Activation of mucosal Th-17 cells in human nasopharynx-associated lymphoid tissue by *Staphylococcus aureus* and *S. pneumoniae*

Thesis submitted in accordance with
the requirements of the University of Liverpool
for the degree of Doctor in Philosophy
by

Lualuaa Saad Zaki

March 2018

University of Liverpool
Institute of Infection and Global Health
Department of Clinical Infection, Microbiology and Immunology

Abstract

Bacterial colonisations are common and can lead to invasive diseases, including *pneumonia, meningitis and bacteraemia.* The common nasopharyngeal carriages *include Staphylococcus aureus* (*S. aureus*) *and Streptococcus pneumoniae* (*S. pneumoniae*). *S. aureus* colonises 20% of the human population persistently and infects around 60% of the population during their lifetime. *S. pneumoniae* can cause significant morbidity and mortality in humans especially in younger children and elderly. There is evidence to show an age-*associated* inverse correlation between these two bacterial carriages in the human nasopharynx. As *S. pneumoniae* carriage rate peaks around one to two years and then decrease, *S. aureus* carriage rate is low at younger children but increase with age to older children around 10-16 years old and adults. Both innate and adaptive immunity may mediate bacterial carriage. Innate lymphoid cells 3 (ILC3) were shown to be responsible for Nasopharyngeal maintaining tissue homeostasis and for providing early protection against bacterial colonisations, which may be particularly important in early life. With age, adaptive cellular immunity including Th17 cells becomes more mature and play a more important role in protection against pathogen colonisation.

In this PhD project, Firstly, the carriage rates of both *S. aureus* and *S. pneumoniae* were analysed, using nasopharyngeal swab samples and the relationship between the bacterial carriage (e.g. *S. aureus*) and local Th17/IL-17A activation by *S. aureus* was examined in children and adults. Secondly, to determine if some staphylococcal antigens activate Th17 cells in NALT, the capacity of a number of *S. aureus* recombinant protein antigens to activate Th-17/IL-17A responses was investigated. Finally, the relationships between ILC3 and Th17 in NALT and bacterial carriage were examined.

The bacterial carriage was examined by culturing nasopharyngeal swabs on blood agar plates. *S. pneumoniae* carriage rate was the highest in younger children aged around 2 years old and then gradually decreased to into adults. On the other hand, *S. aureus* carriage rate was lowest in young children and then increased in adolescents. The relationship between salivary IL-17A levels and the carriage rates revealed a significantly higher salivary IL-17A concentrations in *S. aureus* carriage-positive samples. This indicated that a local Th-17 response was activated in

patients with *S. aureus* bacterial carriage. This hypothesis was supported by subsequent experiment using flow cytometry that showed stimulation of tonsillar cells (MNC) by *S. aureus* culture supernatant (CCS)elicited a marked increase in Th-17 frequency when compared to unstimulated controls.

To examine the potential *S. aureus* recombinant proteins to activate a Th-17 immune response, flowcytometric analysis of CFSE labelled adenotonsillar MNC were stimulated with nine *S. aureus* recombinant proteins (IsdA, ClfB, SdrC, SdrD, CnaBE3, EsxAB, HlaH35L, Csa1A and FhuD2). Results suggested that HlaH35L, a non-toxic α-haemolysin derivative, was capable of stimulating CD4+ T cell proliferative response and activate Th-17 response in the tonsillar MNCs. The measurement of cytokines in stimulated MNC supernatant by both ELISA and cytometric beads array (CBA) indicated that HlaH35L elicited the production of Th-17 related cytokines IL-17A and IL-17F with IL-6. The ability of HlaH35L to stimulate CD4+ T cell proliferation and Th-17 response may indicate a vaccine potential of this antigen. The finding that a stronger Th-17 response after stimulation by the wild-type *S. aureus* CCS (containing Hla) than that by CCS derived from its isogenic Hla-/- mutant, indicating a Th17-activating property of Hla protein. Induction of Th-17 response from naïve T cells of tonsillar MNC was also studied using tonsillar MNC depleted of activated and memory T cells. Stimulation with *S. aureus* CCS induced Th-17 and IL-17A production from naïve T cells in tonsillar MNC.

A subsequent study was carried out to examine the relationship between ILC3 and Th-17 cells and their effect on *S. pneumoniae*. Frequency of Th-17 cells in tonsillar tissues was shown to be significantly higher in adults than children, which may be related to the accumulation of memory Th-17 cells in NALT. The frequency of IL-22+ ILC3 was compared in children and adults, results showed that the frequency of IL-22+ ILC3 in children was significantly higher than in adults. A significant increase in the number of NKp44+ ILC3 was shown to be associated with *S. pneumoniae* carriage.

To determine whether the potent Th-17 response activated by *S. aureus* CCS could be inhibited, GSK805 (a RORγt antagonist) was used to be co-cultured with MNC,

followed by stimulation with *S. aureus* CCS. The results showed that GSK805 selectively reduced the Th-17 activation but not ILC3. This may have therapeutically implications in reduction of Th17-mediated inflammatory pathology.

In summary, the activation of Th-17 response in human NALT (e.g. adenotonsillar tissues) may be common in humans during bacterial carriage in the nasopharynx. While the balance of the ILC3/Th-17 cells may contribute to the bacterial carriage status in the nasopharynx, and ILC3 may promote carriage and Th17 promote clearance of carriage. In addition, ILC3/Th-17 relationship development in different age groups may also impact the pattern of bacterial colonisation in the human nasopharynx. Non-toxic recombinant protein HlaH35L was shown to activate and promote Th-17 cells so maybe a vaccine candidate against bacterial carriage in the human nasopharynx. Understanding the development of natural immunity against *S. aureus* and to *S. aureus* carriage may provide important information on the development of protein-based vaccines against respiratory tract infections in humans.

Acknowledgements

First of all, the ultimate thanks and gratitude always and forever is for ALLAH for the continuous mercifulness during joys and strains. Thanks that cannot be translated into words for my parents and family; my mother in special, for all the support and love she was providing also for my companion in the PhD journey. The second thank is for my sponsors the Higher Committee of Education Development in Iraq (HCED) for funding my project and for offering the opportunity of development.

I would like to express my appreciation to my primary supervisor Dr Qibo Zhang for all his knowledge, support, flexibility and for always being approachable and available. Also for helping me in the technical learning and to offer help with data analysis whenever the tools I should use were not available. I would also like to thank my secondary supervisor Dr Neil French for his offering advice whenever needed.

Thanks to all IGH/University of Liverpool members of staff, technicians and *students whom I* would also like to thank them all for their technical support, knowledge and help in various situations during the PhD. Special thanks for Ms Caroline Broughton for the help she provided whenever I requested. Here I would like to refer to the role of Dr Ahmed Shamsher for the guidance and sharing technical knowledge when I started this journey. Not missing all our group members for sharing the journey and the experience.

I would like to thank all of the participating patients and the ENT surgeons who collaborated in providing the samples needed to carry-on this study from Alder Hey Children's Hospital, and of Royal Liverpool and Broadgreen University Hospitals.

Gratitude for Baghdad (Iraq) the original home, Liverpool (UK), the journey towards myself and last but not lease for everyone I have not mentioned accidentally or on purpose including my true friends!

.

.

.

Dedication

To the one how brought me up well….

To my core of strength…

To the homes that I had and to those I am still live within….

Table of Contents

List of figures

List of Tables

Abbreviation list

Abb	Name
AHR	Aryl Hydrocarbon Receptor
BAP	Blood agar plate
BCA	Bicinchoninic acid assay
BFA	Brefeldin A
BSA	Bovine serum albumin
CCS	Concentrated Culture Supernatants
CFSE	Carboxyfluorescein succinimidyl ester
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	The enzyme-linked immunosorbent assay
GATA-3	Trans-Acting T-Cell-Specific Transcription Factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hBD	Human β-defensin
HBSS	Hank’s Balanced Salt Solution
HLA-DR	human leukocyte antigen-D related
IBD	inflammatory bowel disease
IC	DNA Internal Control
IL	Interleukin
ILC	innate lymphoid cells
Ion	Ionomycin
MNC	Mono nuclear cell

MRSA	Methicillin-resistant Staphylococcus aureus
MRSA	Methicillin-resistant *S. aureus*
NCR	Natural cytotoxicity receptor
PBMC	peripheral blood mononuclear cell
PBS-T	PBS containing 0.05% Tween20
PMA	Phorbol 12-myristate 13-acetate
RT-PCR	Real-time polymerase chain recycler
Rag	Recombination activating gene
RORγt	Retinoid-related orphan receptor γt
scmRNA seq	Single cells mRNA sequencing
STAT3	Signal transducer and activator of transcription 3
STGG	Skim milk-Tryptone-Glucose-Glycerol
TSB	Tryptic soy broth
TSST-1	toxic shock syndrome toxin 1
WCP	Whole-cell population

Chapter 1

GENERAL INTRODUCTION

Chapter one: Introduction

1.1 Bacterial colonisation and immune tissues in the nasopharynx

The nasopharynx is an important site of bacterial infection and spread out to other parts of the human body. Nasopharynx includes a Nasal associated lymphoid tissue (NALT). Because of continuous exposure pathogens, the immune properties are widely developed. NALT is a secondary lymphoid tissue consisting of waldeyer's ring and has major and minor impact structures. The major part includes two pairs of palatine tonsils and two prominent unpaired adenoids (as reviewed in Per Brandtzaey et al., 2015). (figure 1.1). The minor parts represented with tubal tonsils and lateral pharyngeal bands (Dolen et al., 1990). Due to its' junctional location between the nasopharynx and oesophagus (the respiratory tract and digestive tract). It is considered the induction site of pathogenic infections that spread-out to more the upper respiratory tract infection (URT), ear infection or more serious life-threatening diseases including meningitis and bacteraemia (Garcia-Rodriguez, and Martínez, 2002). The most common bacterial colonisers of the nasopharynx including four common bacterial pathogens: *S. pneumoniae*; non-typable *Haemophilus influenzae*; *Moraxella catarrhalis* and *Staphylococcus aureus* (O'Brien et.al, 2009, Van der Poll, and Opal, 2009). In addition to all the above health complications, nasopharyngeal infections have a major impact on younger children due to the problem of recurrent infections. Recurrent infections in young children might be linked to the hypertrophy of tonsils and obstructive sleep apnea (OSA) (Hung et al., 2018) and as a subsequence may force to the tonsillectomy and adenoidectomy. The carriage rates of the pathogenic bacteria influenced by numerous factors including geographic area, age, host immune response (Gannarsson et al., 1998; Auranen et al., 2010). The nasal bacterial colonisation affected hugely by age. In early childhood, around 0-2 years old, *S. pneumoniae* showed higher carriage rates among other common bacterial groups. That started as early as two weeks and peaked at 2 years old (Bogaert, et al, 2004; Simll, et al., 2012). The high pneumonia carriage associated with higher respiratory infection (Gorcea Rodriguez et. al, 2002). The pneumonia infections might be related to the developing immune system in younger children as well as to the close contact that combined with a high transmission rate. In contrast, the reported rates

of *S. aureus* nasal colonisation were relatively low in younger children and raised to peak at 10-16 years children (Bogaert et. al., 2004). This conversed relationship between *S. pneumoniae* and *S. aureus* was first presumed to the antagonist interactions between these two genera but later it was shown to be associated with the host immune response (Archer et al., 2013).

The interactions between the four common colonisers pathogens in the URT were in earlier studies. There is a noticeable positive association between *S. pneumoniae* and *M. catarrhalis* and between *S. pneumoniae* and H. influenzae (Bae et al., 2012; Jacoby et al., 2007). In contrast, *S. aureus* co-carriage tended to be rare and *S. aureus* colonisation appeared to occur separately (Bae et al, 2012). When the dynamic of infection between *S. pneumoniae* and *S. aureus* was analysed. There is a direct antagonism

related to neuraminidase, H_2O_2 or bacteriocin production (Pericone et al., 2000). Therefore, it was first presumed that these factors control the relationship between them in the host and direct their adversative carriage patterns in NALT. But the investigation showed that H_2O_2 produced by *S. pneumoniae* has no effect on *S. aureus* (Marglis, et al., 2009). These results turned the focus on other factors that might be the source of the opposed carriage relationship. Recently, the researched focused on the relationship of host immune response with the bacterial colonisation with *S. aureus* and *S. pneumoniae* colonisation.

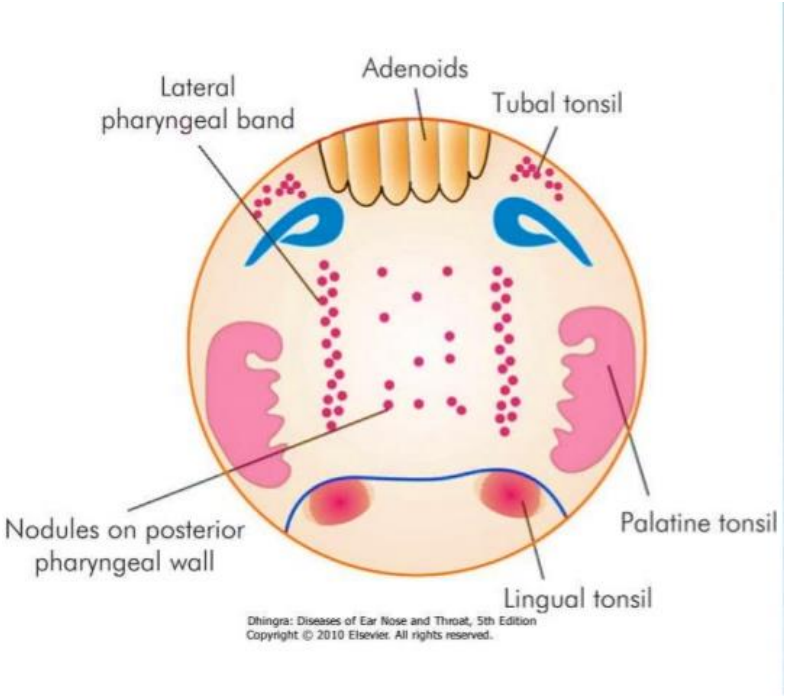


Figure 1.1: The composition of Waldeyer's ring in human Nasopharynx.

Waldeyer's ring consists of palatine tonsils and adenoids along with paired of tubal tonsils and lingual tonsils. Adapted from (Dhingra, 2010).

1.2 *Streptococcus pneumoniae*

Under microscope, *S. aureus* is 0.5-1.25 µm in diameter, non-motile, non-spore former, Gram-positive cocci (purple), arranged in a diplococcus and sometimes appear in short chains (Brooks and Carroll, 2007). On blood agar plates, they can be differentiated from other group-A Streptococci by their characteristic α-haemolytic colonies, glistening, raised colonies, measures 1 mm in diameter. When colonies aged, they become flattened with a depression in the middle of the colony giving them a differential 'Draughtsmen's' shape. Laboratory identification of *S. pneumoniae* primitively depends on the optochin sensitivity test that differentiates them from other α-haemolytic (Viridans) along with inulin hydrolysis and bile solubility (Ryan, 2003). In addition to the morphological and biochemical test, *S. pneumoniae* sub-typed according to their polysaccharide capsular antigenic properties. Various antisera react with the different types of *S. pneumoniae* with quelling reaction (Henrichsen, 1995). The genomic analysis recently was adapted for more accurate identification and differentiation from S. viridians. The molecular

identification involved performing real-time PCR using genes like lytA (responsible for autolysis) (Wang, et al., 2011), ply (the pneumolysin encoding gene) and psaA (pneumococcal surface adhesion gene) (Carvalho, et al., 2007).

1.3 *Staphylococcus aureus*

S. aureus is one of the most medically important infection and health-burden causative agents form the Gram-positive Staphylococci family. Under a microscope, *S. aureus* is 0.5-1µm, non-motile, gram-positive cocci (purple), arrange in a grape-like cluster and sometimes appear individually or in pairs (figure 1.X b). On agar plates, grow well in both aerobic and microaerophilic conditions but do not survive a non-aerobic environment (et al., 2017). On the agar surface, colonies are large (6-8 mm in diameter), smooth, raised, glistering, and most of the strains are white in colour or golden-yellow pigmented. Laboratory identification of *S. aureus* primitively depends on the colony's appearance and under microscope examination along with biochemical tests. Biochemical tests as catalase and coagulase distinguish *S. aureus* from another Staphylococcus spp. (Holt, and Bergey, 1994). More recently, molecular identification using real-time PCR amplification for distinguishing the MRSA genes like *mecA* (Peterson, et al. 2010) and *femA* (Sabet, et al., 2007).

