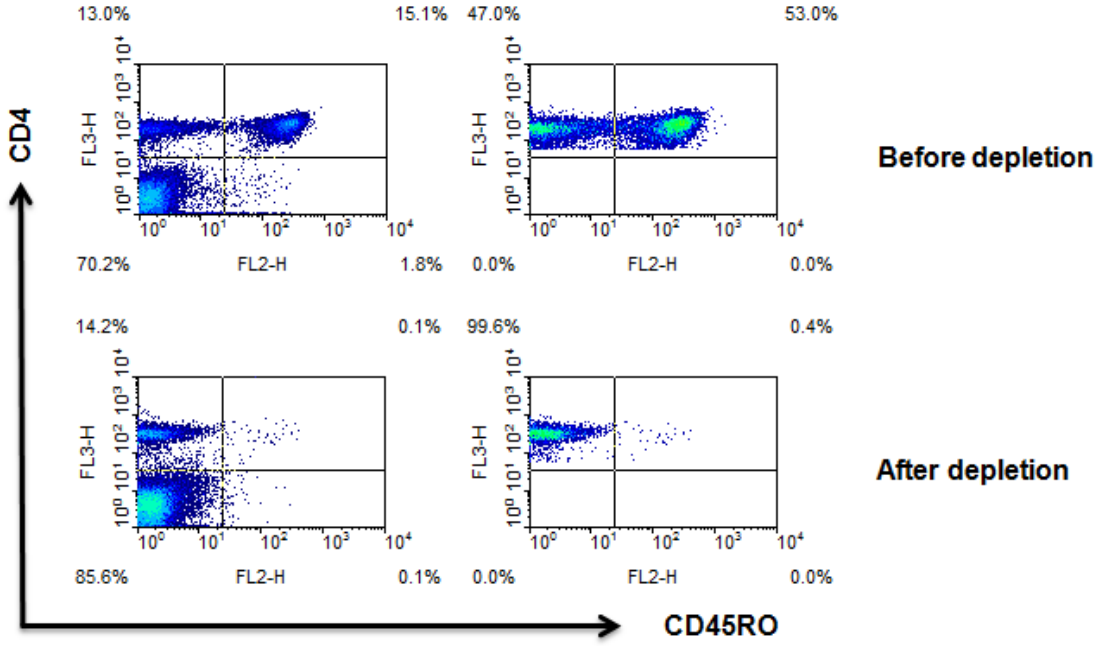


Figure 5.1 Efficiency of CD45RO+ cell depletion from tonsillar MNC.

The depletion of CD45RO+ cells from tonsillar MNC was performed by MACS negative depletion. Anti-CD4 and -CD45RO anti-human antibodies were used in cell staining. The efficiency of $CD45RO^{+}$ T cell depletion was shown by the disappearance CD45RO+ cells.

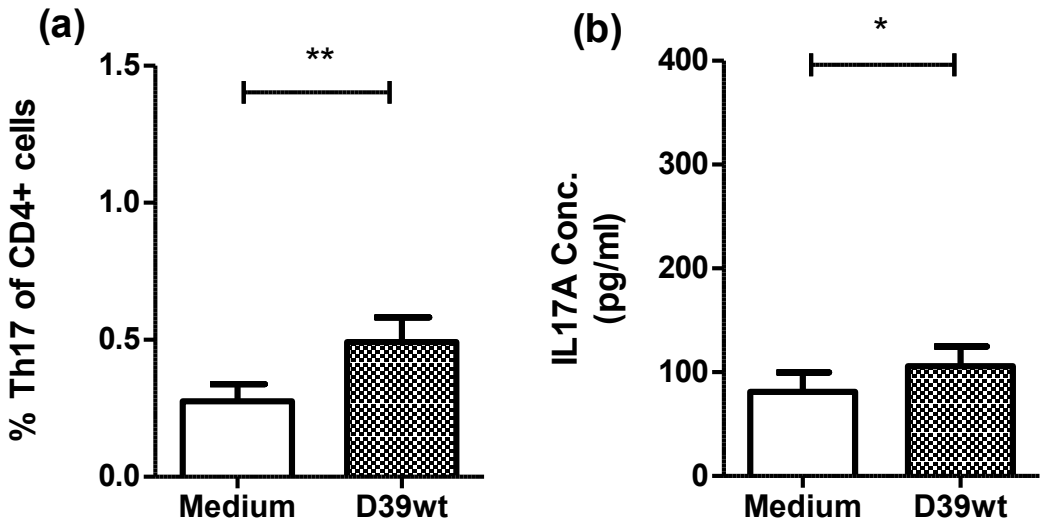


Figure 5.2 Induction of Th17 cells by D39wt pneumococcal CCS.

(a) The frequencies of Th17 (n=6) after 7 days of stimulation by D39wt pneumococcal CCS (*p<0.05, compared with medium control). Mean+ SEM are shown. Paired-t test was used. (b) Cytokine levels of IL17A (n=8, *p<0.05) in tonsillar RO+ cell depleted MNC culture supernatant after 7 days of CCS stimulation when compared with medium control. Data represent mean+ SEM of cytokine levels (pg/ml) measured by ELISA.

5.4.2. The effect of Th17-favouring environment on Th17 induction by D39wt pneumococcal CCS

From above experiments, it was shown the level of Th17 induction by pneumococcal CCS stimulation alone to be low. A number of exogenous cytokines were then tested in order to amplify Th17 induction. A series of experiments were done to optimise the cytokine concentrations and combinations for Th17 induction. The optimal cytokines and concentrations were determined at TGF-β (2.5ng/ml), IL1-β (50ng/ml) and IL21 (50ng/ml). There was a significant effect of TGF-β, IL1-β and IL21 combination on Th17 induction in the presence of D39wt pneumococcal CCS. Stimulation with the cytokines alone did not show a significant increase in Th17 numbers. The mean proportion of Th17 cells in total experiments performed after stimulation with D39wt increased by two-fold (1.1%) compared with unstimulated control 0.5% (Fig.5.3.b, n=17, ***$p < 0.001$).

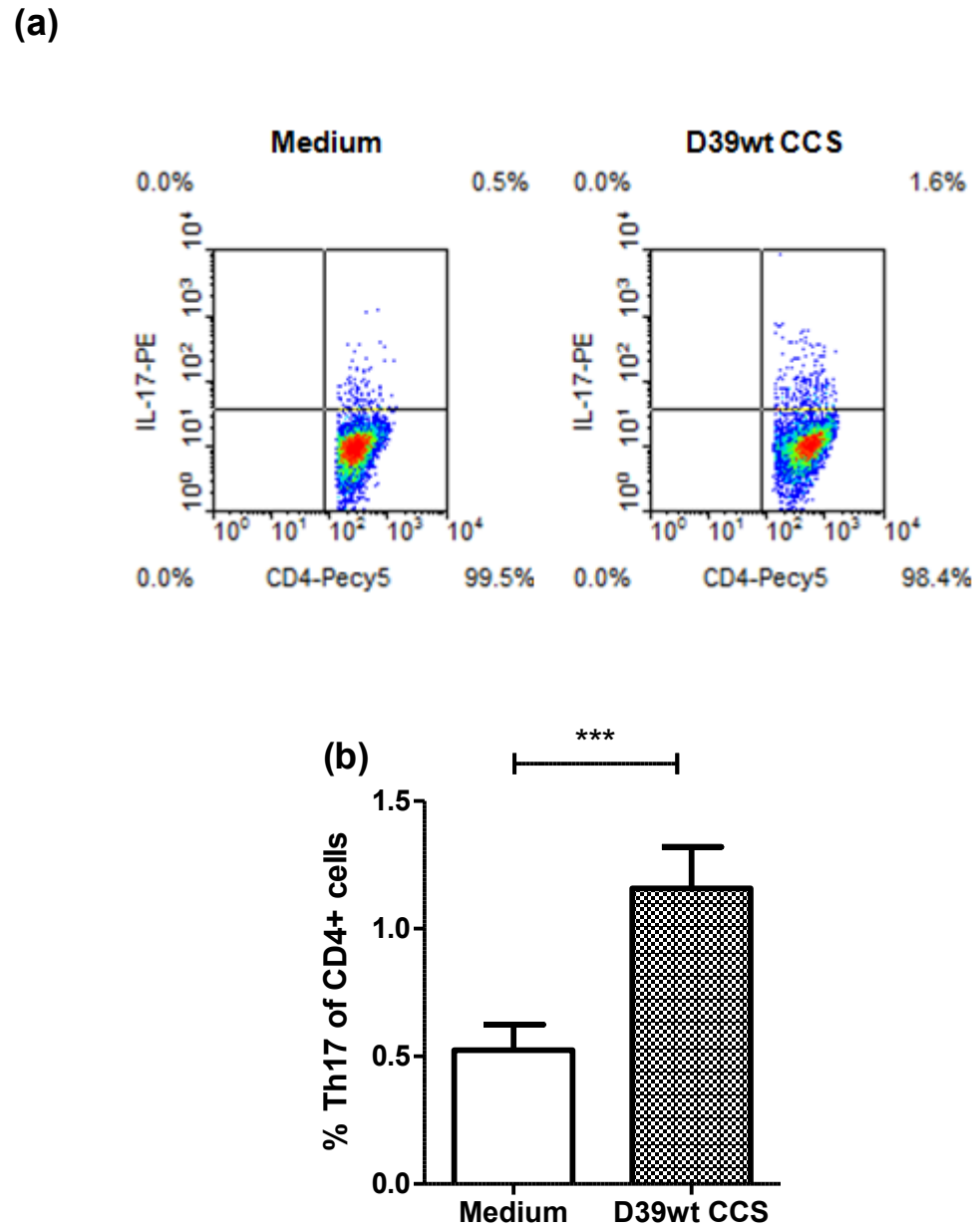


Figure 5.3 Induction of Th17 by D39wt pneumococcal CCS in the presence of cytokines.