1.3.1The virulence factor of *S. aureus*

S. aureus possesses a wide range of virulence factors that are essential for the adhesion to the host tissue, colonisation and the invasiveness to the adjacent tissue inside the host. The virulence factors are divided into; cell wall polysaccharides, cytoplasmic membrane integrated proteins, extracellular toxins, and enzymes (figure 1.2).

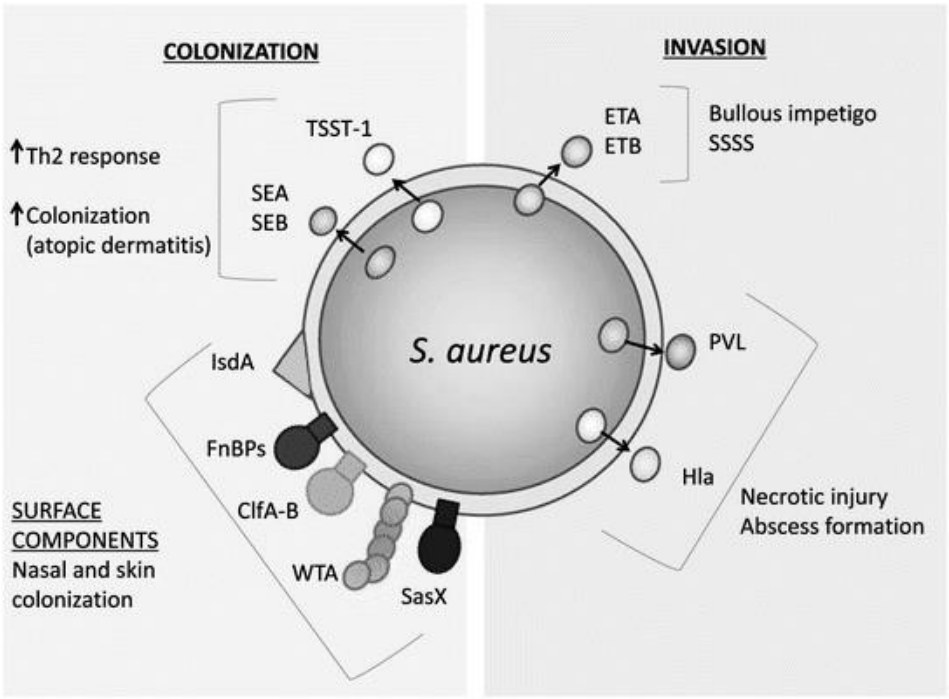


Figure 1.2: The virulence factors of *Staphylococcus aureus*.

The figure shows some of the virulence factors that support the colonisation and invasiveness of *S. aureus*. Virulence factors responsible for colonisation include cell surface components; IsdA, fnBps, CifA-B, WTA, SasX, and some of the secreted toxins and proteins like TSST-1, SEA and SEB. While invasion responsible virulence factors include the ETA, ETB, Hla, and PVL.

1.3.1.1 Cell-wall-anchored proteins

S. aureus possesses several cell wall anchored proteins. They are characterised by the presence of the R region which contains multiple serine-aspartate repeats (Foster and Hook 1998; Ní Eidhin et al., 1998). These proteins are divided into two main groups: first group are molecules called microbial surface components recognising adhesive matrix molecules MSCRAMM and the second group are Sdr proteins (from SD Repeat).

1.3.1.2 Iron-regulated surface determinant A (IsdA)

Iron-regulated surface determinant A (IsdA) has wide-spectrum adhesion ligands including fibrinogen and fibronectin and control iron uptake (figure 1.3). The *isdA* gene is only expressed in iron-limited environment. When the bacteria are grown under iron-limited conditions, the C-terminal domain of IsdA defends *S.*

aureus against human skin bactericidal fatty acids and antimicrobial peptides by making the cell surface hydrophilic (Clarke, et al., 2007). The immunogenetic properties of IsdA were discovered by the founding antibodies against IsdA in both healthy individuals (higher frequencies in non-carriers than in commensal carriers) and in *S. aureus* infected individuals (Clarke, et al., 2006; Verkaik, et al., 2010). Immunogenetic properties of IsdA were shown by both active immunisation with IsdA and passive immunisation and protected mice against abscess formation and intravenous challenging (Stranger-Jones et al., 2006; Kim, et al., 2010; Arlian and Tinker, 2011).

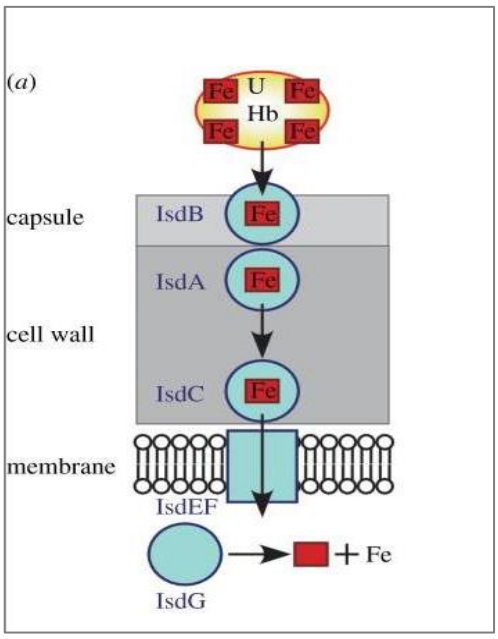


Figure 1.3: Iron-regulated surface determinants of *S. aureus*.
Isd proteins that responsible for undertaking iron during iron derivation. Adapted from Schneewind, and Missiakas, (2012).

1.3.1.3 Clumping factor A and B (ClfA and ClfB)

Clf A and B are part of a group of surface adhesions molecules called microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Ni Eidhin et. al, 1998). That binds *S. aureus* to the extracellular matrix components including collagen, fibrinogen and fibronectin and heparin-liked polysaccharides (Patti et al., 1994; McAdow et al., 2011; Walker et al., 2013). ClfA and ClfB are MSCRAMMs that bind to fibrinogen (Hawiger et al., 1982; O'Brien et al., 2002). They are expressed by reserved *clf* genes among *S. aureus* strains. Their binding

affinity to fibrinogen is quite strong so the interaction with a low concentration of fibrinogen in solution results in immediate clumping of bacterial cells. The binding difference between ClfA and ClfB is in the recognition of the binding domain within the fibrinogen molecule. ClfB recognizes the a- and b-chains (Ní Eidhin et. al, 1998), while ClfA binds with the γ-chain through Ca+2-dependent ligand binding (O'Connell et al., 1998). Studies showed the role of ClfB in *S. aureus* in nasal epithelial cell adherence by binding to cytokeratin 10 (O'Brien et al., 2002). Murine model of both active immunisation with ClfB and passive immunisation with monoclonal antibodies (MAB) to ClfB showed a significant reduction in the nasal colonisation by *S. aureus* compared to the control group through the reduction in binding to cytokeratin 10 (Schaffer et. al, 2006).

1.3.1.4 Serine-aspartate repeats (Sdr) proteins (SdrC, SdrD and Sdr E)

The Sdr proteins are part of *S. aureus* cell-wall anchored proteins. Sdr term derived from the amino acid serine –S- and aspartic acid –D- repetition. The characteristic feature of the Sdr family is the multiple serine-aspartate repeats in the R region (Foster and Hook 1998; Josefsson et al. 1998; Ní Eidhin et al. 1998). There are three Sdr proteins (Sdr C, D, and E). Sdr proteins are encoded by sdr locus within *S. aureus* genome. Notably, although not all of them present in all *S. aureus* strains (Sabat et al. 2006), genetic analysis reviled that the SdrC gene is predominantly present in the sdr locus unlike sdrD and sdrE (Peacock et al., 2002; Sabat et al., 2006). Each Sdr protein composed of N-terminus peptide chain followed by an interactive A domain then repetitive B domain that consist of two, three, or five repeated sequences (for SdrC, SdrE, and SdrD, respectively), The C-terminal region of the Sdr proteins contain the SD repeat domain followed by an LPXTG motif (Josefsson et al., 1998) (figure 1-4).

Clinically, SdrD was shown to be able to inhibit innate immune-mediated bacterial killing by neutrophils and this effect was independent of other *S. aureus* proteins (). Since the identified fragments incorporated the second, the third and fifth B repeat domains of SdrC, SdrD, and SdrE respectively, the reserved amino acid chains were termed as CnaBC2, D5, and E3 domains respectively. Clinically, researches showed a correlation between the presence of SdrE and carriage/invasiveness of *S.*

aureus strains (Peacock et al., 2002). That encouraged the investigation of CnaB, a conservative domain, conserved among the three Sdr proteins. CnaBE3 showed a resistance wards trypsin lysis and demonstrated to be expressed during *S. aureus* infection as the serum antibody levels against CnaBE3 were significantly higher during *S. aureus* infections also active immunisation with CnaBE3 significantly reduced bacterial load (Becherelli et al., 2012).

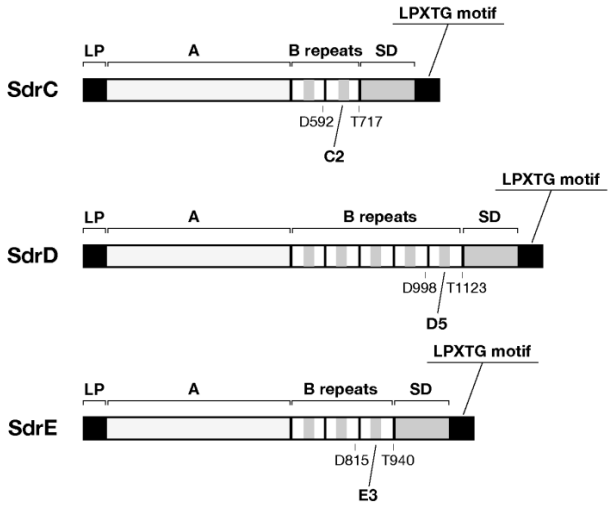


Figure 1.4: Schematic structure of the Serine-aspartate repeats (Sdr) proteins (SdrC, SdrD and Sdr E). The Sdr molecule consists of N-terminal that have domain A and distinguish repetitive B sequences. These B repeats vary among Sdr structures in SdrC two B repeats, SdrD contains five B repeats while SdrE contains three B repeats. The carboxyl ends consist of SD and LPXTG motifs.

1.3.2 ESAT-6 Secretion System (ESS)

S. aureus has a Sec-dependent secretion system (SEC). ESS responsible for the secretion and the exportation machinery that transport and secrete proteins through the cytoplasm membrane of the bacterial cells. *S. aureus* SEC is resembling the type VII secretion system in Mycobacterium tuberculosis (Pallen et al., 2002). In *S. aureus*, ESAT-6 secretion system (ESS) encoded by eleven essential genes are esaA, esaB, essA, essB, essC, esxA and esxB genes (Kneuper et. al, 2014). The ESS encoded apparatus consists of structural membrane-located proteins of the secretion apparatus, regulatory proteins, and exported effector proteins. EsaB is a regulator protein while the EsxA, EsxB, EsxC (EsaC), and EsxD are small exported

effector proteins with N-terminal (Burts et. al, 2005; Burts et. al, 2008; Kneuper et. al, 2014). Esx proteins function as homo or heterodimers usually, EsxA and EsxC dimerizes with itself or with heterodimers whereas, EsxB and EsxD, appear to function as heterodimer only (Anderson et al., 2013).

In addition to their role in the transporting apparatus, some of these proteins shown to have an impact on the colonisation and disease symptoms caused by *S. aureus* infections. ESS has been shown to affect nasal colonization and pneumonia (Kneuper et. al, 2014). Also, when using murine infections with *S. aureus* mutated in esxA, esxB, and essC, the results reviled that these proteins contribute to abscess formation by *S. aureus* (Burts et. al, 2008; Anderson et al., 2011). In addition, results from a recent study specify the role of EsxA and EsxB in the modulation of apoptosis and release of ingested *S. aureus* from epithelial cells (Korea et al., 2014).

1.3.3 Conserved Staphylococcal Antigen 1A (Csa1A)

Cas1A is putative lipoprotein belongs to a recently discovered family of conserved staphylococcal antigens (Csa). Cas family is highly similar in structure and they are restricted to Staphylococci. Cas was found to be expressed in multiple loci although Csa1A is processed and released extracellularly after transcription. Because Csa paralogues structurally interact that suggested similar functions in structure and pathogenicity of *S. aureus* (Schluepen, et al., 2013). *In vivo* murine models of staphylococcal infections showed that Csa1A elicited a protective immune response when applied individually (Schluepen, et al., 2013) or within combined formula (Bagnoli, et. al, 2015; Mancini et. al, 2016).

1.3.4 FhuD1 and FhuD2

FhuD1 and FhuD2 are post-transcriptionally lipoproteins that anchored in S. aureus cytoplasmic membrane and involve in the transportation of iron (III)-hydroxamate complexes under iron-restricted growth conditions. FhuD1 and FhuD2 encoded with gene termed *fhuD1 and fhuD2* respectively and their transcription downregulated with exogenous iron supplementation. FhuD2 is involved in the transport of iron (III) in complex with ferrichrome, ferrioxamine B, aerobactin, and coprogen. While FhuD1 only transports iron (III)-ferrichrome and iron (III)-ferrioxamine B in *S. aureus (*Sebulsky, and Heinrichs, 2001*).* Several

attempts of FhuD2 vaccination showed antibodies mediated protection associated with opsonophagocytosis. Passive immunisation with those antibodies were able to block FhuD2-mediated siderophore uptake (Mishra, et al., 2012).

1.3.5 Extracellular toxins

S. aureus produces several toxins that can be divided depending on their effect on the host cellular level. Cytotoxins, like alpha-toxin, produced by *S. aureus* and cause pore formation to induce proinflammatory changes within host cells. While the pyrogenic-toxins like toxic shock syndrome toxin (TSST-1) and enterotoxins function as superantigens by binding to major histocompatibility complex (MHC) class II proteins, causing strong T-cell proliferation and cytokine release (Foster 2001, Tuffs et al., 2018). The effect of the superantigens on the infected individual could cause a series of effect including toxic shock syndrome (Ramachandran, 2014) and food poisoning (Foster 2001, Tuffs et al., 2018).

1.3.5.1 Superantigens

Superantigens (SAgs) are the most powerful T cell mitogens ever found. *S. aureus* possesses around 26 SAgs including enterotoxin A (SEA) and toxic shock syndrome toxin (TSST-1) (Tuffs et al, 2018). Their function as superantigens achieved by binding directly to major histocompatibility complex (MHC) class II proteins on Antigen-presenting cells (APC). The binding occurs in the heavy region instead of the high variability region that most antigens bind to MHC-II structure in APC and subsequentially bind to the T cell receptor in the variable region of the β-chain (Vβ) (Herman et al, 1991; Seth et al, 1994) (figure 1.5). Because the numbers of a specific Vβ in a host consist of approximately 50 of the entire repertoires when they are stimulated by even low concentration of superantigen, it results in an extensive T-cell proliferation (activating approximately 20% of the T cell population) combined with cytokines release. In contrast, the responding frequency to an antigen through the conventional antigen presentation approach usually stimulated less than 1 in 10 000 of the T cell population. Uncontrolled inflammatory response results in a massive systemic release of pro-inflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α) and interleukin-beta (IL-1b), resulting in fever, shock and death moreover promoting autoimmune responses

(Krakauer and Stiles, 2013). Consistent exposer to an extremely low amount of SAgs can cause multiple inflammatory diseases with features including infiltration of MNCs in lung, liver, and kidney (Vaildehi et. al, 2012).

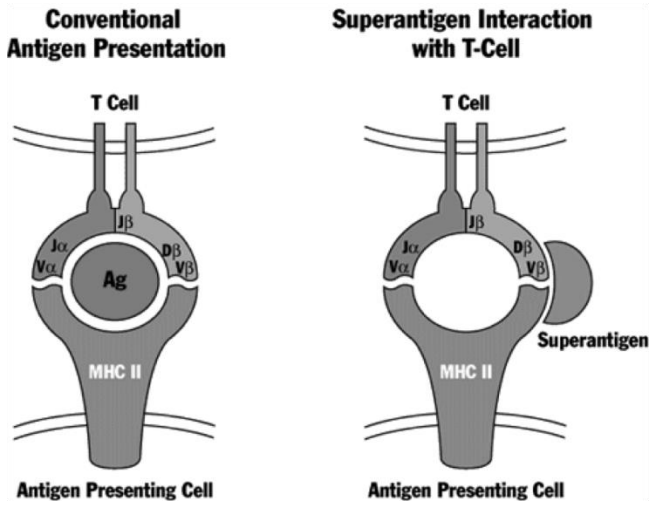


Figure 1.5: Schematic model of *S. aureus* Superantigen binding to T cell and MHC-II. The interaction between *S. aureus* SAgs to T cell in the Vβ region and linked to the MHC-II receptor on the antigen-presenting cells (APC). This directed a massive activation to the T-cell population and the release of the effector mediators. In contrast, the conventional Ag presentation, the Ag bind to the high variability grove in the MHC-II receptor and then presented to the T cell receptor as seen in the figure.

1.3.6 alpha-haemolysin (Hla)

Hla (α-toxin) is a cytotoxin that was the first discovered as a pore-forming toxin (Bhakdi, and Tranum-Jensen, 1991). Hla is secreted as a water-soluble monomer of 33 kDa. Upon interaction with specific receptors on the vulnerable host cells, it forms a cylindrical, heptameric, β-barrel structure of 1 to 2 nm in diameter within-host membranes (figure 1.6).

Hla permeabilizes liposomes composed of phosphatidylcholine or sphingomyelin and cholesterol. A high-resolution crystallography study conducted by Galdiero and

Gouaux in 2004 showed that each Hla subunit binds to, the high-affinity binding site, phosphocholine head group in a position between the rim and the stem domains of the heptamer (figure 1.7).

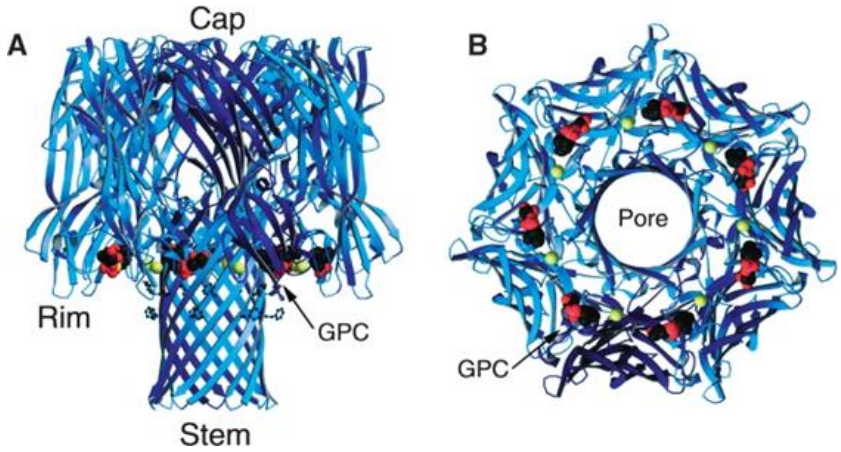


Figure1.6: The schematic 3D structure of Hla toxin. Crystal structure of α-toxin showing heptametrical, β-barrel structure of combined Hla monomers when attached to the phosphocholine head group. Hla heptamer unit consists of Cap, the region of the toxin that demarcates the entry of the pore; the Rim, the membrane-interfacing region, and the membrane punching stem. (Galdiero and Gouaux, 2004).

When initially discovered, the Hla- receptors interactions on Eukaryotic cells were unspecified. It was not clear until 2010 when Wilke and Wardenburg reported that the expression of the protein receptor a disintegrin and metalloprotease 10 (ADAM10) is crucial for Hla toxin to initiate assembling in cholesterol/sphingolipid-rich caveolar rafts within the plasma membranes. It is hypothesised that ADAM10 is activated by the influx of extracellular Ca2+ through the toxin pore (Reiss et al, 2005). When the concentration of Hla toxin is significantly low, the interaction-clustering of Hla with ADAM10 raises Hla concentration in the local area and initiates toxin oligomerization with the help of caveolin-1 (CAV1) (figure 1-7). Furthermore, Hla-ADAM10 complex interaction promotes Hla toxin by interrupting the host-cellular-barriers forming the β-barrel through the lipid bilayer to form a hydrophilic transmembrane channel (Seilie and Wardenburg, 2017). This pore will enhance the invasiveness of *S. aureus* by increasing the superantigen permeability into the affected cell (Brosnahan et al. 2009).

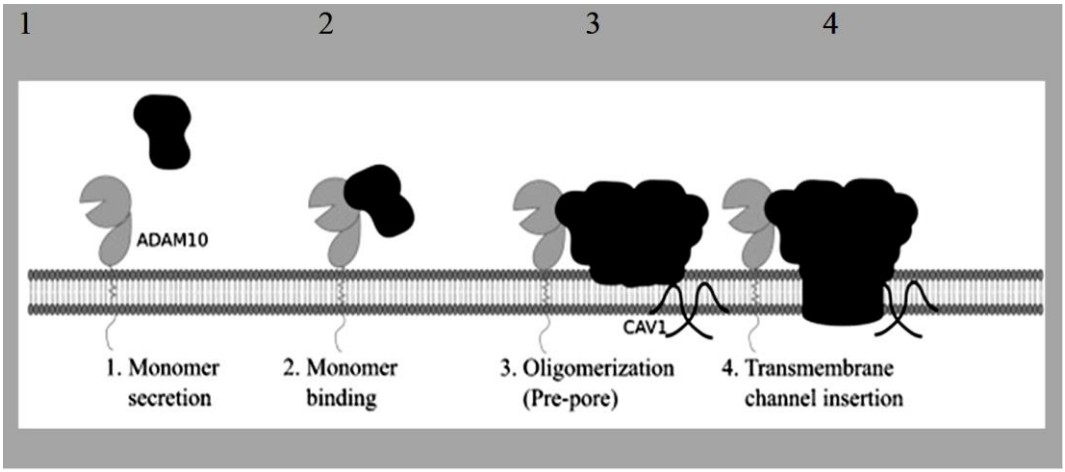


Figure 1.7: the mechanism of pore-formation by Hla toxin in the host plasma membrane. The steps of Hla pore-formation includes first: attachment of Hla monomer with ADAM10 receptor on the susceptible cell (1 and 2). Then, the oligomerization occurs by the mediation of caveolin-1 (CAV1) (3). Finally, the B-barrel structure transfers through the cytoplasmic membrane and form a hydrophilic channel through the infected cell (4). (Seilie and Wardenburg, 2017).

In addition to its role as a pore-forming toxin, Hla possesses additional characteristics of binding to other host-cell-receptors and activation of intracellular signals. The role of Hla in triggering host cells by chemokines secretion. those chemokines mainly responsible for recruiting neutrophils to the site of infection and mediating inflammatory response. By the mean of syndecan-1 ectodomain shedding through ADAM10 (Berube and Wardenburg, 2013).
At high concentrations, Hla toxin binds non-specifically to the lipids of the cellular membrane and prime to massive cellular disruption and eventually cell death via apoptosis (Bhakdi, and Tranum-Jensen, 1991).

1.3.6.1 Role of Hla in the immune response

The antibodies mediated response towards the mature toxin, or its non-toxic genetically modified proteins were previously studied (Ragle and Wardenburg, 2009). Previous studies liked the level of Hla antibodies titration in different populations with the type of the *S. aureus* strain and clearly showed a higher

antibodies titer in patients with invasive strains infection. (Granstrom, et al., 1983, Julander, et al., 1983). Later on, a flow-up study documented two monoclonal antibodies recognized specific epitope within the first 50 amino acid residues of HlaH35L and lead to the inhibition of alpha-hemolysin oligomer formation on the target cell surface. They also showed that both active and passive immunization with this Hla50 showed promising protection and good vaccine potentials for *S. aureus* pneumonia infections (Ragle and Wardenburg, 2009).

Hla toxin has been shown to induce an inflammatory immune response generated by triggering specific host immune cells to produce a range of chemokines (Wardenburg and Schneewind 2008). Hla toxin induces the secretion of inflammatory cytokine lL-1β by macrophage and monocytes. IL-1β is cleaved to produce the active form that recruits neutrophils to the site of infection. The neutralization by anti-Hla toxin antibodies reduced IL-1β secretion during pneumonia caused by *S. aureus* (Wardenburg and Schneewind 2008). In addition to the role of Hla in innate immunity, there is increasing evidence to show Hla toxin modulation of the adaptive immune response. Hla toxin was shown to affect the adaptive immune response either by directly evoking programmed cell death or via the stimulation of cytokine secretion, which controls the interaction between adaptive and innate immune cells, specifically, via the interleukin 17A (IL-17A) produced by Th-17 lymphocytes. Th-17 cells function by recruiting neutrophils to the site of infection and regulate pathogen clearance in the epithelium. Frank and colleagues (Frank, et al. 2012) conducted a comparative study of the immune responses in mice infected with wild-type *S. aureus* to those infected with Hla - mutant *S. aureus* and have suggested that Hla expression was associated with induction of the IL-17A response.

1.4 Nasopharynx-associated lymphoid tissue (NALT)

NALT is consisting of Waldeyer's ring and their location between the nasopharynx and oropharynx contribute to its importance as an immune protection site against both airborne and ingested potentially harmful pathogens. It has both effector and induction sites for local and systematic immunity.

1.5 Nasopharyngeal mucosal immunity

1.5.1 Innate immune response

1.5.1.1 Dendritic cells

One of the innate cellular immune system that involves in the processing of the pathogen upon infection and the processing of damaged/apoptotic cells during incidence like inflammation. DC possesses several functions in the mucosal site. When exposed to a specific apoptotic cell, DC goes through the maturation process in which they increase their dendrites and activate the expression of MHC-I, MHC-II, and co-stimulatory molecules like CD40. Upon the processing and presentation of the specific antigen, the attract naïve T cells through binding with their surface receptors (Stoll, et al., 2002). Cytokines-derived priming process requires DC secretion of cytokines combination that direct Th cell induction like IL-6 and TGFβ that prime Th-17 (Ascota-Rodreguez et al., 2016; Bettetti et al., 2006; Mangan et al., 2006; Veldhon et al., 2006) in contrast the production TGFβ by DC stimulates Treg cells priming (Lombardi, et al., 2012). In addition to their role in Th cells induction and activation, DC was also associated with the DC Furthermore, DC was shown to stimulate other innate immune compartments as ILC. For ILC3 activation DC production of IL-23 was associated with the production of IL-22 from ILC3 in mucosal tissue (Satpathy, et al., 2013).

1.5.1.2 Innate lymphoid cells

Innate lymphoid cells (ILC) are tissue innate resident cells. Mostly localised adjacent to the mucosal barriers like in lamina propria and in mucosal and non-mucosal tissue. There are three subtypes of ILC populations ILC1, ILC2, and ILC3. These groups mimic the adaptive cellular Th-1, Th-2 and Th-17 groups by the transcriptional factors and cytokines profile in each group. Interestingly, there is substantial plasticity between these groups (Brnink *et al.*, 2015, Artis *et al.*, 2015). ILC has been associated with supporting mucosal homeostasis and during inflammation (figure 1.8) (Arits and Spit, 2018). ILC are rarely found in circulation, although, in some autoimmune cases like psoriasis patients, distinct populations were found in the blood (Klein et al., 2012).

1.5.1.2.1 Innate lymphoid cells type-1 (ILC1)

ILC1 are distinguished from other ILCs with the expression of T-bet transcription factor. So far, two ILC1 populations were categorised according to CD127 expression: intra-epithelial CD127low ILC1 which express CD56, NKp44 and CD103 and responsive to IL-12 and IL-15 (Fuchs *et al*. 2012). On the other hand, CD127high ILC1 express CD56, NKp44 and CD94 and response to IL-2 and IL-18 by producing IFNγ (Brnink *et al.*, 2013).

1.5.1.2.2 Innate lymphoid cells type-2 (ILC2)

ILC2 express GATA for differentiation and function, and responsive to IL-25 and IL-33 cytokines. ILC2 produce IL-4 and IL-13. Both Th-2 and ILC2 are producing IL-2 when infections occur. GATA and STAT5 activation signals result in the production IL-2 (Mjosberg *et al*, 2011). Their roles are amplified in Asthma and lung inflammatory disease (Alkhverdi, 2009).

1.5.1.2.3 Innate lymphoid cells type-3 (ILC3)

ILC3 were first discovered as immature NK cells (iNK) but recently were categorised as innate lymphoid cells and distinguish by the transcription factor RORγt and resembles Th-17 cells by transcriptional factors and cytokine production. The main transcriptional factor for ILC3 is RORγt (Eber *et al*, 2004; Luci et al., 2009). RORγt also regulates Aryl hydrocarbon receptor transcriptional factor (Ahr). *Ahr* is a ligand-dependent transcriptional factor activated by environmental toxins, bacterial toxins, and food allergens) (Veldhoen *et al.*, 2008; Quintana *et al.*, 2008). In mice, ILC3 sub-population seems to involve the activation of transcription factor Notch (Satoh-Takayama et al., 2008) and T-bet as well. These cells can also be activated by bacterial, toxins and certain substances in food (Kiss *et al*, 2011; Lee *et al*, 2012; Qiu *et al.*, 2012). ILC3 also resembles Th-17 in the expression of CCR6 (Cella *et al.*, 2009). There are several types of ILC3: Lymphoid tissue inducer (LTi) are CD4+ cells that found during the fetal stage and responsible for the organogenesis while in adults they contribute in the protection of infection (Ederl and Littman, 2004; Mebius *et al.*, 1997; Yoshida *et al.*, 1999). The second type of ILC3 is NCR2, NKp44+ cKit+ (Bjorklund *et al.*, 2016).

NKp44+ ILC3 produce IL-22 but not IL-17A. The third distinguished type of ILC3 has represented a naive ILC3 population. These cells express CD62L and CD45RA and response to IL-23 and IL-1β but not capable of IL-22 or IL-17F production (Serafini et al., 2014 and Yogi et al., 2015). Another ILC3 subpopulation was found to express CCR6 and HLA-DR markers and are also responsive to IL-23 and IL-1β by producing IL-17A, IFNγ, and a lower concentration of IL-22 (Buonocore *et al.,* 2010; Hepworth *et al.,* 2013; Klose *et al.,* 2013 and Sciume *et. al.,* 2012) and IL-17F (Bjorklund et al., 2016). These cells are suggested to participate in maintaining homeostasis and regulation of the T helper cell response (Hepwarth et al., 2013; Hepwarth et al., 2015).