Dot plots showing IL17A-producing cells gated for CD4+ T lymphocytes (a). CD45RO+ cell-depleted tonsillar MNC were stimulated by pneumo CCS for 7 days followed by PMA/Ion for 5 hours. One representative experiment is shown. (b) Mean frequency of Th17 cells after stimulation by D39wt CCS was shown (n=17 ***p< 0.001, paired-t test, compared with unstimulated control). Mean+ SEM are shown.

5.4.3. CbpA- and Ply- mutants derived pneumococcal CCS induced less IL17A production than wild type CCS

Induction of Th17 cytokine (IL17A) was analysed in CD45RO+ cell-depleted tonsillar MNC after 7 days of stimulation with pneumococcal CCS derived from wild type (D39wt), or from CbpA- and Ply- mutants in the presence of TGF-β, IL1-β and IL21. As can be seen in Fig.5.4, concentrations of IL17A induced by isogenic mutant CCS, CbpA- and Ply- were lower than D39wt CCS (n=8, *p< 0.05, **p< 0.01).

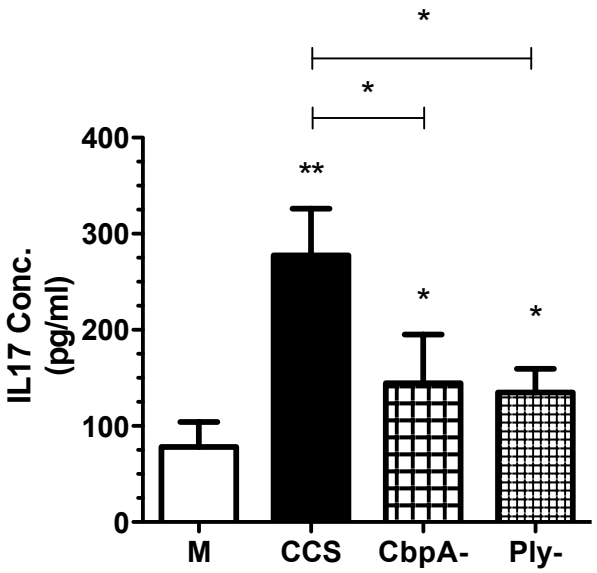


Figure 5.4 IL17A concentrations induced by CCS from D39wt, CbpA- or Ply- mutant strains

IL-17A was measured after 7 days of stimulation with D39wt, CbpA-, or Ply- CCS in the presence of TGF-β, IL1-β and IL21. *p<0.05, **p<0.01, compared to medium control. *p<0.05, mutants compared to wild type pneumococcal CCS, n=8. Paired-t test was used. Mean+ SEM are shown.

5.4.4. Pneumolysin and toxoid (PdB) promote Th17 induction

Purified recombinant pneumococcal proteins pneumolysin (50ng/ml) and its toxoid (PdB: 250 ng/ml)) were cocultured with tonsillar MNC containing naive T cells in the presence of TGF-β, IL1-β and IL21. Th17 frequencies were analysed on day 2, day 5 and day 7 following stimulation. The Th17 frequencies were shown to increase gradually over the course of 7 days (Fig.5.5.).

PdB and rPly were shown to induce Th17 cells from naive cells in the presence of optimal Th17-inducing cytokines (TGF-β, IL1-β and IL21). The increase was shown in figure (Fig.5.6.) by both protein antigens rPly, (n=8, **p< 0.01) and PdB (n=8, **p< 0.01) compared to unstimulated cells.

Concentrations of IL17A, IL17F and IL22 were measured in the cell culture supernatants after 7 days of stimulation. As can be seen in Fig.5.7, both pneumolysin and PdB induced an increase in the concentrations of the 3 cytokines.

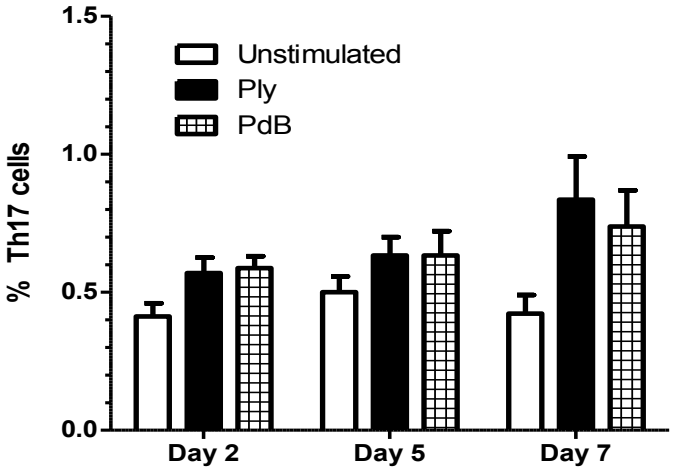


Figure 5.5 Time course of Th17 induction by rPly and PdB.

Tonsillar CD45RO+ cell-depleted MNCs were stimulated by Ply (50ng/ml) and PdB (250ng/ml) with TGF-β, IL1-β and IL21 and analysed for Th17 frequency at Day 2, 5 and 7.

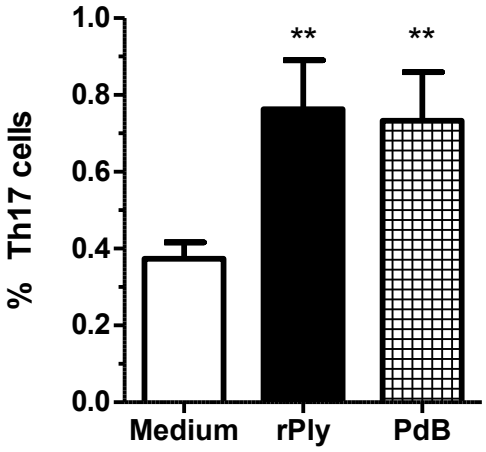


Figure 5.6 Recombinant pneumolysin and toxoid (PdB) promote Th17 induction.

Mean frequency of Th17 cells after 7 days of stimulation by rPly (a) and PdB (b) was shown (n=8, **p< 0.01, compare with unstimulated control). Mean+ SEM are shown.

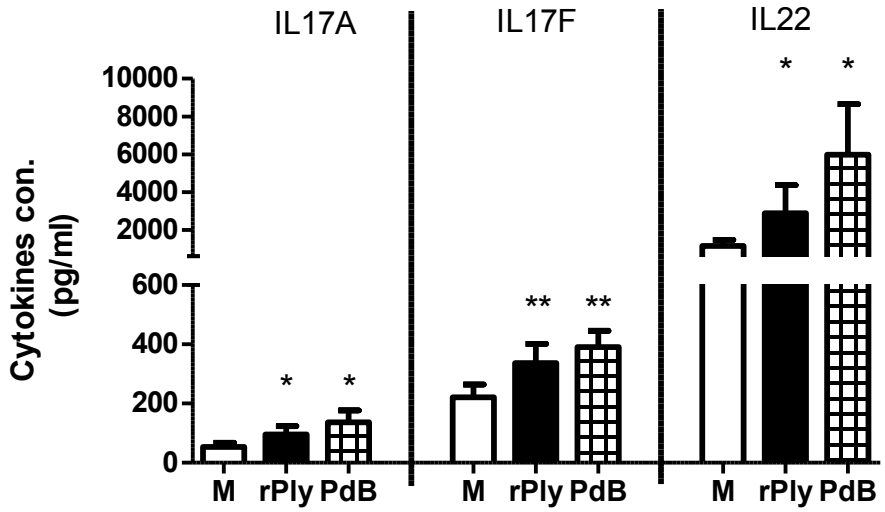


Figure 5.7 Concentrations of IL17A, 17F and IL22 following stimulation by rPly and PdB in the presence of TGF-β, IL1-β and IL21.