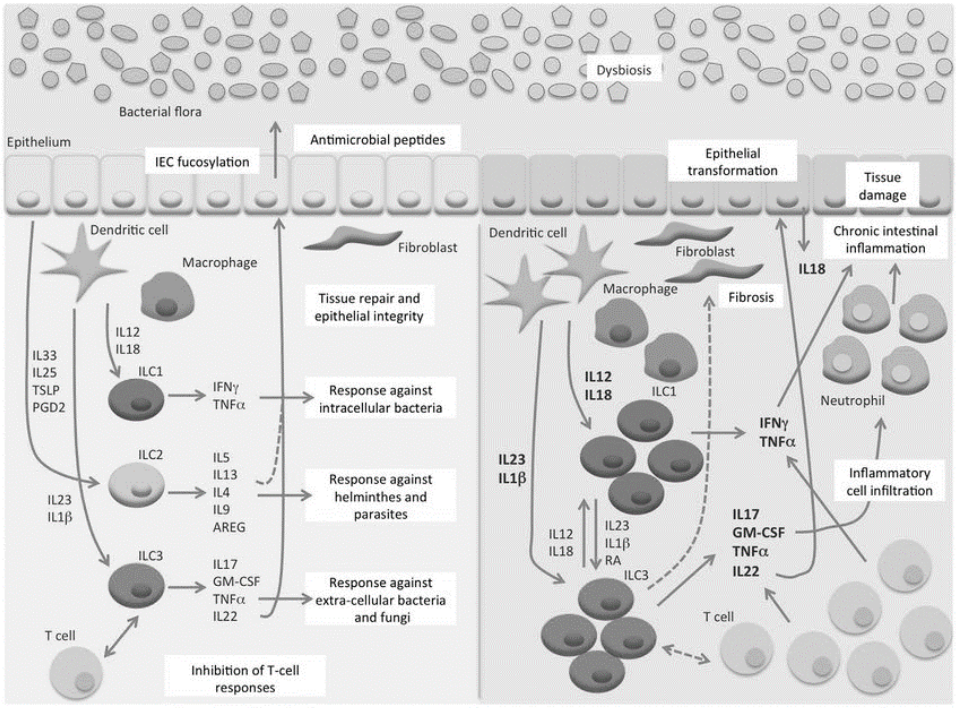


Figure 1.8: The role of Innate lymphoid cells (ILC) in homeostasis and chronic inflammation. In the healthy intestine mucosa (left), ILC role in maintaining homeostasis and integrity of the mucosal tissue and in protection against pathogens. During the infection, dendritic cells (DC) and macrophages produce IL-12 and IL-18 and that stimulate ILC1 against intracellular pathogens. In response to an extracellular pathogen, macrophage, and DC produce IL-23 and IL-1β that stimulate ILC3 and produce effector cytokines IL-17 and IL-22. On the other hand, in response to helminths, epithelial cells produce factors such as IL33, IL25, TSLP, and prostaglandin D2 (PGD2) and that induce ILC2 activation to secrete type 2 cytokines. During chronic intestinal inflammation, such as Crohn's disease (CD) (right), it combined with distorted normal flora and with the excessive immune response lead to the extensive production of IL-12. IL-18. IL-23 and IL-1β from DC and macrophages. This will activate ILC1 and ILC3 to secrete a high amount of type 1 and type 17 cytokines. This effect gathers with Th-1 and Th-17 due to the prolonged inflammation such attract neutrophils and result in chronic inflammation and tissue damage. Furthermore, prolonged inflammation promotes the secretion of inflammatory cytokines like IL-18 and that deteriorates the integrity of the mucosal barrier (Adopted from Geremia, and Arancibia-Cárcamo, 2019).

1.5.1.2.3.1 The role of ILC3 in the immune response.

The role of HLA-DR+ ILC3 was first observed in the human gut as NKp44+ ILC3 population that are responsive to the stimulation with IL-23 and IL-1β, IL-2 and IL-7 and produce IL-22 (Creillin *et al.*, 2010). Human gut-ILC3 induces the proliferation of epithelial cells by the expression of Leukaemia inhibitory factor (Cella *et al.,* 2009). ILC3 induce gut epithelial cells to produce TNF and IL-8. they negatively regulate epithelial cells by the production of IL-26 (Dambacher, *et al.,* 2008). Another role of ILC3 is in Th-17 the regulation due to their expression of HLA-DR that leads to the stimulation of commensal microbiota-specific T helper cell response despite the fact of lacking the expression of the co-stimulatory markers (Hepworth *et al.,* 2013). Furthermore, IL-22+ ILC3 was shown to regulate the Th-17 immune response (in particular, suppression of pathogenic Th-17) through the upregulation of the Aryl hydrocarbon receptor (Ahr) (Qiu, J. and Zhou, L. 2013). Ahr is a ligand-dependent receptor activated by numerous derivatives from bacterial toxins, microbial flora, and food. The absence of Ahr in a murine model showed to be linked with the reduction in IL-22 from ILC3 and with encouraging the proliferation of segmented filamentous bacteria and this triggered a significant inflammatory response represented by the expansion of pathogenic Th-17 (Qui *et al.,* 2013). Furthermore, in some inflammatory diseases like Crohn's disease, HLA-DR +ILC3 appeared to stimulate cell apoptosis in commensal-bacteria specific CD4+ Th cells. It appeared that HLA-DR+ ILC3 compete with CD4+ T helper cells on binding with IL-2 (Hepworth *et al.,* 2015). HLA-DR+ ILC3 controls homeostasis through several mechanisms including apoptosis and negative selection of microbiota specific CD4+ Th cells. Compared with healthy individuals, in IBD patients, a downregulation in HLA-DR + in ILC3 was associated with an increase in Th-17 (Hepworth *et al.,* 2015).

1.5.2 Adaptive immune response

1.5.2.1 Cellular adaptive immune response

cellular components of the adaptive immune response two major types of white blood cells (lymphocytes) are B-cells and T-cells. Both cell groups share characteristics of an adaptive immune response including:

- the expression of unique cell surface markers that enable them to recognize the antigen.
- The robust expansion upon the recognising of a specific antigen as well as the induction of lineage-specific cells subtype that possess lineage-specific cell markers and secreted molecules for bacterial clearance.
- Finally, after the clearance, they possess a long-term memory that persist and enable the host to develop faster respond in the second exposure to the same agent (Russ, et al., 2013).

T-lymphocytes separate into two lineages according to their surface marker expression, are CD8+ and CD4+ T-cells. CD8+ T-cells recognise antigens peptides presented by MHC-I (expressed on all nucleated cells) to form effector cytotoxic T lymphocyte (CTL). While CD4+ T-cells recognise MHC-II processed antigens (a receptor expressed on APC). CTL are inducted from naïve CD8+ T-cells in the presence of IL-2. unlike CD4+ T-cells, CD8+ T-cells expand massively upon the exposure to primary antigens due to their binding to antigens presented by MHC-I. which are available on all nucleated cells (reviewed in Broere, et al., 2011).
There are two major subsets of CD4+ T cells, T helper cells (Th) and T regulatory cells (Treg). CD4+ Th cells are categorised into several subsets including Th-1, Th-2, Th-17, Th-9 and T follicular helper (Tfh) although they all share the main function of orchestrating the immune response towards various pathogens. The Th-1 subset produces IFNγ upon the activation of their transcriptional factor T-bet. Th-2 produces IL-4, IL-5, and IL-13 and have Trans-Acting T-Cell-Specific Transcription Factor (GATA3). Th-17 produce IL-17A/IL-17F and IL-22 following the transcription factor RORγt activation and Tfh cells produce IL-21 and depend on Bcl-6 (reviewed in O'Shea and Paul, 2010) while Th-9 secrete IL-9 and depend

on different expression factors including Etr5 and Pu.1 (Koh et al., 2016). In contrast to CD4+ Th cells, Treg cells and their function is the regulation of the inflammatory immune response. Treg manly produce IL-10 and depend on FoxP3. The other regulatory subset is type 1 regulatory T cells (Tr-1) and they found to be activated by several transcriptional factors including Ahr (Mascanffoni et al., 2015) and EMOES (Zhang et al., 2017). The differentiation process is illustrated in figure 1.9. Regarding the functional role, Th-1 cells are involved in immunity against intracellular pathogens, while Th-2 cells are more involved in immunity asthma and helminths infections. Besides these two subsets, Th-17 cells are more involved in immunity against extracellular pathogens and involved in the development of autoimmune diseases.

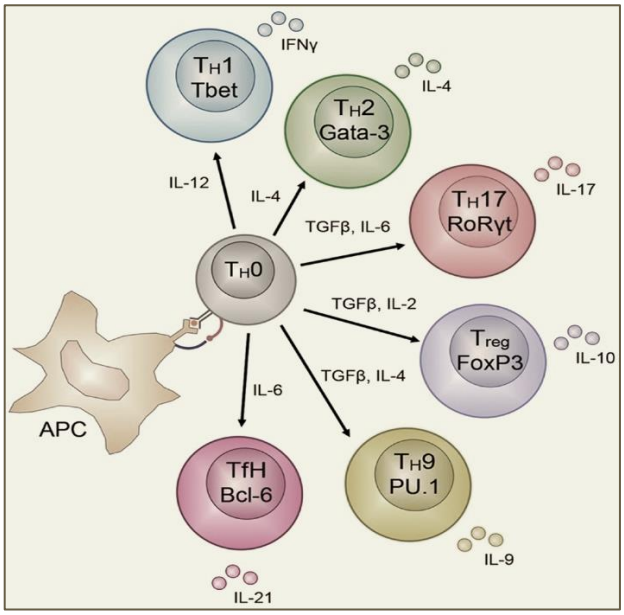


Figure 1.9: CD4+ T lymphocytes subset differentiation. CD4+ T cells show extraordinary plasticity to differentiate into various subsets. Differentiation of naïve T cells based on the secreted substance when contact with the APC (during required for Th-1 cells. The different subsets can be distinguished and regulated by the transcription factors T-bet for Th-1 cells. Cytokines secreted by these subsets, e.g., IFN-γ for Th-1 cells, are directed to the control of the pathogen that initiated the induction of the clone (Russ, et al., 2013).

1.5.2.1.1 Th-17 cells

Th-17 cells were differentiated as a distinctive group of T-helper cells. Although they were from the same naive CD4+ Th cells, they are differentiated into a distinct lineage that differs from Th-1 and Th-2 cells by the expression of transcriptional factor and the effector cytokines secretions. Th-17 produce a range of effector cytokines including IL-17A, IL-17F, and IL-22. Their main functions are the attracting of neutrophil and the stimulating of epithelial cells to produce antibacterial peptides against bacterial colonisation. The first time Th-17 became known as a different subset of CD4+ Th cells was in 2000 when CD4+ Th cells differentiated from naïve CD4+ recognised to secret IL-17A but not IFNγ in response to mycobacterial infection (Infante_Duarte *et al.,* 2000) and was first recognised as Th-17 subset by Park, 2005. The differentiation of Th-17 cells involves several transcriptional factors including Retinoic acid receptor-related orphan receptor γt (RORγt) (Kao *et al.,* 2006 and Wilson et al., 2007), RORα (Yang *et al.*, 2008), Signal transducer and activator of transcription 3 (STAT3) (Yang *et al.*, 2007), and Aryl hydrocarbon receptor (Ahr) (Veldhoen *et al.*, 2008; Quintana *et al.,* 2008). First, priming of naïve CD4+ Th cells to Th-17 requires the upregulation of Signal transducer and activator of transcription 3 (STAT3). STAT3 upregulated in response to IL-6 and IL-23 cytokines. Upon STAT3 activation, RORγt upregulation takes place (O'shea and Murray *et al.*, 2008). In addition, Ahr was also found to be required for priming IL-22 producing Th-17 rather than IL-17A production by primed Th-17 cells. The differentiation of Th-17 from naïve CD4+ Th cells requires priming cytokines (figure 1.10). Polarizing cytokines cocktails are controversial and previously studied in vivo and in vitro experiments (Bettetti *et al.,* 2006; Mangan *et al.,* 2006; Veldhon *et al.,* 2006). One of these cytokines' combinations required for Th-17 cells priming is IL-6 in combination with a low concentration of TGF-β (Ascota-Rodreguez et al., 2016; Bettetti *et al.,* 2006; Mangan *et al.*, 2006; Veldhon *et al.,* 2006). As a result of the apoptosis upon the pathogenic bacterial infection, TLR-ligand promoted the production IL-6 and TGF-β by APC. Of note, the production of TGF-β alone directed the naïve CD4+ Th cells to Treg cells priming. Another cytokine required for Th-17 differentiation is IL-1β. IL-1β along with the support of IL-6 was required for the

naïve Th cell priming to Th-17. Interestingly this process was suppressed by IL-12 or TGF-β (Ascota-Rodreguez *et al.,* 2016). IL-23 was first thought to be responsible in the priming process of Th-17. Recently it showed that it is not essential in the priming of Th-17 but it is essential for the colonic expansion of Th-17 because IL-23R expressed on Th-17 cells but not naïve CD4+ Th cells and it was necessary for the maturation of Th-17 cells to their full potential of IL-17A secretion (Kastelian *et al.,* 2007; McGoachy *et al.,* 2007).

1.5.2.1.2 T helper-17 function

In addition to the role of Th-17 autoimmune disease like rheumatoid arthritis, or Crohn's disease, Th-17 cells have a prominent role in infectious disease and the clearance of bacterial infections. Its role is emphasised by the release of pro-inflammatory cytokines that is essential for the recruitment of innate immune cells like neutrophils to the site of infection. In addition to the stimulation of epithelial cells in the local affected site to produce antimicrobial peptides (AMP) and β-defensins (BD) (Korn et al., 2009), B-cell recruitment (Jaffar, et al., 2009) may be connected to the expression of B-cell chemoattractant CXCL13 on Th-17 cells (Takagi, et al., 2008).

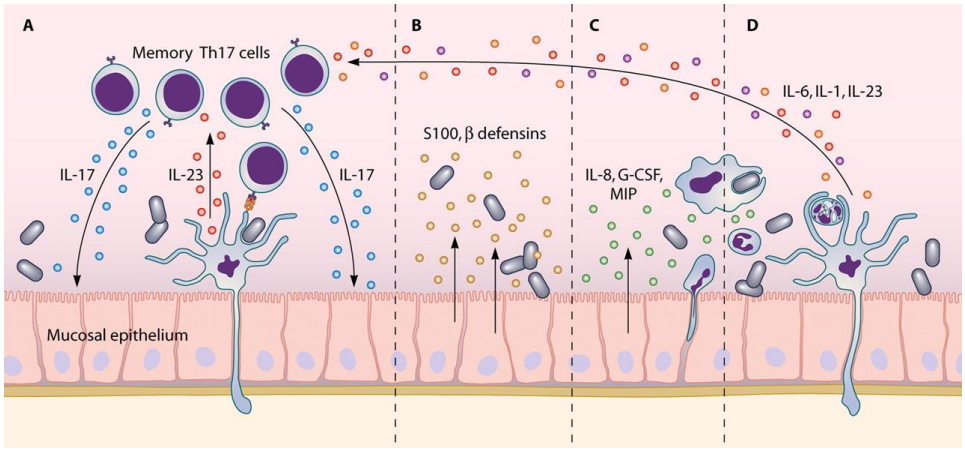


Figure 1.10: The role of Th-17 in mucosal infection.

When mucosal DCs activated by extracellular pathogen produce IL-23 causing the Th-17 activation by secretion of IL-17A and IL-17F (A). The pro-inflammatory cytokines induce the secretion of the antimicrobial peptides and β defensins (B). IL-8 and G-CSF production by neutrophils leads to apoptotic (C). When DC engulf the apoptotic cell, release IL-6, IL-1, and IL-23 cytokines and prime Th-17 induction (Peck and Mellins, 2009).

1.5.2.1.3 The role of T helper-17 in *S. aureus* infection

During the event of bacterial infection, APC process the invader pathogen and present the antigens through MHC-II molecules. The activation of antigen-specific Th-17 cells requires the direct contact of APC through MHC-II and the co-stimulatory molecules with the complementary receptors on the Th-17 cell surface. This binding stimulates Th-17 to produce their effector pro-inflammatory cytokines including IL-17A, IL-17F, and IL-22 to the site of infection. These cytokines eventually initiate the recruitment of neutrophils and stimulate the epithelial cells to produce antibacterial β-defensins (BD) and AMP. During *S. aureus* infection, several BD and cathelicidin were observed to be activated. It was reported that in keratinocytes, *S. aureus* colonisation triggered the production hBD-2. While during human nasal infection with *S. aureus*, hBD-3 and CAD18 (the precursor of LL-37) were found to have greater activity against *S. aureus* (Midorikawa *et al.*, 2003). The effect of hBD-3 was effective in *S. aureus* infection at higher concentrations

(Bals *et al.*, 1998). In contrast, any defects in the regulatory genes responsible for hBD-2 and hBD-3, come with a low concentration of hBD-3 and associated with the persistence of *S. aureus* infection (Nurjadi *et al,* 2013).

The roles of IL-17A and IL-17F in nasal *S. aureus* infections were studied in several previous researches (Shigame, et al., 2009; Archer, et al., 2013; Archer et al., 2016; Reiss-Mandel et al., 2018). In a murine model of *S. aureus* infections, IL-17A and IL-17F were together responsible for promoting the production of BD and deficient mice from both IL-17A and IL-17F were unable to clear the infection (Archer *et al.*, 2016). A previous study by Shigame and colleagues (2009), showed that the depletion of IL-17A and IL-17F coupled with abscesses developed around mouth and nose in response to *S. aureus* colonisation. Furthermore, the antibacterial effect of AMP in nasal secretions was shown to have heat-sensitive anti-Staphylococcal properties (Archer *et al.*, 2016).