Th17-related cytokines (IL17A, IL17F and IL22) were measured after 7 days of stimulation of CD45RO-depleted MNC by rPly and PdB. Both rPly and PdB were shown to induce production of all three cytokines (pg/ml) (*p<0.05, **p<0.01, compared to medium control, n=8). Mean+ SEM are shown.

5.4.5. Effect of TGF-β, IL1-β and IL21 on the induction of Th17-related cytokines - IL17A and IL17F

Production of Th17-related cytokines (IL17A and IL17F) were analysed in tonsillar CD45RO-depleted MNCs culture supernatants after 7 days of stimulation with pneumococcal D39wt CCS. Without TGF-β, IL1-β and IL21, pneumococcal CCS had little effect on the level of IL17A (Fig.5.8.a, n=11, *p< 0.05) and IL17F (Fig.5.8.b, n=9). The addition of TGF-β, IL1-β and IL21 into medium alone has little effect on the production of IL17A, IL17F, but their effects became apparent after stimulation together with pneumococcal CCS. Also, incubating CD45RO+ cell-depleted MNCs with pneumococcal CCS in the presence of TGF-β, IL1-β and IL21 induced higher IL17A (**p<0.01) and IL17F (*p<0.05) than stimulation with pneumococcal CCS alone.

However, it has been demonstrated that the addition of TGF-β, IL1-β and IL21 appeared to reduce the induction of IFN-γ-producing CD4+T cell (Th1) from naïve T cells in CD45RO-MNC when analysed by flowcytometry. As can be seen in figure 5.9, the addition of these cytokines reduced the mean frequencies of IFN-γ+ CD4+ Th1 cells in the presence or absence of pneumococcal CCS stimulation (n=6, *p<0.05, **p<0.01, compared to without TGF-β/IL1-β/IL21).

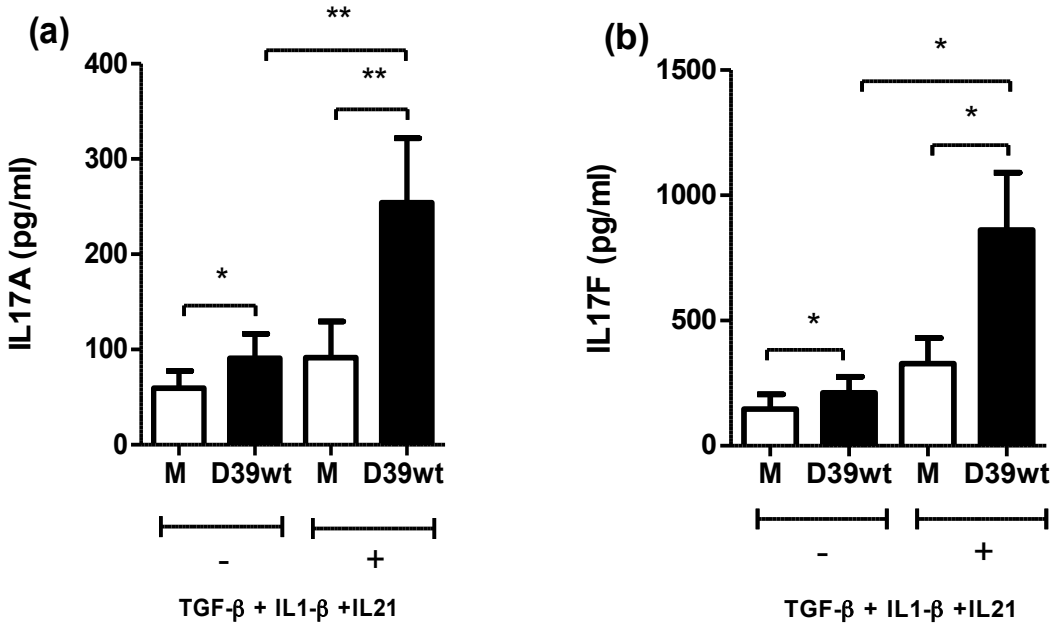


Figure 5.8 The effect of TGF-β, IL1-β and IL21 on the production of IL17A and IL17F.

Cytokine levels of IL17A (a) (n=11) and of IL17F (b) (n=9) in tonsillar CD45RO+ cell depleted MNC culture supernatant after 7 days of CCS stimulation with cytokines (+) or without (-). Significant differences (*p< 0.05 and **p< 0.01) are shown on graph. Mean+ SEM are shown. Paired t test was used.

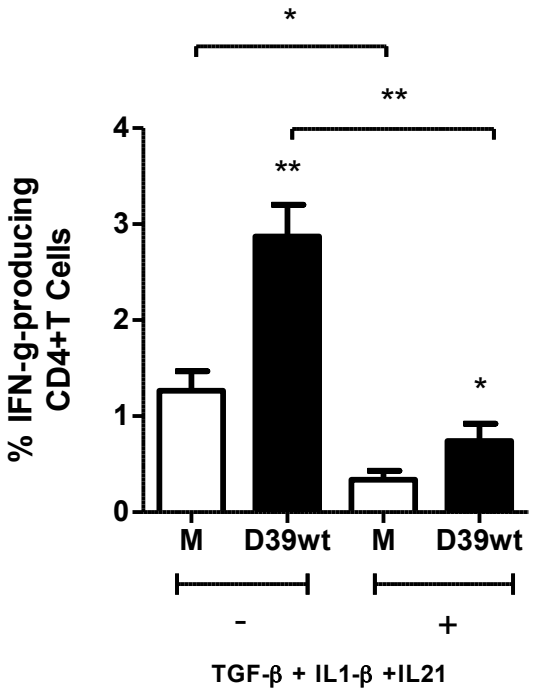


Figure 5.9 The effect of TGF-β, IL1-β and IL21 on IFN-γ producing CD4+ T cells.

Mean frequency of IFN-γCD4+ T cells in tonsillar CD45RO+ cell depleted MNC after 7 days of CCS stimulation with cytokines (+) or without (-). Significant differences (*p< 0.05 and **p< 0.01, n=6) are shown to compare with medium control. Mean+ SEM are shown. Paired-t test was used.

5.4.6. Induction of Foxp3+Treg cells by pneumococcal CCS with or without TGF-β

To assess the induction of Foxp3+Treg cells, CD45RO+ and CD25+ cell depleted tonsillar MNC (Fig.5.10.a) were stimulated by D39wt pneumococcal CCS for 7 days with (+) or without (-) TGF-β added. Intracellular staining of Foxp3 was performed as described in the materials and methods. There was a gradual increase in Treg cells numbers after stimulation with pneumococcal CCS and that was associated with TGF-β concentrations (Fig.5.10.b).

As can be seen in Fig.5.11, in the presence of TGF-β, Treg cell numbers were significantly elevated by pneumococcal CCS (n=18, ***$p<0.001$, compared to unstimulated cells). In the absence of TGF-β, there was a small increase in the mean percentage of Foxp3+Treg cells (n=7, *$p< 0.05$, compared to unstimulated cells).

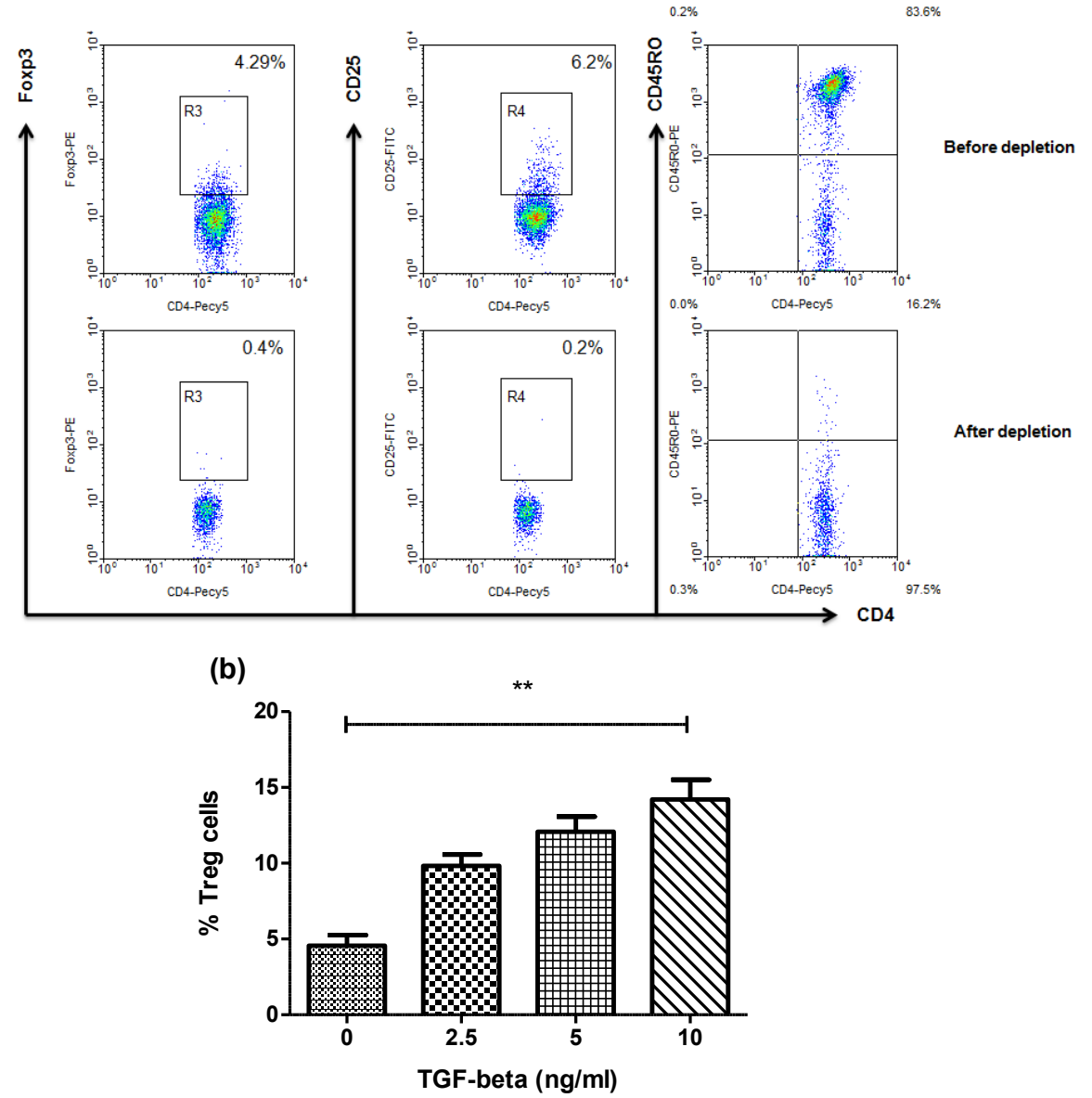


Figure 5.10 Efficiency of CD45RO+ CD25+ cell depletion from tonsillar MNC.

The depletion of both CD45R0+ and CD25+ cells from tonsillar MNC was performed by MACS negative depletion. The cells were stained with CD4, CD45RO, CD25, Foxp3 anti-human antibodies. The efficiency of $CD45RO^{+}$ CD25+ cells depletion was shown by the disappearance of most CD45RO+CD25+ cells (a). (b)The mean percentage of Treg cells was increased gradually with high concentrations of TGF-β after 7 days stimulation by pneumococcal CCS.

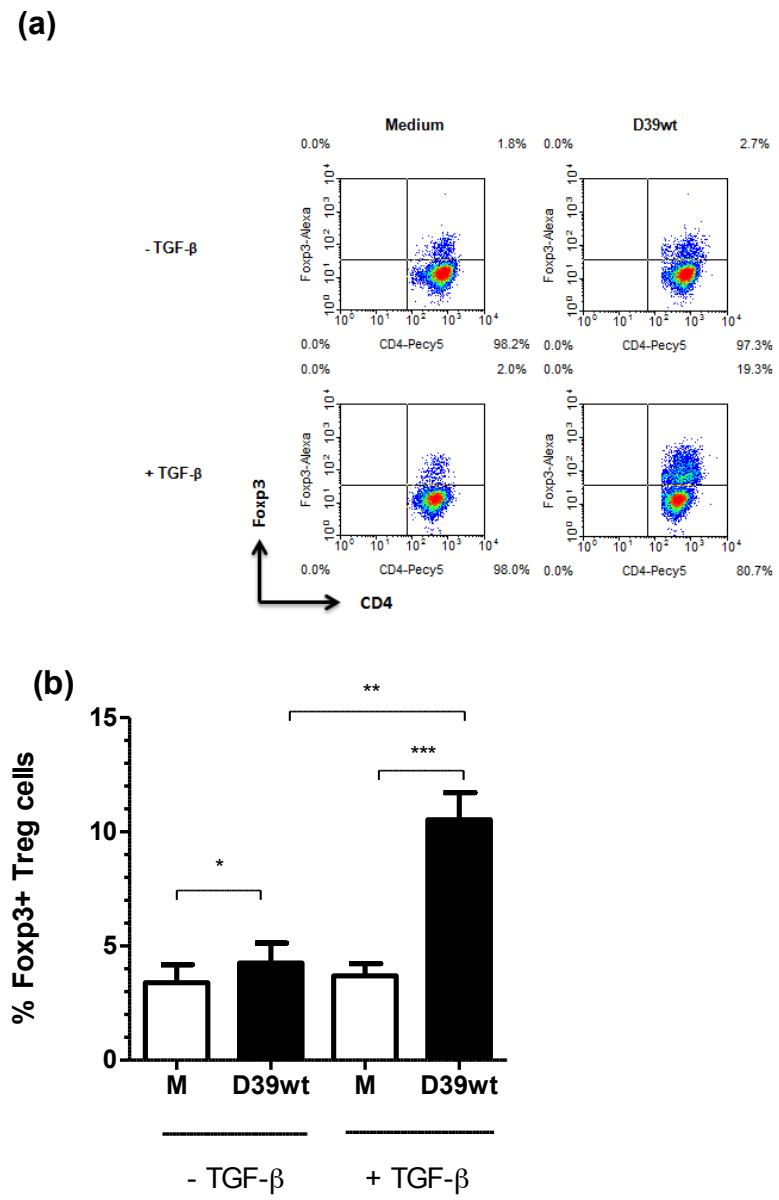


Figure 5.11 Effect of TGF-β on frequency of Foxp3+ Treg cells.

Density plot shows expression of Foxp3 in Treg cells gated for CD4+ T lymphocytes in one representative experiment (a). Mean frequency of Foxp3+Treg cells in tonsillar CD45RO+ and CD25+ cells depleted MNC after 7 days of CCS stimulation with TGF-β (n=18) or without (n=7). Significant differences (*p< 0.05 and ***p< 0.001) are shown to compare with medium control. Paired t test was used. **p< 0.01, compare D39wt CCS + TGF-β to D39wt CCS. Unpaired-t test was used. Mean+ SEM are shown.

5.4.7. Induction of Treg by stimulation with pneumococcal CCS derived from wild type, Ply- or CbpA- mutants

The number of Foxp3+ Treg cells was analysed in tonsillar MNC depleted of CD25+ and CD45RO+ cells after 7 days of stimulation with pneumococcal D39wt CCS or with CbpA- and Ply- CCS. As can be seen in figure 5.12, pneumococcal CCS derived from the Ply- and CbpA- mutants induced lower numbers of Foxp3+ Treg (n=10, $^{**}p<0.01$).

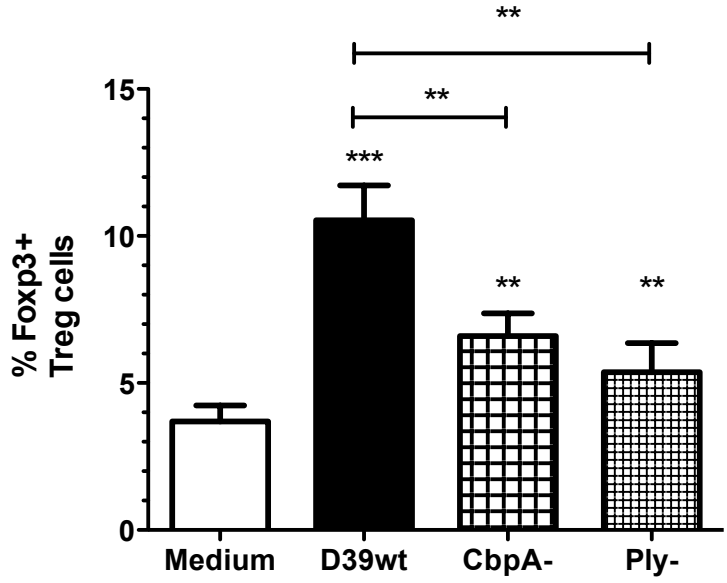


Figure 5.12 Frequencies of tonsillar Treg cells following stimulation by CCS derived from D39wt, CbpA- or Ply- mutant strains.

The mean frequency of Foxp3+Treg cells was measured after 7 day of stimulation with D39wt, CbpA-, or Ply- CCS in the presence of TGF-β. $^{***}p<0.001$, $^{*}p<0.05$, compared to medium control. $^{**}p<0.01$, mutants compared to wild type pneumococcal CCS, n=10). Paired-t test was used. Mean+ SEM are shown.

5.4.8. Effect of pneumolysin and toxoid (PdB) on expression of CTLA4 on tonsillar Treg cells

PdB enhanced the induction of Treg at optimised concentration of 250ng/ml in the presence of TGF-β. Samples were collected at day 2, day 5 and day 7 (n=6). As seen in figure 5.13, the number of Treg cells gradually increased by time.