In addition to the role of IL-17A in neutrophilic chemotaxis, IL-17A enhanced the expression of CXCL-1, CXCL-2, CXCL-8 (IL-8) on neutrophils and attracted neutrophils to the site of infection (Kolls, J., and Linden.; Laan et al., 1999; Ye et al., 2001). Furthermore, upon the activation with IL-17A, epithelial cells were stimulated to produce GM-CSF (Laan et al., 2003; Starne et al., 2002) and that enhances the neutrophils regeneration and survival and also increases the elastase activity in neutrophils (Hoshino et al., 2000).

1.6 The role of Inverted RORγt agonist in Th-17 response

RORγt is an essential transcription factor for proinflammatory CD4 + T-helper 17 (Th-17) induction and the production of IL-17 (Kao *et al.*, 2006 and Wilson et al., 2007). IL-17 expression following bacterial is important for the clearance of bacteria while prolonged inflammation may be associated with autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, and psoriasis (Korn, et al., 2009). Thus, RORγt is an attractive drug target for autoimmune diseases (Bronner et al., 2016). One of the resent Th-17 suppressors is GSK805. GSK805 is RORγt selective inverse agonist that functions as an isoform of the RORγt receptor. Thus, it binds with RORγt and blocks the transcriptional process. Nevertheless, GSK805 blocking does not affect the RORγt binding with DNA. Therefore, it directed RORγt to bind with GATA3 and this will downregulate the Th-17

inflammatory response especially in patients with autoimmune disease. Recently, the ex-vivo experiment of GSK805 to tonsillar MNC showed a selective inhibitory effect to RORγt from Th-17 but not for ILC3 group (Withers, et al, 2016). This encouraging finding highlighted the possibility to use this molecule during bacterial infections and experiment it selective suppression for Th-17 in adenotonsillar MNC towards both Th-17 and ILC3.

1.7 Aims of the study

1. Study the carriage percentage of *S. aureus* and *S. pneumoniae* in the nasopharynx and the association of their percentages with age. Then, study the relationship between *S. aureus* carriage, and the local IL-17A levels by measuring of the salivary IL-17A. After that, study the effect of *S. aureus* CCS in stimulating IL-17A in isolated MNCs.
2. Screening the effect of *S. aureus* recombinant peptides (antigens) on Th-17 stimulation and whether they elicit Th-17 cytokine production in NALT. Then, the effect of *S. aureus* antigens (HlaH35L) on Th-17 stimulation will be studied by the activation MNC with the recombinant protein or with comparing wild-type (Hla-producing strain) with a non-Hla-producing mutant strain.
3. In the third section, the frequency of ILC3 in NALT will be analysed and investigate the relationship between ILC3 and Th-17 through the NKp44+ ILC3 and HLA-DR+ ILC3 and their relationship with both *S. aureus* and *S. pneumoniae* respectively. The last section involves studying the effect of GSK805 RORγt inhibitor on both Th-17 and ILC3 in NALT.

Chapter 2

Materials And Methods

Chapter two: Materials and methods

2.1 Subjects and study ethics

2.1.1 Subjects recruitment and sample collection

Samples used in this study were from patients who underwent tonsillectomy and/or adenoidectomy recruited from Alder Hey Children's Hospital and Royal Liverpool and Broadgreen University Hospitals. Operations were due to either upper airway obstruction or recurrent tonsillitis.

2.1.2 Study ethics

The local research ethics committee approval for this study was obtained before the study. Also, all the patients and children's parents or custodians have been asked for their approval to participate in this study and to sign informed consent. The exclusion criteria for the study included that patients received antibiotics or systemic steroids within three-weeks of the surgery or who had known immunodeficiency. All the laboratory experiments were performed in the laboratories of Clinical infection, Microbiology, and Immunology department at the University of Liverpool.

2. 2 Patients Samples

2.2.1 Adenotonsillar Tissues

Pre-prepared 25ml universal tubes, containing 10ml Hank's Balanced Salt Solution (HBSS) (Sigma Aldrich, UK) supplemented with 10µg/ml gentamycin (Sigma) and 1% L-glutamine (Sigma), were used for the transfer of adenotonsillar tissues after the operation to the laboratory for processing.

2.2.2 Peripheral Blood

Up to five millilitres of peripheral blood sample were collected into a 25ml universal tube containing anticoagulant (heparin 100µl, LEO Pharma, UK), and transported to the laboratory.

2.2.3 Nasopharyngeal Swabs

Nasopharyngeal swab samples were obtained using a nasopharyngeal swab (and placed into a sterile vial containing 1ml transportation media, Skim milk-Tryptone-Glucose-Glycerol (STGG) broth (Appendix-I), as described previously (Katherine L. O'Brien et al., 2001). The swabs were stored in a -80°C freezer before use.

2.2.4 Saliva

Saliva samples were collected via a sterile 'Oracol' sponge swab (Malvern Medical Developments Limited, UK). Briefly, the sponge was placed into one side of the patient's oral cavity and left for about 1 minute to sock the accumulated saliva. Upon saturation, swabs were secured in test tubes and transport to the laboratory. In the lab. the sponges were squeezed with the aid of a 20 ml plastic syringe into 1.5ml Eppendorf tubes. Samples were immediately stored at a -80°C freezer for further analysing at a later point.

2.3 Detection of bacteriological carriage

2.3.1 Nasopharyngeal swab culture on 5% blood agar plates

Nasopharyngeal swab samples were cultured on 5% blood agar plates (BAP) following a standardized procedure as described previously (Bae et al., 2012; K. L. O'Brien et al., 2003). First, the thawed swab samples were mixed thoroughly and then 40µl from each swab sample was pipetted into a sterile blood agar plate, after that drops were spread into the plate with a sterile 5µl plastic loop (Technical Service Consultants Ltd, UK) using standard colonies isolation streaking procedure. All inoculated plates were incubated overnight at 37°C, in 5% CO2 for the growth results to be examined and recorded. Data collected were including the general morphological characteristics, density of the bacterial spp. In the sample and the haemolysis activities of each strain. Following the identification of the colonies based on their morphology, the colonies of interest were further identified with biochemical tests. After incubation, colony morphological characteristics of the growth, growth density and haemolytic activity were examined and recorded.

Purified colonies were further examined by Gram staining and essential biochemical tests. The schematic diagram below summarised the identification process of the bacterial carriage (Bae et al., 2012).

2.3.2 Morphological characteristics of bacterial growth and density measurement:

Morphological identification of the commonly isolated bacterial species was identified following the UK Standards for Microbiology Investigations in combination with the results of gram staining (Appendix-II) as the following (figure 2.1):

- *S. aureus* colonies are gram-positive cocci that aggregate in distinctive grape-like clusters under the microscope. On blood agar, colonies are 2-3mm in diameter following 24hr incubation. They usually translucent colonies but often with a golden pigment. Although most of the isolates are β-haemolytic, αhaemolytic as well as non-haemolytic isolates could be isolated. They appear as glistening, smooth, entire, and raised.
- *Streptococcus pneumoniae* are gram-positive cocci that occur in pairs and some are encapsulated. On the blood agar plate, colonies are small 1-2mm at 16-24hr, although they might grow bigger and mucoid under anaerobic conditions. They show typical greenish α-haemolytic activity. Due to its autolytic activity during incubation, they may appear as ‘draughtsman' colonies.

Growth density was measured semi-quantitatively as described in (O'Brien and Nohynek 2003). Growth was categorized by dividing the plate into four quadrants, from 1 to 4 as the following category:

· One plus (1+) if the colonies number were <10 in Quadrant 2

· Two pluses (2+) when colonies were <10 colonies in Quadrant 3

· Three pluses (3+) if the colonies were <10 in Quadrant 4

· Four pluses (4+) if >10 colonies were in Quadrant.

2.3.3 Biochemical testes

2.3.3.1 Catalase test/Slide method

Hydrogen peroxide (3%) was used to demonstrate the capability of a bacterial spp. for catalase production, a catalysing enzyme that catalyses oxygen from hydrogen peroxide (H2O2). This test was used for the differentiation between the bacteria that produces catalase, such as *staphylococci*, and *Moraxella* from non-catalase producing *streptococci* in nasal swabs. For a slide test, a drop of 3% H2O2 was placed on the surface of the clean, dry slide. Then by a sterile plastic loop, a small amount of isolated colony was transferred on the drop. The formation of oxygen bubbles was an indication of a test positivity.

2.3.3.2 Coagulase tube test:

For the detection of coagulase-positive *S. aureus* among other *Staph spp.* tubes were prepared by reconstituting of lyophilized rabbit plasma with EDTA (Sigma, UK) in 3 ml of sterile distilled water, and then 0.3 ml of the rabbit plasma was distributed into a sterile culture tube using a sterile pipette. To conduct the test, a colony from BAP were inoculated into the plasma tubes and incubated at 37°C. Then, the tubes were examined every hour for coagulation by gently tipping to the side without shaking them after 30 min to 4-6 hrs. The coagulase test was considered positive if a coherent clot was formed in more than 75% of the tube. If the test is negative after 4-6 hours, tubes were incubated for 24 hours and then the results of the coagulase were recorded. In each test, negative control of a non-inoculated tube was included.

2.3.3.3 Optochin susceptibility test

Blood agar plates were used for Optochin testing. Optimal *S. pneumoniae* colonies were picked by sterile loop and streak into the blood agar plates. Then, optochin disks were instilled on the streaking place by sterilize forceps. Plates were incubated overnight at 37°C, in 5% CO2 for the growth inhibition around the optochin disks were recorded. Susceptibility was conformed when the zone of inhibition was ≥14

mm (Chandler, Reisner, Woods, & Jafri, 2000). Isolates susceptible to optochin were confirmed as *S. pneumoniae* (figure 2.1).

2.3.4 Storage of bacterial isolates

After screening and identification of nasopharyngeal bacterial carriage, bacterial colonies of interest were re-cultured on BAP overnight at 37°C, in 5% CO2. Then, 3-5 colonies from each strain were picked up by sterile 5µl plastic loop and then inoculated into screw-capped Protect Plus bacterial preservative vials (Technical Service Consultants Ltd, UK). Bacterial isolates were then stored at -80°C for future analysis.

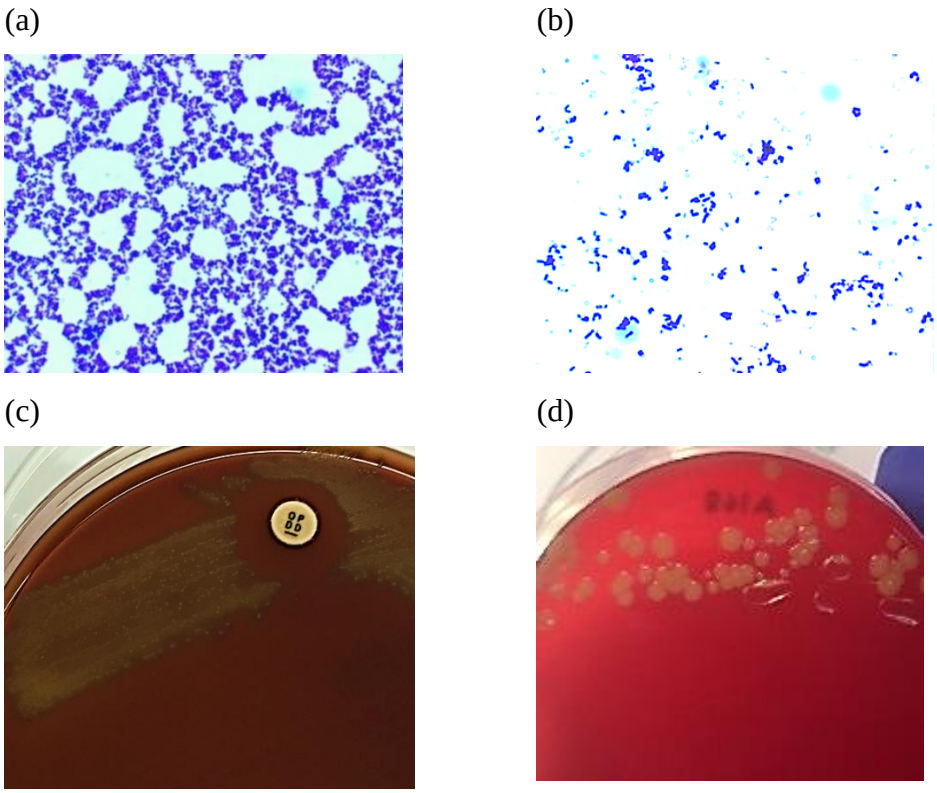


Figure 2.1: Identification of Bacterial growth in the nasal swab, *S. aureus* (a) and *S. pneumoniae* (b) under a microscope. Morphologies of colonies on blood agar plates, *S. aureus* golden colonies (d) and optochin sensitivity test (c).

2.4 Quantitative PCR of FemA gene for identification of *S. aureus* in nasopharyngeal swabs.

2.4.1 DNA extraction from nasopharyngeal swab samples

The extraction of bacterial DNA was performed using the QIAamp UCP Pathogen Mini kit (QIAgen, UK). This robust protocol has several advantages as it enables the extraction of bacterial DNA from small sample volumes as 400µl. Also, it ensures pure bacterial DNA yield along with the facility of using pre-mechanical treatment for biological samples, which provides a simpler protocol to extract DNA from Gram-positive rigid cell wall bacteria. The protocol conducted according to the suppliers' instructions with minor modifications. Briefly, the mechanical breakdown of the bacterial cell wall within the sample involves the use of Pathogen Lysis Tubes (QIAgen, UK). 400 µl of nasal swabs' STGG media suspension was added to the tube and vigorously vortexed for 11 min. Then, spinning down tube for 5 s at 8,000 x g to remove drops from the lid. After that, the supernatant was transferred into a fresh 2 ml tube avoiding the beads. Purification procedure followed the last step that included the addition of 40 µl Proteinase K mixed by vortexing for 10 s. Sample incubated at 56°C for 10 min, then 200 µl of Buffer APL2, mix by pulse-vortexing for 30 s, followed by incubation at 70°C for 10 min. After a short spin, 300 µl ethanol was added to the lysate and mix thoroughly by pulse-vortexing for 15–30 s. Next, 600 µl apply of the mixture to the column (in a 2 ml collection tube), avoiding wetting the rim, and centrifuged at 8000 rpm for 1 min. Columns were placed in a clean 2 ml collection tube. This step was repeated for the remaining mixture. Following filtration, 600 µl of APW1 buffer were added without wetting the rim and the columns centrifuged at 6000 x *g* for 1 min. Next, 750 µl APW2 Buffer was added without wetting the rim and centrifuge at 20,000 x *g* for 3 min. before the elusion step, columns were added into a new 2 ml collection tubes and incubated the assembly at 56°C for 3 min with an opened lid to dry the membrane completely. Finally, elution of DNA samples was done by placing the column into a clean 1.5 ml elution tube and carefully 50–70 µl of AVE Buffer were added to the centre of the membrane, incubated at room temperature for 1 min. then, columns were centrifuged at full speed 20,000 x *g* for 1 min to elute

the DNA. As a positive control DNA sample from pure *S. aureus* strain was extracted.

DNA concentrations were measured using Nanodrop and the ratio of 260/280 was detected as the purity indicated of the extracted DNA. The concentration of DNA samples was adjusted to 2ng/µl then DNA samples were kept at -80°C to be used for RT-PCR.

2.4.2 Primers and probe design

The primers were designed to amplify *fem A* gene as a conserved gene as described previously (Sabet et al., 2007). Primers were validated by NCBI primer BLAST and with 20–80% of G/C ratio contents. The final amplicon size is less than 150 bp as intentionally primers were designed to amplify short segments within the target gene sequence. The probe used was a Taqman probe that has been designed and labelled with FAM fluorophore. Detailed information of the primers and the probe (Sigma Aldrich, UK) is summarised in table 2.1.

Table 2.1: The *FemA* primers and probe used in the RT-PCR for the detection of *S. aureus* in nasopharyngeal swab samples.