Co-expression of CD152 and Foxp3 was analysed following cell stimulation by pneumolysin or PdB in the presence of TGF-β. One representative experiment was shown in Fig.5.14.a. The mean proportion of CD152+Foxp3+ Treg cells after stimulation with PdB and Ply increased significantly to around 4.7% and 4.6 respectively, compared with unstimulated control (Fig.5.14.b, n=14, ***$p < 0.001$).

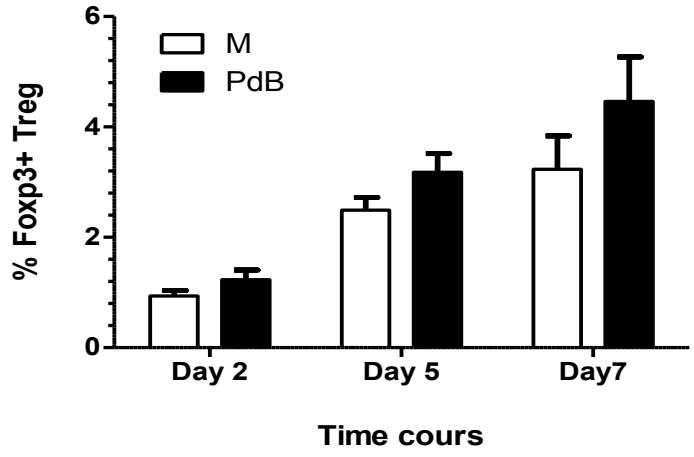
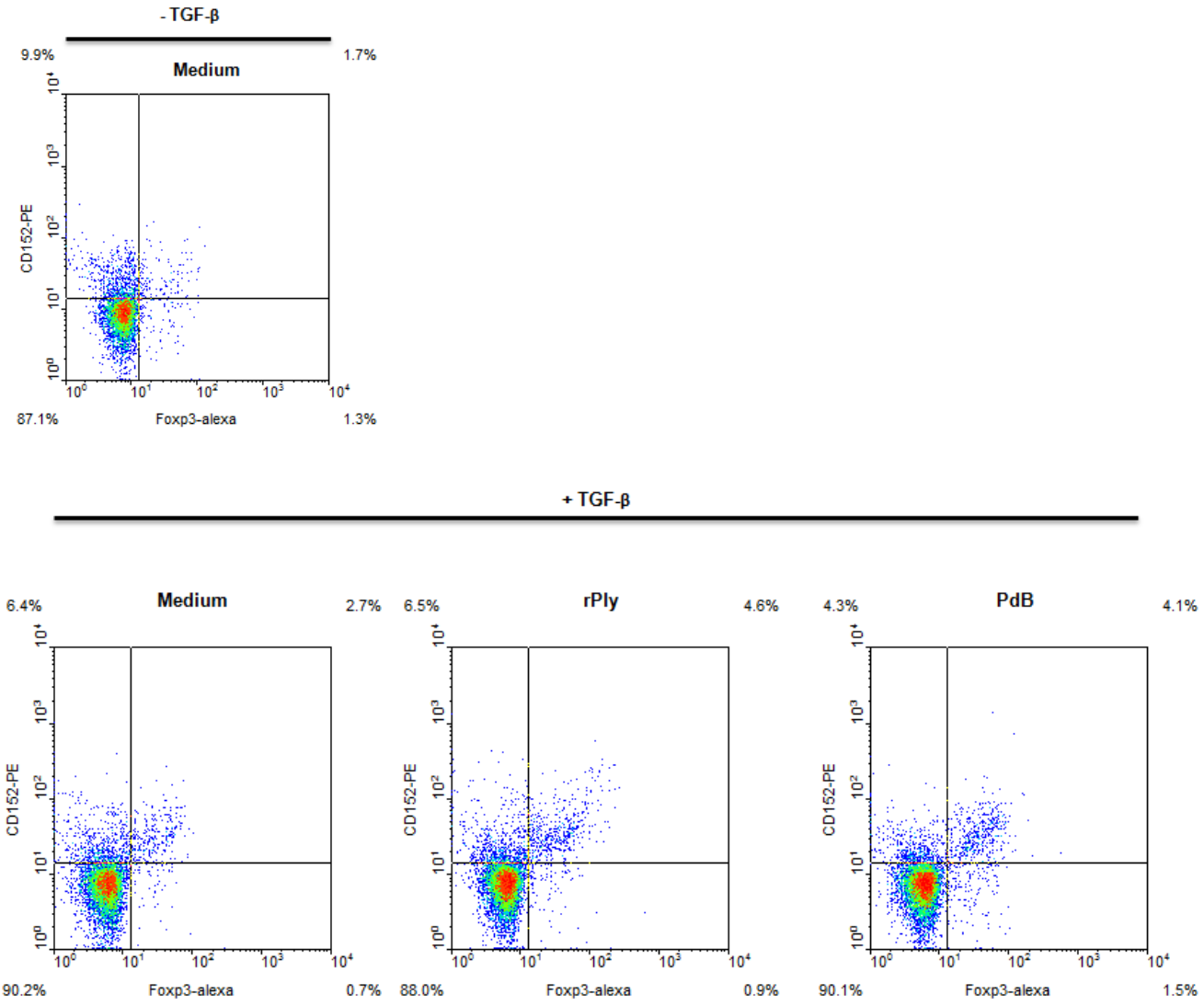


Figure 5.13 Time course of Treg induction by PdB in tonsillar MNC depleted of CD25+and CD45RO+ cells.

Tonsillar CD45RO+ cell-depleted MNCs were stimulated by PdB (250ng/ml) in the presence of TGF-β and analysed for Treg cell frequency at day 2, 5 and 7).

(a)


- TGF-β
9.9%
1.7%
Medium
CD152-PE
87.1%
Foxp3-alexa
1.3%
+ TGF-β
6.4%
Medium
2.7%
90.2%
Foxp3-alexa
0.7%
6.5%
rPly
4.6%
88.0%
Foxp3-alexa
0.9%
4.3%
PdB
4.1%
90.1%
Foxp3-alexa
1.5%

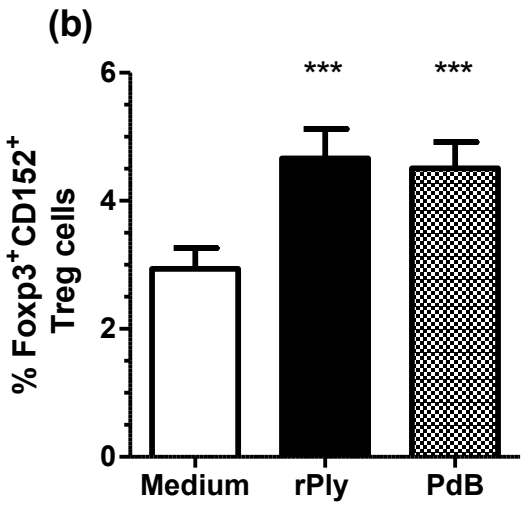


Figure 5.14 Pneumolysin and toxoid (PdB) increased the CTLA4 expression in tonsillar cells in the presence of TGF-β.

Density plot shows expression of CTLA4 (CD152) in Foxp3^{+}Treg cells in one representative experiment (a)**.** CD25+ and CD45RO+ cell-depleted MNC were stimulated by rPly at 50 ng/ml and PdB at 0.25µg/ml (n=14) for 7 days (b). ***p< 0.001, rPly and PdB compared with un-stimulated cells (medium control). Mean+ SEM are shown.

5.5. Discussion

In this study, it has been sought to examine the induction of Th17 from tonsillar MNC that were depleted from activated and memory T cells. As our earlier studies demonstrated that the majority of Th17 cells were from CD45RO+ CD4+T cells, we therefore used CD45RO+ cell depletion to remove activated/memory cells containing Th17. The remaining tonsillar MNC containing only naïve T cells were then used to study the induction of Th17 by pneumococcal protein antigens in the presence of relevant cytokines. We also assessed the effect of TGF-β, IL1-β and IL21 on the induction of Th17.

Naïve and memory cells are mainly two distinct subsets of CD4+ T cells in peripheral blood. Memory T cells are capable to mediate a rapid and effective response to pathogen. These memory cells are divided into major subsets with regard to homing capacities and effector function, CD4+ central memory T (T_{CM}) cells and CD4+ effector memory T (T_{EM},) cells in both humans and mic (Sallusto, Geginat & Lanzavecchia 2004; Sallusto et al. 1999). The expression of chemokine receptors and adhesion molecules, CCR7 and L-selectin (CD62L) are the main characterisation of these memory cell subsets. CCR7 and CD62L are both expressed in human T_{CM} cells and home T cell areas of the secondary lymphoid organs such as tonsils. T_{CM} cells can proliferate and differentiate into effector cells upon antigenic stimulation but have limited effector function (Onoda et al. 2007).

However, human T_{EM} cells lack the expression of lymphoid homing receptor, CCR7 but they heterogeneously express CD62L (Onoda et al. 2007). Also, they express inflammatory receptors such as CCR5 and CXCR3 in order to enter the inflamed peripheral tissues(Qin et al. 1998). In terms of distribution, these cells are mainly localised in the lung, liver and gut (Campbell et al. 2001). T_{EM} cells have much ability to differentiate and polarize than T_{CM} and produce effector cytokines following antigenic stimulation which leads to immediate immune protection (Lanzavecchia & Sallusto 2000; Onoda et al. 2007).

The induction of Th17 in CD45RO- MNC T cells was assessed by co-incubation with D39wt pneumococcal CCS with or without exogenous cytokines. Stimulation by pneumococcal CCS induced a small increase in Th17 cells. The results were consistent with IL17A concentrations measured in culture supernatants (Fig. 5.1).

With the addition of cytokines including TGF-β, IL1-β and IL21, Th17 numbers were significantly higher compared to stimulation by pneumococcal CCS alone, supporting the role of the cytokines in promoting Th17. The presence of TGF-β, IL1-β and IL21 cytokines alone showed little effect on Th17 induction and on the production of IL17A and IL17F, but together with pneumococcal CCS enhanced the effect (Fig.5.3.). Furthermore, up-regulation of RORC2 and IL17A mRNA expression and differentiation of human Th17 from naive CD4+ T cells can be enhanced by IL21 and TGF-β in serum-free medium (Yang et al. 2008). Both cytokines can induce the expression of IL23R and RORγt and subsequently secretion of IL17A from naive CD4+ T cells (Nurieva et al. 2007; Zhou et al. 2007).

However, the combination of IL1-β together with IL23 or IL6 was shown to promote the secretion of IL17 by human central memory CD4+ T cells (Yang et al. 2008).

It was shown that a low concentration of TGF-β was important in the induction of Th17 cells from naive cells in the presence of pneumococcal antigens. This is in agreement with previous reports in Th17 induction (Manel, Unutmaz & Littman 2008; Volpe et al. 2008; Yang et al. 2008). Some reports showed that TGF-β may downregulate human Th17 cells whereas IL1β, IL23 or IL6 promote Th17 (Acosta-Rodriguez et al. 2007; Chen et al. 2007; Wilson et al. 2007). The different results reported may be related to the observation that TGF-β were used, as we observed low concentration promoted Th17 whereas higher concentrations inhibited Th17. This finding was consistent with the study carried out by Mills (Mills 2008).

It has been also demonstrated CCS of CbpA- and Ply- mutant strains induced less IL17A. Also, purified pneumolysin (rPly) and its toxoid PdB were shown to induce Th17 cells in the presence of the cytokines. This suggests pneumococcal proteins such as pneumolysin can promote Th17 cells in NALT. These data were consistent with our previous finding that pneumolysin domain 4 can promote Th17 differentiation in the presence of TGF-β and IL21. (Gray et al. 2014).

Our finding revealed that pneumolysin and PdB could induce the production of Th17-signature cytokines (IL17A, IL17F and IL122). It has been suggested that these cytokines are crucial mediators of mucosal defence (Aujla & Kolls 2009). Moffitt et al. has demonstrated that IL17A was elicited from splenocytes in

response to several recombinant proteins of pneumococcus and suggested that IL17-producing cells (Th17) may have the ability to provide protection against pneumococcal colonisation (Moffitt, Malley & Lu 2012).

It is known that IL17A, IL17F and IL22 have a role in immune response against many pathogens. These cytokines are able to recruit and activate neutrophils through inducing the release of chemo-attractants and G-CSF from epithelial cells or fibroblasts. Once these cells are activated then killing are mediated by neutrophils. Also, these cytokines have the capability to elicit the expression of antimicrobial peptides such as β-defensin 2 and S100 family and the secretion of HCO_3^- along with CCL20 can kill the microbes directly and draw effectors, memory T cells and immature dendritic cells (DCs) into site of infected tissue (McAleer & Kolls 2011; Wilson et al. 2007). IL22 is reported to be crucial for tissue defences at mucosal surface by controlling the microorganisms at the site of infection (Volpe et al. 2009).

The addition of cytokines TGF-β, IL1-β and IL21 was shown to promote Th17 induction (Fig.5.8) but appeared to suppress the induction of IFN-γ+ Th1 cells (Fig.5.9). This was in agreement with the previous reports (Yang et al. 2008) (Hebel et al. 2011) that showed IL21 and IL1-β can significantly induce the production of IFN-γ from human naive T cells whereas the addition of TGF-β/IL21/IL1-β inhibit the production of IFN-γ and promote the induction of Th17 cells. Findings herein are also consistent with that induction of Th17 by TGF-β/IL21/IL1-β was associated with decreased secretion of IFN-γ through

suppressing the expression of T-bet and Th1 development (Annunziato et al. 2008; Volpe et al. 2008; Yang et al. 2008). Induction of Th17 just expressing IL17 was shown to result from TGF-β-polarising treatment, whilst Th17 cells secreting IL17A and IFN-γ+ were shown in the absence of TGF-β (Hakemi et al. 2011; Valmori et al. 2010).

Tonsillar MNC depleted of CD45RO+ and CD25+ cells were used to test for the ability of pneumococcal CCS stimulation or proteins to induce Foxp3+Tregs, in the presence or absence of TGF-β. It has been shown that Treg cells frequencies were associated with higher concentrations of TGF-β following pneumococcal CCS (Fig.5.10.b). As can be seen in Fig.5.11, stimulation by D39wt pneumococcal CCS induced a marked increase in Treg in the presence of TGF-β, and that was significantly higher than stimulation by either CCS or TGF-β alone.

It has been shown that stimulation by pneumococcal CCS derived from Ply- and CbpA- mutant strains appeared to induce less Foxp3+Treg cells compared wild type CCS. This suggests that Ply and CbpA proteins may play a role in induction of Treg. It has been shown the induction of Treg from naive T cells requires TGF-β and TCR stimulation and both are needed for the induction (Chen et al. 2003).

The expression of CTLA-4 in Treg cells was also shown to be upregulated by stimulation of D39wt CCS and TGF-β. CTLA is an important marker for Treg function in regulating an immune response; therefore the increased Treg may have upregulated inhibitory activity following stimulation by pneumococcal antigens. It

has been demonstrated in mice that TGF-β possessed the potential to generate CD4+CD25+Foxp3+ Treg population from CD4+CD25-Foxp3- non-Tregs and the generated cells showed a high level of CTLA-4 and Foxp3 expression. Likewise, in humans the increased numbers of Foxp3+ Treg cells with $CD25^{high}$ and CTLA-4 were shown following stimulation of human CD4+ T cells by TGF-β (Zheng et al. 2002). The secretion of IL35 by Treg cells induces Treg population from conventional T cells, named iTreg35 and that leads to IL35 mediated suppression (Collison et al. 2010). According to Chaturvedi et al., human Treg cells have the capability to express and secrete IL35 and that has a significance role in the suppressive activity on T cells when compared to IL10 and TGF-b. This was confirmed by the addition of neutralising antibodies and the effective suppression by Treg was mediated by IL35 but not with IL10 and TGF-b (Chaturvedi et al. 2011). Taken together, TGF-β, and IL35 have an important role in Treg induction in humans, which leads to suppress other T cell population.

5.6. Summary

It has been shown that pneumococcal CCS stimulation induced Th17 and Treg in tonsillar cells in the presence of some cytokines. Pneumococcal protein antigens including Ply and CbpA may contribute to the induction of both cells. A combination of TGF-β, IL21 and IL1 β may provide a favourable cytokine environment for Th17 induction from naïve cells in tonsillar tissue. Also, TGF-β appeared to be crucial in Treg induction. Consequently, the induction of Th17 or

Treg in human tonsillar tissue may be common in humans especially in children during natural infection/carriage, and the balance of the two may determine the clearance or carriage of pneumococcus.

Chapter 6: General Discussion and Conclusion

6.1. Discussion

Globally, *Streptococcus pneumoniae (pneumococcus)* is a major cause of morbidity and mortality in humans among risk groups (O'Brien et al. 2009). Pneumococcus is a common cause of respiratory tract infections in both developing and developed countries. Annually, pneumococcal diseases are responsible for around 1 million deaths of young children under 5 years (Obaro & Adegbola 2002). Healthy individuals can be colonized in the upper respiratory tract by pneumococcus and nasopharynx is the main reservoir for *S. pneumoniae*. Colonisation with pneumococcus in the nasopharnyx is very common in infants, up to 90% in some populations (Bogaert, De Groot & Hermans 2004).

It was hypothesised that mucosal Treg and Th17 cells in NALT may play a crucial role in mediating the persistence or clearance of pneumococcal carriage in human nasopharynx. Thus, in this study we investigated the association of human mucosal Th17 cells and Treg cells with pneumococcus through analysing the numbers of Th17 and Treg cells in tonsillar tissues of both adults and children; and their relationship to pneumococcal carriage status.