Oligo Primer	**Primer sequence (5′-3′)**	**Reference**
femA-F forward primer	ACT GTG ACG ATG AAT GCG ACA A	(Sabet et al., 2007)
femA-R reverse primer	ATG TTG TGG TGT TCT TAT ACC AAA TCC	(Sabet et al., 2007)
femA- probe	FAM 5′ CGACAACTGGCACATTGGCTATCGCT TT 3′- (BHQ-1)	

2.4.3 Real-time PCR (RT-PCR) procedure

The QuantiFast Pathogen RT-PCR +IC Kit (QIAgen, UK) was used for *femA* amplification. The DNA Internal Control (IC) was used to monitor the performance of RT-PCR amplification. The amplification mixture was prepared with a final volume of 10µl. The volumes and concentrations for each component were specified in the table (2.2). Samples were prepared and tested in triplicates. Each RT-PCR experiment involved running three controls first control was containing only internal control DNA (IC) to ensure a successful amplification process and exclude the presence of amplification inhibitors in negative results, the second control contained a DNA sample extracted from purified *S. aureus* isolated strain according to the method mentioned earlier with IC. While the third control (the negative control) was a reaction without the addition of IC DNA and contain DNA/RNA-free water as a replacement for DNA template and IC.

Table 2.2: *femA* RT-PCR reaction mix components: working concentrations and final volumes of each component.

Component	Final Conc.	Volume/test
5X Quontifast Pathogen Master Mix	1x	2µl
10 µM *femA* forward primer	0.3 µM	0.3 µl
10 µM *femA* reverse primer	0.3 µM	0.3 µl
10 µM FAM Probe	0.2 µM	0.2 µl
10X IC assay	1x	1 µl
10X IC DNA	1x	1 µl
Water	-	0.2 µl
DNA Sample	2ng/ul	5µl

2.4.4 The detection of RT-PCR products by agarose gel electrophoresis.

One-percent agarose gel (60 ml) was prepared and 6µl of SYBR® Safe DNA Gel Stain (ThermoFisher, UK) then added to gel, and the mixture was left to cool down before pouring it into the gel tray. 10ul of each RT-PCR product were mixed with 2ul of 6x loading buffer () then loaded into the gel. A 100 bp DNA ladder (Biolab,

UK) was used as the size of the post-amplification product was about 150 bp. DNA samples were run in 1x TBE buffer (ThermoFisher, UK) at constant 100v for 90-110 min. Finally, DNA bands were visualised by a ChemiDoc XR system (BioRad, UK) using a protocol including a blue filter tray (figure 2.2).

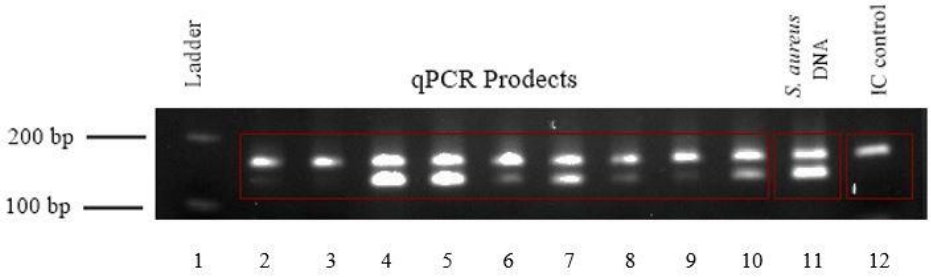


Figure 2.2: Gel electrophoresis of Nasal swab DNA after femA RT-PCR amplification with the expected band size of 150 bp. The figure shows the DNA ladder in lane 1, RT-PCR amplification products of nasal swaps samples in lanes 2-10, *S. aureus* purified DNA as a positive control in lane 11 and Internal control (IC) only as a successful amplification indicator in lane 12.

2.5 *S. aureus* Concentrated Culture Supernatants (CCS)

2.5.1 Bacterial strains

Three *S. aureus* strains were used to produce *S. aureus* CCS, the MRSA strain USA300 LAC (Wt) was kindly provided by Fabio Bagnoli (F. Bagnoli et al., 2015) including both isogenic mutant strain hla::ermB (Hla-) (Popov et al., 2015).

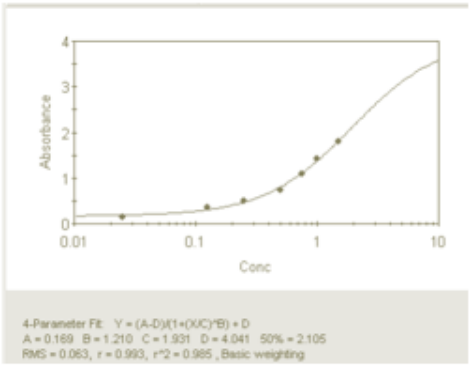
2.5.2 Preparation of *S. aureus* CCS

S. aureus CCS were produced following a previously described procedure (Zhang, Choo, & Finn, 2002) with minor modifications (Popov et al., 2015). First, strains were thawed at room temperature. Then each strain was sub-cultured on sterile blood agar plates and incubated for overnight in 5% CO2 at 37°C. After that, each strain was transferred into Tryptic soy broth (TSB) by inoculation of 3-5 colonies of each strain into a sterile TSB (3ml). Subsequently, several colonies were picked up by a sterile loop and cultured them for overnight. Medium turbidity was examined as an indicator of bacterial growth. In addition to turbidity, the optical

density (O.D) of bacterial growth was read at 620nm using a spectrophotometer. Then, 0.5ml of each culture was added into 250ml conical flask containing 100ml of fresh TSB media. The growth was incubated at the same condition for about 2–4 hr and O.D was measured each half an hour until the O.D value reaches 0.5. the culture was transferred in to 50ml secured tubes and centrifuged at 3000×g for 30 min to spin down bacteria. The culture supernatant was filtered through 0.4µm and 0.2µm sterile Millipore filter subsequently and collected into new sterile 50ml tubes. The filtered supernatants were concentrated to about 10-fold by using Vivaspin15 concentrators (Sartorius Stedim Biotech, Germany) and centrifuging at 3000×g for 30-50 min. Finally, the generated CCS was aliquot in (1.5ml) and stored in freezer -80°C.

2.5.3 The measurement of CCS protein concentration

The protein concentration was measured by using Pierce BCA (bicinchoninic acid assay) (Thermo Scientific, USA). 96-wells round plate was used and the assay was conducted according to the manufacturer's instructions. Briefly, a serial dilution of bovine serum albumin (BSA) that ranges from (0.025 mg/ml- 2 mg/ml) was prepared as a protein standard curve. The preparation of two dilutions (1:100 and 1:1000) for each CCS was also prepared by dilution in distilled water. After the standards and samples were made, a reaction mix was prepared. All standards and samples were pipetted to the plate in duplicate with distilled water as blank, and the reaction mixture was added to the wells, followed by incubation at 37°C for 30 min. O.D. was measured at 562 nm Distilled water. *S. aureus* CCS protein concentration was calculated were deducted from the standard curve. (Figure 2.3 and Table 2.4).



BSA standard	Conc. (mg/ml)	Mean OD
1	0.025	0.15
2	0.125	0.361
3	0.25	0.495
4	0.5	0.742
5	0.75	1.084
6	1	1.425
7	1.5	1.801

Figure 2.3: BSA standard curve measured by Pierce BCA assay.

Table 2.3: The Protein concentrations of *S. aureus* CCS measured by Pierce BCA assay.

***S. aureus* strain CSS**	**Conc (mg/ml)**
Wt.	6.083
Hla -	7.307

2.5.4 The detection of Hla protein in *S. aureus* CCS by Western blot.

Immunostaining of Hla protein in *S. aureus* Wt (which express Hla) and its isogenic mutant (Hla-) and the bacterial free CCS was detected by Western blotting using mouse anti-HlaH35L antisera (Novartis vaccine, Sienna, Italy) to confirm the expression/ absence of Hla protein in the generated CCS (Popov et al., 2015).

2.5.4.1 Sample Preparation

For denaturation of proteins, *S. aureus* CCSs samples and HlaH35L recombinant proteins were blocked by using Laemmli buffer as a reducing buffer. CCSs were diluted in 1:2 ratio while HlaH35L was diluted in 1:100 with Laemmli buffer (Bio-Rad, UK). Then, the sample mixtures were treated on a heat block at 100°C for 5 minutes to denature proteins. After reduction, 30µl of each sample was loaded into the 12% mini protean precast TGXTM gel (Bio-Rad, UK) and precision plus protein kaleidoscope ladder (Bio-Rad, UK) was loaded in designated wells. After

sample loading, gel electrophoresis was performed for 95 min at a constant 250v in 100mA.

2.5.4.2 Protein Transfer by Western blotting

Protein transfer by western blotting was performed by a Transblot TurboTM transfer system (Bio-Rad, UK). Following the gel electrophoresis, proteins were transferred into a 0.2µm nitrocellulose membrane (Transblot turbo transfer pack). Immediately after gel electrophoresis, the gel was assembled with a nitrocellulose membrane and then the gel-membrane was positioned on the bottom of the ion reservoir (anode) stack. Top ion reservoir (cathode) stack was installed over the gel and run for 10 min at a constant 25v in 1000mA.

2.5.4.3 Detection of HlaH35L protein in *S. aureus* CCS

For the detection of HlaH35L, the nitrocellulose membrane was blocked with 5% skimmed milk in PBS containing 0.05% Tween20 (PBS-T) (appendix I) for 2 hr at room temperature (RT). After that, the membrane was washed 3 times in PBS-T and incubated overnight at 4°C with mouse anti-HlaH35L antiserum antibody diluted (1:10000) in blocking solution. After washing for 5 times in PBS-T, the membrane was incubated with secondary antibody EasyBlot anti-Mouse IgG (HRP) (GeneTex, USA) prepared by dilution (1:2000) in blocking buffer and 2µl Streptactin-HRP (1:30000) (Bio-Rad) were added for ladder visualization for 1 hr at RT. The membrane was washed 5 times in PBS-T and then once in PBS. The visualization of protein bands was carried out by incubating the membrane with the substrate solution for 5 min in the dark. The substrate solution was prepared by mixing equal volume (1:1) of Immun-star WesternC chemiluminescence reagent A and B (BioRad, UK). After removal of the excess substrate with PBS, the membrane was imaged using Chemi-DocXRS system (BioRad, UK) (Figure 2.4).

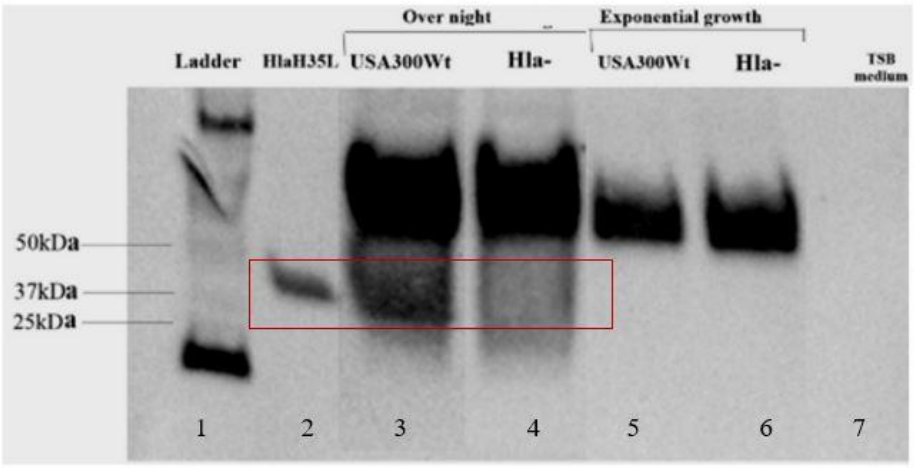


Figure 2.4: Western blotting showing the presence or absence of Hla production of *S. aureus* strains Wt and Hla-. Lane 1 Ladder, HlaH35L recombinant protein in lane 2, lanes (3-4) CCS produced during lag phase: Wt (lane 3) and Hla- strains (lane 4) 4 and they showed the production of Hla in Wt strain while in Hla- strain it was absent. All compared to the TSB medium control (lane 7).

2.6 Staphylococcus recombinant antigens:

Nine recombinant proteins were used as antigen stimulants for tonsillar MNCs. These protein antigens have been studied as possible vaccine candidates. The recombinant proteins were produced in *Escherichia coli* using the respective cloned genes and subsequently purified (kindly provided by Dr Fabio Bagnoli, Novartis vaccine, Sienna, Italy). The general characteristics for each protein were listed in the table (2.5). Optimization of protein concentrations was performed to select the optimal concentrations for cell stimulation.

Table 2.4: The general characteristics of *S. aureus* recombinant proteins.

Protein	name/ Descriptions	Mwt/a.a.	Role in *S. aureus* infection	References
IsdA	(Iron-responsive) surface determinant A	36-38 KDa	Adhesion. Immunogenic, antibodies found in healthy individuals	(Arlian and Tinker 2011)
ClfB	Clumping factor B Fibrinogen receptor B Fibrinogen-binding protein B	124 kDa ~ 940 a.a	Nasal colonization and elicit ads response in a rodent murine model of immunization	(Ní Eidhin et al., 1998) (Schaffer et al., 2006)
SdrC	Serine-aspartate repeat-containing protein C	102,9KDa ~ 947 a.a	Colonization	(Feuillie et al., 2017)
SdrD	Serine-aspartate repeat-containing protein D Serine-aspartate repeat D	146 KDa	Colonization	(Askarian et al., 2016)
CnaBE3	Conservative 3 Repeated sequences within SdrE	110-113 a.a		(Becherelli et al., 2013)
EsxAB	The fusion of the two ESAT-6-like secreted virulence factors EsxA and EsxB	7-14 kDa	Abscess formation	(Sundaramoorthy, Fyfe, & Hunter, 2008)
HlaH35L	alpha haemolysin toxoid	~35 KDa	Cell invasion	Bagnoli, Fontana et al. 2015)(Bhakdi & Tranum-Jensen, 1991)
FhuD2	Ferric hydroxamate uptake D2	~32.7 kDa MONOMERIC	Binding lipoprotein involved in iron uptake and in early stages of invasive *S. aureus* infection	Bagnoli, Fontana et al. 2015)
Csa1A	Conserved staphylococcal antigens	~30-35 KDa	a putative lipoprotein	(Fabio Bagnoli et al., 2015)

2.6.1 Western blotting of Staphylococcus recombinant proteins.

To confirm the identity of each recombinant protein, these protein preparations were examined by Western blotting protein transfer using mouse anti- IsdA, anti-ClfB, anti- SdrD, anti- CnaBE3, anti- EsxAB anti- HlaH35L anti-FhuD2 and anti-Csa1A antisera (Novartis vaccine, Sienna, Italy). The following steps of the western blot were resampling the procedure of Western blot for *S. aureus* CCS described above with minor alterations: Each protein was first reduced in Laemmli reducing Buffer (1:20) 5µl of each protein with 95 µl of the buffer. After heat treatment with a heat block at 100°C for 5 min, 20µl of each protein were loaded into the 12% mini protean precast TGXTM gel (BioRad, UK). After running the gel and proteins blotting to the membrane transfer, the membrane was blocked by the incubation with 5% skim milk PBS-T blocking buffer. Then successively primary and secondary antibodies were prepared with blocking buffer and the secondary antibodies were used for this experiment were Goat anti-mouse IgG-HRP (1:10000) (Santa Cruz Biotechnology, Germany) (Figure2.5).

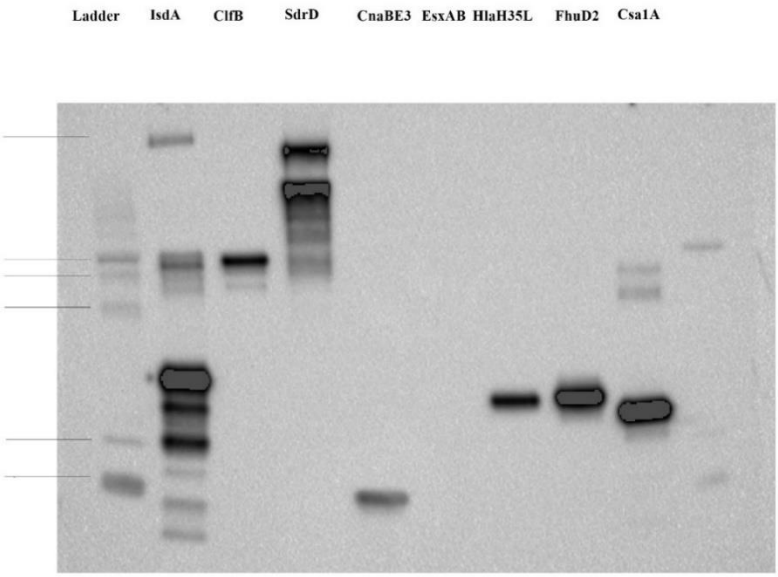


Figure 2.5: Western blotting of recombinant IsdA, ClfB, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A proteins. The figure shows the MW marker ladder in lane 1. While *S. aureus* recombinant IsdA, ClfB, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A proteins distributed in lanes 2-9 respectively.