It has been shown that the frequency of Treg and Th17 cells in tonsillar tissue in each subject was generally higher than in peripheral blood. This may suggest the mucosal compartment within human nasopharynx develops a higher number of Treg due to the exposure of microorganisms than in peripheral blood, and also suggest the presence of antigen-specific (inducible) Treg in human NALT. Also it is likely that the mucosal Th17 in human NALT were primed largely by previous

microbial infection/colonisations in the nasopharynx. Data in previous work showed that the Th17 detected in NALT tissues were primarily memory Th17 cells, as the Th17 cells in tonsillar MNC were abrogated following depletion of CD45RO+ T cells (Gray et al. 2014).

Furthermore, it has been shown that there was a difference between adults and children in the frequency of Treg and Th17 in tonsillar tissues. The frequencies of tonsillar Treg of children were generally higher than in adults, and there appeared an age-associated decrease in tonsillar Treg numbers (Fig.3.1). However, the frequency of tonsillar Th17 appeared to increase with age. There appeared to be a positive correlation between the ratio of Th17/Treg in tonsillar tissues and ages. The mean ratio of tonsillar Th17/Treg MNC was shown to be significantly higher in adults than in children (Fig 3.9). Overall, younger children <10 years were shown to have higher Treg numbers than those subjects >10 years. This would support the hypothesis that extensive exposures to microorganisms in childhood are associated with the induction of Treg. It may be that the local colonisation with microorganisms could induce antigen-specific Treg cells thus leading to high numbers of Treg in NALT tissues, and this may contribute to the persistence/carriage of specific microbes (Zhang et al. 2011). Whilst numbers of tonsillar Th17 in younger children <10 were significantly lower than those subjects >10 years (Fig 3.7.).

Pneumococcal carriage is common in children especially in younger children. With increasing age, pneumococcal carriage rate gradually decreases (Bogaert, De

Groot & Hermans 2004; Granat et al. 2009). During carriage, antigen-specific immunity develops to protect against the subsequent colonisation (Cohen et al. 2011; Richards et al. 2010; Zhang, Clarke & Weiser 2009). It has been hypothesised that mucosal Th17 in the nasopharynx may play a role in the age associated decrease in pneumococcal carriage because the human nasopharynx is a site of colonisation of many microorganisms that may prime for Th17 in NALT.

There appeared to be a difference in the frequency of Treg cells which was higher in culture-positive than in culture-negative children and it has been showen earlier that mucosal Treg frequency also appeared to be associated with pneumococcal carriage rate. Indeed, the mean ratio of Th17/Treg in NALT was shown to be higher in culture-negative children than culture-positive children. These suggest that the induction/promotion of Treg in tonsillar tissues may be contributed by pneumococcal colonisation in the nasopharynx. Also, the delayed clearance or persistence of pneumococcal colonisation in children may occur in the existence of Treg cells (Zhang et al. 2011). This was in agreement with another study which revealed that the pneumococcal response at mucosal surface was highly regulated by the presence of Treg cells, and thus, it prolonged the nasopharyngeal colonisation (Glennie et al. 2012). On the other hand, the ratio of Th17/Treg may be a crucial determinant of pneumococcal clearance. This finding would support that a higher Th17/Treg ratio in NALT contributes to clearance of mucosal carriage of pneumococcus. There results are consistent with the hypothesis that Th17 may play a role in mucosal defence against microbial infection (Wu et al. 2007).

It is unclear what components of pneumococcus contribute to the activation of Treg and Th17 cells in tonsillar tissue. It would be useful to identify what pneumococcal components activate Treg or Th17 cells as that may add important information to novel vaccination strategies against pneumococcal infection. In this study, stimulation with pneumococcal CCS wild type (D39wt) was shown to elicit a significant increase in Th17 and Foxp3+ Treg number with high level of IL17A and TGF-β respectively. Our previous work suggested that there were memory/effector Th17 and Treg within the adenotonsillar tissue (Gray et al. 2014; Zhang et al. 2011), as specific stimulation by pneumococcal antigens could activate these cells. Repeated exposure to pneumococcus in childhood may contribute to the increase in inducible Treg cells in early childhood, and that may delay the induction of Th17 and contribute to the persistence of pneumococcal carriage. Therefore, priming for Th17 cells in early life that may inhibit induction of Treg in NALT might be an attractive vaccination strategy against pneumococcal colonisation (Gray et al. 2014).

In contrast, pneumococcal CCS derived from wild type pneumococcus, the CCS from mutant strains (Ply- and CbpA-) were shown to elicit a lower increase in Treg and Th17 frequencies than wild type CCS. Moreover, these isogenic mutant strains appeared to induce less Foxp3+Treg cells (with TGF-β) and IL17A (with IL21/IL1-β/ TGF-β) compared to wild type CCS. These results suggest that pneumococcal Ply and CbpA proteins may contribute to the activation of Foxp3+ Treg and Th17 cells in tonsillar MNCs and also may play a role in their induction

from naive T cells. Our findings demonstrated that purified individual pneumococcal protein antigens such as rPly, PdB and CBP were capable to prime and activate Foxp3+Treg and Th17 cells which were enriched in adenotonsillar tissue. These results support the presence of antigen-specific Treg and Th17 in human NALT, and they may play an important role in the modulation of pneumococcal carriage or clearance.

In addition, purified pneumolysin (rPly) and its toxoid PdB were shown to induce Th17 cells and Th17-signature cytokines (IL17A, IL17F and IL122) in the presence of Th17-polarising cytokines. This suggests pneumococcal proteins such as pneumolysin can promote the induction of Th17 cells in NALT. These data were consistent with our previous finding that pneumolysin domain 4 can promote Th17 differentiation in the presence of TGF-β and IL21. (Gray et al. 2014). It has been suggested that these cytokines are crucial mediators of mucosal defence (Aujla & Kolls 2009). Moffitt et al. has demonstrated that IL17A was elicited from splenocytes in response to several recombinant proteins of pneumococcus and suggested that IL17-producing cells (Th17) may have the ability to provide protection against pneumococcal colonisation (Moffitt, Malley & Lu 2012). Studies in mice showed that the response of IL-17A following immunisation is associated with decreased pneumococcal carriage which supports a critical role of IL17A in pneumococcal clearance (Lu et al. 2009). The secretion of Th17 cytokines in human tonsillar tissue lead to the recruitment and activation of neutrophils that is associated with phagocytic killing (Lu et al. 2009), and likely act through the

induction of pro-inflammatory mediators such as G-CSF and chemokines from epithelial cells (Aujla, Dubin & Kolls 2007b; Kolls & Linden 2004). Also, IL17A, IL17F and IL22 have the capability to elicit the expression of antimicrobial peptides along with CCL20 which can control the pathogens directly and draw effector T cells, memory T cells and immature dendritic cells (DCs) into site of infected tissue (McAleer & Kolls 2011; Wilson et al. 2007). IL22 is reported to be crucial for tissue defences at mucosal surface by controlling the microorganisms at the site of infection (Volpe et al. 2009).

As it was demonstrated that the majority of Th17 cells were from CD45RO+ CD4+T cells, we therefore used CD45RO+ cell depletion to remove activated/memory cells containing Th17 to study the induction of Th17 in NALT. Stimulation by pneumococcal CCS induced a small increase in Th17 cells and their relevant cytokine (IL17A and IL17F) from tonsillar MNC containing naïve cells. However, with the addition of cytokines including TGF-β, IL1-β and IL21, results showed Th17 numbers were significantly higher compared to stimulation by pneumococcal CCS alone, supporting the role of the cytokines in promoting Th17. In the presence of TGF-β, the ability of pneumococcal CCS stimulation was shown to induce marked numbers of Foxp3+Tregs from tonsillar MNC depleted of CD45RO+ and CD25+ cells. The expression of CTLA-4 in Treg cells was also shown to be upregulated by stimulation of D39wt CCS or pneumococcal protein antigens in the presence of TGF-β. It has been demonstrated in mice that TGF-β possessed the potential to generate CD4+CD25+Foxp3+ Treg population from CD4+CD25-Foxp3- non-Tregs and the generated cells showed a high level of

CTLA-4 and Foxp3 expressions. Likewise, in humans the increased numbers of Foxp3+ Treg cells with $CD25^{high}$ and CTLA-4 were shown following stimulation of human CD4+ T cells by TGF-β (Zheng et al. 2002).

Results presented here have concluded that pneumococcal proteins including Ply, PdB and CBP are immunogenic and capable to activate antigen-specific Th17 and Treg cells, and thus might be implicated for vaccine development. In the future, it is required to consider novel approaches that can be used to mimic natural immunity and provide mucosal protection. Immune response induced by vaccine immunisation should provide an effective mucosal protection against all serotypes of pneumococcus during colonisation as well as invasive disease. Candidate vaccines for preventing nasopharyngeal colonisation should drive the mucosal response toward Th17 cells rather than Treg cells. A Th17-predominant response over a Treg cell response would likely lead to a reduction or clearance of colonisation. A reduction or clearance of pneumococcal carriage may reduce the transmission of pneumococcus and leads to a reduction of pneumococcal invasive infections.

6.2. Conclusion

Results presented in this study suggest that there were marked numbers of Th17 and Treg cells in tonsillar tissues which may play an important role in mediating nasopharyngeal carriage of pneumococcus. Pneumococcal simulation of tonsillar cells elicited a significant increase in numbers of both Th17 and Foxp3+ Treg cells suggesting the presence of antigen-specific Th17 and Treg in human NALT, presumably primed by previous colonisation of pneumococcus. In general, the ratio of tonsillar Th17/Treg was shown to increase with age and shown to be higher in pneumococcal culture-negative children than in culture-positive children. It suggests the balance of Th17/Treg in nasopharynx-associated lymphoid tissue is a critical determinant of pneumococcal clearance or persistence/carriage in human nasopharynx. Moreover, results from this study provided supporting evidence that pneumococcal proteins including pneumolysin, its toxoid and choline binding proteins may contribute to the induction and/or activation of Th17 and Treg cells in human NALT, and that may contribute to the modulation of pneumococcal carriage or clearance in human nasopharynx.

A combination of different pneumococcal proteins including the use of pneumolysin toxoid/derivatives is currently being studied in pneumococcal protein-based vaccines. This implication should be performed in mouse models but instead of using one single pneumococcal protein, it very important to use two or three different pneumococcal proteins beside pneumolysin protein. Also, the use of the proper adjuvant in vaccine is considered very crucial for optimal response. The

ability of protein-based vaccines to induce an optimal T cell response at the mucosal level including a significant Th17 response may offer in near future effective vaccination strategies against pneumococcal diseases. Understanding the natural immune response to these protein antigens may have important implications in novel vaccination strategies such as mucosal vaccines against pneumococcal infection in humans.

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Appendices

Appendix-1: Preparation of different media and buffers

Skim milk, Tryptone, Glucose, and Glycerol Transport Medium (STGG)

STGG transport medium was prepared using the following procedure:

1. 40 ml 10% milk was prepared by adding 4 g skimmed milk powder (Oxoid, UK) into 40 ml dH_2O, then it was autoclaved in 121°C for 5 minutes.
2. In another sterile 500 ml glass bottle, 6 ml Tryptone soya broth (Oxoid, UK), 2.2 ml 45% D-Glucose solution (Sigma Aldrich, UK), 20 ml Glycerol (Sigma Aldrich, UK) were taken. Then, 131.8 ml dH_2O was added into the bottle to make the final volume 160 ml.
3. After that the bottle with ingredients was autoclaved in 121°C for 10 minutes
4. Then the previously prepared sterile 40 ml of 10% skimmed milk was added into the 500 ml bottle to make 200 ml 2% skimmed milk medium.
5. The skimmed milk was mixed thoroughly with the other ingredients by gentle shaking.
6. Aliquot of 1.0 ml media was prepared into 1.5 ml screw-capped vials.
7. The tubes were then kept in a refrigerator (2-8°C) until used for nasal swab inoculation.
8. For sterility of the STGG medium, quality control test was performed periodically by plating a full loop of a homogenized vial from each lot onto a blood agar plate, then incubating the plate at 37°C overnight. If growth of any organism was suspected then the lot was discarded

Todd-Hewitt Broth (THB)

Todd-Hewitt Broth with 0.5% yeast extract was prepared using the following procedure.

1. 36.4 g of Todd-Hewitt Broth (Oxoid, Basingstoke, UK) and 5 g of yeast extract were taken into a sterile 1 litre glass bottle.
2. 1000 ml of distilled water was added to the bottle and mixed thoroughly, until all components were dissolved completely.
3. After autoclaving at 115°C for 10 minutes, the bottle kept in the cold room until used for growing bacteria.

Laemmli Reducing Buffer

For western Blot sample dilution, 10.0 ml Laemmli reducing Buffer was prepared using following recipe:

1.25 ml	0.5 M Tris pH 6.8
2.0 ml	10% (w/v) SDS
0.30 ml	0.5% Bromphenol Blue
2.50 ml	Glycerol
3.55 ml	dH_2O
0.50 ml	β-mercaptoethanol (50 µl for each 0.95 ml)

10x Tris Buffered Saline (TBS) pH 7.4

1. 1 litre 10x TBS was prepared using the following recipe:

30 g	Tris
80 g	NaCl
2 g	KCl
900 ml	dH_2O

2. Then pH was adjusted to 7.4 by adding concentrated HCl, and measurement with a pH meter. When the pH is adjusted then the bottle was topped-up to 1 litre by adding dH_2O.

Running buffer for Western Blot

50 ml (10X Tris-SDS) buffer was added to 450 ml dH_2O

Resolving gel preparation

100 ml resolving gel was prepared using the following recipe:

40 ml	Protogel
26 ml	Protogel buffer
32.9 ml	dH_2O
1 ml	Ammonium persulphate (10 %)
0.1 ml	TEMED

Stacking gel preparation

10 ml of the stacking gel (4 %) was prepared using the following recipe:

1.3 ml	Protogel
2.5 ml	Protogel stacking buffer
6.1 ml	dH_2O
0.05 ml	Ammonium persulphate (10 %)
0.1 ml	TEMED

ELISA and Western Blot Washing Buffer (PBS/Tween20)

1 litre of Western Blot washing buffer (1xTBS with 0.05% Tween20) was prepared using the following recipe:

100 ml	10x TBS
900 ml	dH_2O
0.5 ml	Tween-20

10x Phosphate Buffered Saline (PBS) pH 7.4

1. 1 litre of 10x PBS was prepared using the following recipe:

14.4 g	Na_2HPO_4
2.4 g	KH_2PO_4
80 g	NaCl
2 g	KCl
900 ml	dH_2O

2. Then pH was adjusted to 7.4 by adding concentrated HCl and measurement with a pH meter. The bottle was topped-up to 1 litre by adding deionized water.

P-Nitrophenyl Phosphate (PNPP) Substrate for ELISA

1 litre of p-nitrophenyl phosphate (PNPP) substrate (1M diethanolamine) buffer (pH 9.8) was prepared using the following recipe:

97 ml	Diethanolamine
800 ml	dH_2O
100 mg	$MgCl_2$

Then the pH was measured and adjusted to 9.8 by adding 10M hydrochloric acid. Once the pH is adjusted, dH_2O was added to obtain a final volume of 1000 ml. Thereafter, the buffer was transferred back to the bottle and stored at 4°C. Finally, the substrate was prepared by dissolving p-nitrophenyl phosphate disodium salt (5mg) tablet into 5 ml of substrate buffer to give a PNPP concentration of 1 mg/ml.

Appendix-2: List of publications

Scientific papers

1- Zhang, Q., Leong, S.C., McNamara, P., **Mubarak, A**., Malley, R., Finn, A. 2011. Characterisation of Regulatory T Cells in Nasal Associated Lymphoid Tissue in Children: Relationships with Pneumococcal Ccolonisation. PLoS Pathog 7(8): e1002175. doi: 10.1371/journal.ppat.1002175

2- Gray, C., Ahmed, M.S., **Mubarak, A.,** Kasbekar, A. V., Derbyshire, S., McCormick, M. S., Mughal, M. K., McNamara, P. S., Mitchell, T., Zhang, Q. 2013. Activation of memory Th17 cells by domain 4 pneumolysin in human nasal-associated lymphoid tissue and its association with pneumococcal carriage. Mucosal Immunology, 7 (3, #4). pp. 705-717.

Accepted abstracts

1- **Mubarak, A**., Kasbekar, A., McCormick, M., Beer, H., Cunliffe, N., McNamara, P. S., and Zhang, Q. (2012) Association of Th17/Treg in NALT with pneumococcal carriage in children and adults. Immunology, 137 (Suppl. 1), 185-772.

2- **Mubarak, A**. **S**., Kasbekar, A., McCormick, M., Beer, H., Cunliffe, N., McNamara, P. S., and Zhang, Q. (2013) Induction of Th17 in nasopharynx-associated lymphoid tissue by pneumococcus and the effect of cytokines. Immunology, 140 (Suppl. 1), 39-184.

3- Rider, A., **Mubarak, A**., Derbyshire, S., McNamara, P., Kadioglu, A., and Zhang, Q. (2013) Induction of T regulatory cells by streptococcus pneumonia. Immunology, 140 (Suppl. 1), 39-184.

4- **Mubarak, A**., Casey, V., Upile, C., Sharma, R., Beer, H., McCormick, M., Ahmed, M., McNamara, P., and Zhang, Q. (2014) Relationship between Th17 and Treg in nasopharynx-associated lymphoid tissue and their association with age and pneumococcal carriage in humans. Immunology, 143 (Suppl. 1), 12-42.