2.7 Sample processing and stimulation of tonsillar MNC

2.7.1 Isolation of mononuclear cells (MNC) from adenotonsillar tissues

Adenotonsillar tissues were processed within a class-II Bio-safety cabinet following methods as previously described (Zhang et al., 2006). At first, tissue samples were rinsed with 10ml of fresh HBSS and transferred into Petri-dishes. With the help of a sterile pair of forceps, the tissues were minced by a scalpel to release cells into the medium. With a sterile Pasteur pipette, the cell suspension was filtered through a 70µm cell strainer (BD biosciences, USA). The cells were then layered carefully on to 15ml Ficoll-Paque (GE Healthcare Life Sciences, UK) and isolated by gradient centrifugation (400×g for 30 min). After washing with sterile PBS (400x*g* for 10 min.), cells were re-suspended with (5 to 15ml) fresh RPMI-1640, counted with a haemocytometer and adjusted into 4*106 cells/ml in order to be ready for stimulation.

2.7.2 Depletion of CD45RO+ cells from adenotonsillar MNCs.

In some experiments, CD45RO+ (memory and effector T cell phenotype) cells were depleted from tonsillar MNC, so naïve T cell response could be analysed. The CD45RO+ cell depletion was accomplished by using magnetic cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotec).

First, the initial cells number was determined according to the final cell number needed for each experiment. Then cells were centrifuged at 400xg for 10 min and the supernatant was discarded. Per every 107 pelleted cells, 80µl of depletion buffer (0.5% BSA-PBS) was added to suspend the cells and then 20 µl of anti-human CD45RO+ micro-beads provider was added. Cell-beads mixture was mixed thoroughly and incubated for 15 min. at 4°C. The cells were washed by adding 5ml of depletion buffer and then centrifuged at 400xg for 10 min. For the depletion step, pelleted cells were re-suspended (up to 108) in a final volume of 500µl of the depletion buffer. Magnetic separation using LS column (Miltenyi Biotech) was done by passing the cell-bead mixture through the LS column in the presence of a magnetic field obtained by using a special MACS separator stand. In order to separate the cells, the column was washed with 2ml of depletion buffer. Then, the cell suspension was applied onto the column carefully without allowing bubbles to

form and allow the solution to pass through the column. A sterile collection tube was placed under the column and then another 2 ml of depletion buffer was added to the column and let the elution to pass through. Then, the cells were pelleted at 400xg for 10 min, and re-suspended with RPMI-1640 and adjusted to 4*106 cells/ml in order to be ready for stimulation.

2.7.3 Labelling of adenotonsillar MNC and PBMC with CFSE for assessment of cell proliferation.

T cell proliferation in tonsillar MNC stimulated with *S. aureus* recombinant proteins was assessed by pre-labelling cells with Carboxyfluorescein diacetate succinimidyl ester (CFDASE). The stained cells were then stimulated with the proteins or *S. aureus* CCS for 5 to 6 days. The tonsillar MNC were then harvested and co-stained with cell markers to monitor CD4+ T-helper cell proliferation. 50-60x106 of tonsillar MNC or 20-25x106 PBMC were washed twice with 10 ml of sterile PBS and centrifuged at 400xg for 10 min. Then cells pellet was re-suspended with 3ml PBS. In the same time, CFSE (50µM) was prepared as a working concentration by diluting 5µl of CFSE into 10ml of sterile PBS. 3 ml of this freshly prepared CFSE solution was added to 3ml cell suspension and mixed thoroughly by vortexing. The cells-CFSE mixture was then incubated at 37°C for 8 min in order to quench the reaction, 15ml of ice-cold RPMI-1640 media were added. Thereafter, the cells were spanned down at 400xg for 10 min and the pellet was dissolved in RPMI-1640 medium supplemented with HEPES (Sigma), 10µg/ml gentamycin (Sigma), 1% L-glutamine (Sigma) and 10% FBS (Sigma). For cell stimulation, the cells were counted and adjusted into 4x106cells/ml and distributed into 48 wells plates as 500µl per well. Cells were stimulated with different stimuli depending on the experiment and incubated for 5-6 days. With each experiment, three controls were included: two without any stimulation (as negative control), and a positive control stimulated with anti-CD3 antibodies.

2.7.4 Cell culture and stimulation:

For cell culture and stimulation, adenotonsillar MNC and PBMC were added to 250 or 500 µl into 96 or 48-wells flat-bottom plates (Corning Incorporated, Corning, USA). Cells were either cultured with RPMI-1460 only or stimulated with the

optimum concentration of each stimulant. In some experiments, cells were stimulated with USA300 LAC Wt or Hla- and Hla+ CCSs (2ng/ml) or recombinant proteins.

For detection of the cytokine-producing cells following cell stimulation, tonsillar MNC were stimulated with PMA (100pg/ml) and Ionomycin (Ion) (1ug/ml) in the presence of 1x brefeldin A (BFA) as a protein transport inhibitor (eBiosciences, UK) in the last 4 hours of culture before analysis. For Th-17 induction experiments, PMA/Ion/BFA mixture was added to all stimulated cells at the last 6 hr of the incubation period, before the detection of Th-17 cells by flow cytometry.

For some experiments, GSK805, a RORγt Inverse Agonist II, was used in the cell culture to study what effect it may have on Th-17 cells and innate lymphoid cells, and for which an optimum concentration of 0.5µM was used.

2.8 Flow cytometry analysis

For the analysis of the cells after stimulation by flow cytometry, several markers were used to stain cells as below.

2.8.1 Surface marker staining:

Tonsillar MNCs were first collected into 1.5ml Eppendorf tubes by adding 0.5 ml of staining buffer (0.2%BSA-PBS). Harvested cells were washed twice with FACS staining buffer by centrifuging at 500xg for 8 min and discarding the supernatant. After that pellet was re-suspended and then stained with surface markers including CD3, CD4 (BD biosciences, UK) for 30-60 min. at 4°C according to the experiments conducted. Then, cells were washed twice with FACS staining buffer. At this step, cells were either proceeded to be stained with intracellular staining or if the surface markers were the end step. Cells were then suspended with 400-500 µl of the FACS staining buffer (depends on the cells numbers) and transferred into FACS tube and samples before analysis by Flow-cytometry. Data were analysed using software that indicated in each section. Lymphocytes were gated and based on typical FSC/SSC characteristics.

2.8.2 Intracellular staining:

For the detection of intracellular cytokines, before staining the surface markers, BFA (eBioscience, UK) was added to the cultured cells at the last 5-6hr of the incubation time culture. Positive control cells were stimulated with PMA/ Ion (100 ng, 1ug/ml respectively) for 5-6 hr along with BFA. In the case of the Th-17 induction experiment using CD45RO+ cells-depleted MNC, both unstimulated and stimulated cells were stimulated by PMA/Ion (100 ng, 1ug/ml respectively) at the end of culture period for 5-6 hr along with BFA. Then cells were harvested and stained for the surface antibodies marker as described previously.

For intracellular staining, cells were fixed and/ permeabilized using eBiosciences™ Intracellular Fixation & Permeabilization Buffer Set (eBioscences, UK) and according to the manufacturers' protocol. After the last wash for the surface antibodies, pelleted cells were fixed by adding 100 µl of fixation buffer provided and incubated in a dark place for 30 min at RT. Then, cells were washed twice with 1-2ml of 1x permeabilization buffer. The cells were re-suspended in the remaining volume of permeabilization solution, and fluorescence-labelled monoclonal antibodies (BD bioscience, eBiosciences, and Biolegend, UK) were added, vortexed and incubated for 60min in a dark place at RT. After the final incubation, the cells were washed twice, once with 1x permeabilization buffer and the second time with FACS staining buffer and re-suspended with 0.5 ml of FACS buffer. Cells were kept at 4°C until being analysed by flow cytometry.

2.9 Detection of Th-17 cytokines

2.9.1 Measurements of cytokine production in cell culture supernatants

2.9.1.1 Measurement of cytokine production in cell culture supernatants by ELISA

Sandwich ELISA was commonly used with relatively high sensitivity. Cytokines levels were measured in culture supernatants of pre-stimulated cells with *S. aureus* CCSs and recombinant proteins or and as well as the TH-17 blocking

experiment. ELISA was performed according to the instructions of the manufacturer (eBiosciences).

2.9.1.1.1 Measurement of IL-17A

For the measurement of IL-17A production from adenotonsillar and PBMC stimulated culture supernatant, IL-17A ELISA Ready-Set-Go® set (eBiosciences, UK) was used and the procedure was conducted according to the manufacturer's instructions. Capture antigens were prepared by adding 44µl of purified anti-human IL-17A into 11ml 1x coating buffer (1:250 dilutions). Then, 96-well Costar plates were coated with the capture antibody solution (100µl/well) and incubated overnight at 4°C. The next day, plates were washed 5 times with PBS-T, then blocked with 1x assay diluent (200µl/well) and incubated at RT for 1 hr. at the same time, Standard and samples dilutions were prepared. Standard was first reconstituted in 1 ml of Distilled water and left to dissolve for 10 min before preparing the working concentrations. The top concentration (500pg/ml) of the standard was prepared by adding 30µl human IL-17A stock solution into 870 µl of 1x assay diluent. The samples were also prepared (1:5 for children and 1:10 for adults' samples dilution) in 1x assay diluent. After blocking, the plate washed 5 times with PBS-T. Simultaneously, the top standards (200 µl/well) and samples (100µl/well) were added to the plate and assay diluent (100µl/well) were added in the other standards and blanks allocated wells. A total of 8 standards dilutions were prepared by 2-fold serial dilutions from the top standard. The plate was then incubated overnight at 4°C to maximize the sensitivity. The next day, the detection antibody solution was prepared by adding 44µl of purified anti-human IL-17A Biotin into 11ml 1x assay diluent. Then the plate washed for 5 times with PBS-T. Anti-human IL-17A Biotin (100µl/well) was added to the plate and incubated for 60 min. at RT. Next, the HRP-conjugated antibodies were prepared by adding 44µl of HRP-conjugated antibodies into 11ml 1x assay diluent. The plate was washed 6 times and then, HRP-conjugated antibodies were added (100µl/well) and the plate was incubated for 30 min in the dark, the plate was washed 7 times with PBS-T. The last step was the incubation of the substrate solution for 15 min. and then the reaction was stopped with (1N H2SO4). Finally, the plates were ready for OD

measurement at 450nm, and the concentration (pg/ml) was determined for each sample against the standard curve with Deltasoft PC software (Biometallics).

2.9.1.1.2 Measurement of IL-22

In vitro IL-22 production in adenotonsillar MNC cultures and PBMC was measured with human IL-22 ELISA Ready-Set-Go® set (eBioscience), following manufacturer's instructions. Briefly, ELISA plates were coated overnight at 4°C with a 1x prepared capture antibody solution (prepared by adding 44µl of purified anti-human IL-22 into 11ml 1x coating buffer). Standard and samples dilution (1:10) were prepared by diluting them in with the 1x assay buffer on the following day with the top concentration of the standard (1000pg/ml). Both standards and samples were tested as duplicates and incubated overnight at 4°C. The next step was involving the detection of antibodies incubation for 60 min. followed with HRP-conjugated antibodies for 30 min. and the last step was the incubation of the substrate solution for 15 min. the reaction was stopped with (1N H2SO4) and the OD measurement at 450nm. The concentration (pg/ml) was determined for each sample against the standard curve with Deltasoft PC software (Biometallics).

2.9.1.2.1 Protocol of CBA array analysis

First, the cytokine standard was reconstituted in the Assay Buffer, mixed thoroughly, and allow the vial to sit at room temperature for 10 minutes. This was used as the first concentration of the standard C7 (10,000 pg/ml). Another 6 standard dilutions were prepared by 1:4 dilution from the first standard by transferring12.5 µl of the standard into 37.5 µl of assay Buffer will be to make C6. Other following dilutions were made in the same way and C0 was made containing only assay buffer as a blank (0 pg/ml).

After the standards were prepared, a set of 1.5ml Eppendorf tubes was labelled for both standards and samples. Tests mixture was prepared by adding 25 µl of assay Buffer to all tubes then, 25 µl of each standard and samples were added and 25 µL of thoroughly mixed beads were added to all tubes. 25µl detection antibodies were added to all tubes. Then tubes were incubated in the dark on a plate shaker (600rpm) for two hrs at RT. Next, without washing, 25 µl of streptavidin-PE was added to

each tube and the tubes were incubated at the same conditions for 30 min. At the end of the incubation, the beads were centrifuged at 1000xg for 5 min and the supernatant was carefully removed. The beads were re-suspended with 200µl of washing buffer and vortexed before centrifuging at 1000rpm for 5 min. 400µl of the washing buffer was added to the tubes followed by analysis using flow-cytometry.

2.9.1.2.2 FACS data acquisition and analysis

Before analysing the samples, a template was prepared to detect two beads sizes (Bead A and B). 4000-6000 cell events of each sample were acquired. final data were analysed using LEGENDplex software (Biolegend, UK).

2.9.2 Measurement of IL-17A in serum and saliva samples

Serum and saliva IL-17A level was analysed by Human IL-17A hi sensitive ELISA kit (eBioscience, UK). According to the manufacturer's instructions, per coated strips were washed twice with 400 µl/well of washing buffer allowing 15 sec. socking per each wash. Standard was pre-prepared by dissolving the vial in 1000 µl of distilled water ending by 30 pg/ml concentration. Seven serial (1:2) dilutions of standard prepared with sample diluent; samples were pre-thawed, and the biotin-conjugated antibodies were prepared by diluting it with a 1x assay buffer. In the next step, 50 µl of sample diluent was added to the sample's wells while 100µl were added to some wells and considered as a blank. 100µl/well standard dilutions were loaded and samples (50µl/well) were added to the allocated wells before the addition of 100µl/well of Biotin conjugated antibodies to all the wells, and the plate was incubated at 18-25°C overnight in the dark. The next day, the plate was washed six times and 100µl/well of the streptavidin HRP was added and the plate incubated for 60 min at 18-25°C in the dark on a plate shaker with 150 rpm. Following the incubation, the plate was washed 6 times and the amplification I solution (100µl/well) was added and the plate incubated for 15 min in the same incubation condition. The plate was washed 6 times and the amplification II solution (100µl/well) was added and incubated for 30 min. In the last step, the plate was washed and TMB substrate (100µl/well) was added and incubated for 15-20 min.

The reaction was stopped by adding stop solution (100µl/ml) and the OD was measured at 450nm.

2.10 Detection of Innate lymphocytes 3 (ILC3) in adenotonsillar cells.

ILC3 in the tonsillar MNC was examined using freshly isolated cells by staining Lin1, CD127, cKit (CD117) and NKp44.ILC3 were identified according to the following gating strategy (Figure2.6). Adenotonsillar cells were either stained in freshly isolated MNC or following stimulation with various stimuli (including *S. aureus* or *S. pneumoniae* CCSs and/or bacterial recombinant proteins) for 3 days. For experiments that required the analysis of intracellular cytokine production, cultured cells were stimulated with PMA/Ion and BFA in the last 4 hours of the cell culture/stimulation period. For intracellular cytokine staining, cells were collected and stained with different FACS antibodies (as the experiment required) to be analyses by flow cytometry.

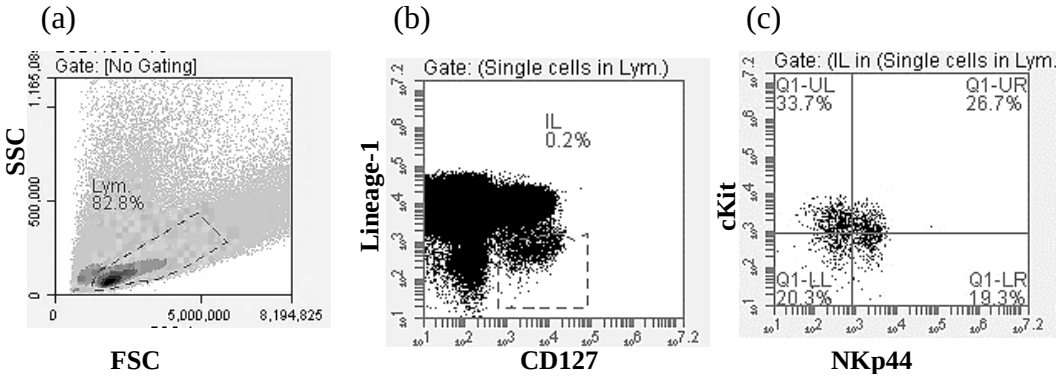


Figure 2.6: Gating strategy of innate lymphocytes 3 population using Lin1, CD127, ckit (CD117) and NKp44 antibodies.

MNCs stained for Lineage, CD127, cKit and NKp44 markers. First, the "lymphocyte" population was gated according to the typical forward (FSC) and side scattered characteristics (SSC) (a). Innate lymphoid cells (ILC) gated as Lin-/ CD127+ population (b). ILC3 cells population were sub-divided by using cKit and NKp44 markers into cKit+/NKp44+ and cKit+/NKp44- ILC3 cells (c).

2.11 Statistic analysis

Data distribution was tested for normality first, and then the differences between the two groups were analysed accordingly by either parametric (e.g. student t-test) or non-parametric (e.g. Mann-Whitney U) test. Paired data were analysed by paired t (parametric) test or Wilcoxon test (non-parametric). Group comparisons (>2 groups) were made by one-way variance (ANOVA). Two-factor correlation analysis was performed by Pearson's correlation test (parametric). The results were considered statistically significant when a *p-value* of < 0.05.

Chapter 3

Nasopharyngeal Carriage Of *S. aureus* And *S. pneumoniae* In Children And Adults And Their Relationship With Th-17 Activation.

Chapter Three: Nasopharyngeal carriage of *S. aureus* and *S. pneumoniae* in children and adults and their relationship with Th-17 activation.

3.1 Introduction

Bacterial carriage in the human nasopharynx is considered a prerequisite of invasive diseases (Shak *et. al.*, 2013 and Micheal, 2015). Among the common colonisers, *Streptococcus pneumoniae (S. pneumoniae) and Staphylococcus aureus (S. aureus)* are key pathogens for invasive diseases including pneumonia, sepsis, and meningitis. The nasopharynx is one of the important sites of immune defence against microbial pathogens. Nasopharynx-associated lymphoid tissue (NALT) comprising adenoids and tonsils are major components of the mucosal immune system in the nasopharynx and are known to be induction sites for natural immunity to pathogens colonising the nasopharynx. It will be important to understand the cellular and molecular mechanisms by which the local mucosal immunity clear the bacterial carriage. The immune mechanisms mediating bacterial nasopharyngeal carriage including *S. pneumoniae* and *S. aureus* in humans are still not well defined.

Several recent studies have demonstrated a negative association between carriages of *S. pneumoniae* and *S. aureus* in the nasopharynx (Bogaert et al., 2004; Regev-Yochay et al., 2004 and Pettigrew et al., 2007). Although the underlying mechanisms are unclear. Bacterial co-culture *in vitro* showed that hydrogen peroxide (H2O2) produced *by S. pneumoniae* inhibited *S. aureus* (Pericone *et al*., 2000). However, neither the H2O2 secretion by *S. pneumoniae* nor the H2O2 sensitivity of *S. aureus* has been shown to correlate with the co-colonisation patterns in children (Regev-Yochay et al., 2004 and Melles et al., 2008) or in animal models (Margolis et al., 2009). In addition, despite *S. pneumoniae* being shown to inhibit the growth of *M. catarrhalis* and *H. influenza* via H2O2 production *in vitro* (Pericone et al., 2000), epidemiological data showed co-colonisation of these bacteria are common (Jacoby *et al*., 2007; Pettigrew *et al.*, 2007 and Mackenzie *et al.*, 2010). Therefore, the bacterial co-culture experiments may not accurately reflect their *in vivo* interactions *in the human nasopharynx*, and host factor(s) may interact with the bacteria and contribute to changes/dynamics of local bacterial carriage status.

Recent evidence from animal studies support a crucial role of IL-17-producing CD4+ T cells (Th-17) in the clearance of mucosal microbial colonisation (Chen K, et al., 2011; Curtis et al., 2009; Dubin, 2008; Happel et al., 2005; Kolls et al., 2004 and O'Connor et al., 2010) including that of *S. pneumonia* (Lu *et al.*, 2008) and *S. aureus* (Archer *et al*., 2013).

The main mechanism of action of Th-17 cells against bacterial infection is through neutrophils and macrophage recruitment to the site of infection and activate bacterial killing by the phagocytes (Matsuzaki and Umemura, 2007 and Oukka, 2008). Furthermore, both Th-17 effector cytokines (IL-17A and IL-17F) contribute to the elimination of bacterial colonisation, e.g. *S. aureus* through the stimulation of antimicrobial peptides production (HBD-3) (Selsted, *et al.*, 1985). Archer *et al*., (2016) suggested that salivary levels of both IL-17A and IL-17F are determinant factors in *S. aureus* colonisation. The level of HBD-3 s in colonised individuals was higher than the non-colonised and this increase correlated with higher IL-17A and IL-17F levels and with antimicrobial properties shown in the saliva. Nasopharyngeal colonisation *of S. pneumoniae* is common in children and considered a prerequisite of diseases including otitis media, sinusitis, bacteraemia, and meningitis (Bogaert and Hermans, 2004; Van der Poll and Opal, 2009).

Earlier studies demonstrated an age-related carriage rate of *S. pneumoniae* with higher carriage in young children followed by a significant decrease of *S. pneumoniae* colonisation in older children and adults (Bogaert, et. al, 2004). Furthermore, Dukers-Muijrers, *et. al*, 2012, compared the nasal colonisation of both *S. aureus* and *S. pneumoniae* in day-care children's centres in the Netherlands. They found an interesting inverse correlation of the nasopharyngeal colonisation pattern of these two bacteria, with the two rarely co-exist. Another study by Bogaert, et. al, (2004) also showed a negative correlation between the bacterial carriage between *S. pneumoniae* and *S. aureus* and showed that in contrast to *S. aureus*, *S. pneumoniae* carriage had a peak prevalence in younger children. Therefore, the inverse association between these two colonising bacteria needs to be studied further by focusing on their interactions with consideration of the local immune response involved.

3.1.1 Aim of the study

The interactions between *S. aureus* or *S. pneumonia* carriage and the local immune responses in humans are not yet clear. We hypothesized that *S. aureus* stimulates a strong Th-17 response in the local mucosal immune tissue in the nasopharynx, i.e. NALT, which may affect bacterial carriage such as *S. pneumonia* in the nasopharynx.

In this chapter we aimed to study:

1. *S. aureus* and *S. pneumoniae* carriages in the nasopharynx of children and adults undergoing adenotonsillectomy, and their relationship to local salivary IL-17A levels.
2. The possibility of *S. aureus* stimulation to activate a strong Th-17 response in human NALT.

3.2 Methods

3.2.1 Experimental design:

The first step of this project was screening the carriage status of both *S. aureus* and *S. pneumoniae* using nasopharyngeal swab samples from our study population including children and adults. For this purpose, Nasopharyngeal swabs were cultured on 5% blood agar plates and bacterial colonies were then characterized depending on several morphological and biochemical unique features. In the next step, RT-PCR analysis was also used for the detection of *S. aureus* in swab samples by extracting the DNA and then detection of *fem* A gene within the extracted DNA samples. we proposed a theory that strong Th-17 response in NALT following *S. aureus* colonisation produced high levels of IL-17A locally in the nasopharynx, which may mediate an IL-17A-mediated bacterial clearance, thus reduction of bacterial carriage, i.e. *S. pneumonia*. To investigate that, we aimed to measure the local IL-17A levels in saliva samples and the correlation with the bacterial colonisation. Then as the last step to confirm whether *S. aureus* activates Th-17 response in NALT, isolated adenotonsillar MNCs were stimulated with *S. aureus* CCS followed by analysis of Th-17 response by flow cytometry and IL-17A production detected through the measurement of IL-17A in culture supernatants by ELISA.

3.2.2 Detection of bacterial carriage using nasopharyngeal swab samples

Nasopharyngeal swab samples were cultured on 5% blood agar plates (BAP) following a standardized procedure as described previously (O'Brien et al., 2003; Bae et al., 2012). Inoculated plates were incubated overnight at 37°C, in 5% CO2. Bacterial growth was examined for the general morphological characteristics and the haemolytic activities of the growth on blood agar. The density of the bacterial load was measured semi-quantitatively according to O'Brien *et al.*, (2003). Then, colonies of interest were purified by transferring a single isolated colony into a new blood agar plate to preform further identification with gram stain and selective biochemical tests (Bae *et al*., 2012).

3.2.2.1 Identification of *S. pneumonia* carriage in adults and children.

S. pneumoniae was identified by their unique colonies' morphology and haemolytic activity on a blood agar plate. Small α-haemolytic colonies which are 1-2mm at 24hr culture with a characteristic 'draughtsman' colonies shape. These colonies were subjected to optochin susceptibility tests. Optochin discs were applied onto the newly streaked bacteria. Then, the plates were incubated overnight at 37°C, in 5% CO2. Susceptibility was confirmed when the zone of inhibition was ≥14 mm in diameter. (Chandler, *et. al*, 2000).

3.2.2.2 Identification of *S. aureus* growth on blood agar plates

According to morphological identification of *S. aureus* on blood agar, colonies were 2-3mm in diameter, translucent glistening, smooth, raised colonies and typically produce golden pigment. The colonies were further stained by gram stain and examined under the microscope. they appeared gram-positive cocci and aggregated in distinctive grape-like clusters. In the next step, single colonies were picked to check by catalase test and coagulase tube test as described in the previous chapter 2. Staphylococcal colonies that were both catalase and coagulase-positive were considered *S. aureus.*

3.2.3 Identification of *S. aureus* by RT-PCR detection of femA gene in nasopharyngeal swab DNA samples.

3.2.3.1 DNA extraction from nasopharyngeal swabs

The extraction of bacterial DNA was performed using the QIAamp UCP Pathogen Mini (QIAgen, UK). Briefly, 400 µl of nasal swab samples suspended in transporting media was first mechanically hydrolysed using specific tubes with beads. Then, the samples were undergoing several treatment steps that included the addition of various solutions and alternative centrifugations. DNA samples were precipitated by ethanol and DNA eluted by adding 50–70 µl of AVE buffer into new clean 1.5 ml elution tubes. As a positive control in the RT-PCR reaction, a DNA sample from pure *S. aureus* strain was extracted. The DNA concentration in the samples was measured using Nanodrop and the ratio of 260/280 was measured

as the purity indicator of the extracted DNA. In the final step, the concentration of DNA samples was adjusted into 2ng/µl and then DNA samples were kept at -80°C to be used for RT-PCR.

3.2.3.2 Primers design

The primers were designed to amplify *femA* gene as a conservative gene described previously (Sabet *et. al,* 2007). Primers were validated by NCBI primer BLAST and with 20–80% of G/C ratio contents. The final amplicon size is less than 150 bp as intentionally primers were designed to amplify short segments within the target gene sequence.

3.2.3.3 RT-PCR reaction

To promote *femA* amplification, the QuantiFast Pathogen RT-PCR +IC Kit (QIAgen, UK) was used for this protocol. The DNA Internal Control (IC) was used to monitor the performance of RT-PCR amplification. The amplification mixture was prepared with a final volume of 10µl. Each test was performed in triplicates. Each RT-PCR run involved a positive control in addition to two other controls. The positive control was a purified DNA from *S. aureus* isolated strain. While the first negative control was including the IC DNA and the other was without the addition of IC DNA and both were containing DNA/RNA-free water as a replacement for DNA template. The implemented RT-PCR reaction conditions were programmed according to a protocol mentioned previously (Sabet et al., 2007). In this protocol, both the hybridization and extension steps were integrated into one annealing step.

3.2.3.4 The detection of RT-PCR products by agarose gel electrophoresis.

Following RT-PCR reaction, DNA containing samples were detected by 1% agarose gel electrophoresis. DNA samples were run in 1x TBE buffer (Thermo Fisher, UK) at constant 100v for 90-110 min. DNA bands were visualised by a ChemiDoc XR system (Bio-Rad, UK) using a protocol including a blue filter tray (figure 3.1).

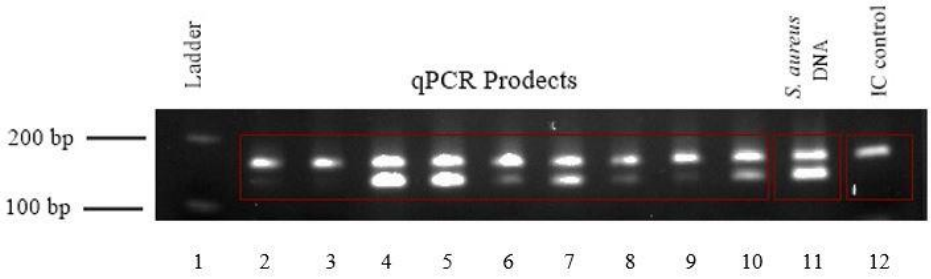


Figure 3.1: Gel electrophoresis of nasopharyngeal swabs' DNA samples after femA RT-PCR amplification. After DNA RT-PCR, DNA samples were detected by gel electrophoresis. The expected band size of 150 bp. The figure shows the DNA ladder in lane 1, RT-PCR amplification products of nasal swaps samples in lanes 2-10. *S. aureus* purified DNA as appositive control in lane 11 while internal control (IC) was as a successful amplification indicator is shown in lane 12.

3.2.4 Measurement of IL-17A concentrations in saliva samples

Local IL-17A concentrations were measured by a Human IL-17A hi sensitive ELISA kit (eBiosciences, UK) according to the manufacturer's instructions. Briefly, pre-coated strips were washed and 50 µl of sample diluent was added to the samples' wells, while 100µl were added to some wells which were used as blank controls. Then, (100µl/well) IL-17A standard dilutions were loaded to the slandered wells and samples (50µl/well) were added to the allocated wells, and then 100µl/well of Biotin conjugated antibodies were added to all the wells and the plate was incubated at 18-25°C overnight in the dark. The next day, the plate was washed six times and 100µl/well of the streptavidin HRP was added and the plate incubated for 60 min at 18-25°C in the dark on a plate shaker with 150 rpm. Following the incubation, the plate was washed 6 times and amplification I solution (100µl/well) was added and the plate incubated for 15 min in the same incubation condition mentioned in the previous step. After that, the plate was washed 6 times and Amplification II solution (100µl/well) was added and the plate incubated for 30 min in the same incubation conditions above. In the last step, the plate was washed and TMB substrate (100µl/well) was added, then the plate was incubated for 15-20 min without shaking. The reaction was stopped immediately by adding stop solution (100µl/ml) and the OD was measured at 450nm.

3.2.5 Stimulation of NALT MNCs with *S. aureus* CCS

S. aureus strains were used to produce CCSs. MRSA strain USA300 LAC (Wt) was kindly provided by Fabio Bagnoli (Bagnoli *et al*., 2015). CCS was produced as described previously and the final protein concentration was adjusted into 1µg/ml. Then, 4x106 cell/ ml of MNCs were stimulated with CCS and incubated for 72 hrs at 37Cº. After incubation, culture supernatants were collected and stored at -80Cº before being used for analysis by ELISA. Then, stimulated cells were harvested and stained for CD4 and IL-17A antibodies and finally analysed by flow cytometry.

3.2.6 Measurement of IL-17A in stimulated culture supernatants

For the measurement of IL-17A production from stimulated adenotonsillar MNC culture supernatant, IL-17A ELISA Ready-Set-Go® set (eBiosciences, UK) was used and the procedure was conducted according to the manufacturer's instructions.

3.2.7. Statistical analysis

Data were analysed using IBM SPSS statistics. First, the normality test was applied for the analysed data, and then differences between different groups were analysed by using the unpaired t-test. The differences between pre- and post-stimulation were analysed with paired t-test. Association between age groups and the bacterial colonisation was analysed by Chi-square ($\chi 2$) test. A *p-value* of <0.05 was taken as a level of statistical significance.

3.3 Results

3.3.1 Nasopharyngeal carriage rates and their association with age.

A total of 218 nasal swabs were analysed in this study from which 148 were from children (age <16 years, P0JUMean±SEM= 5.307±0.2955) and 70 were from adults (16-60 years, Mean±SEM =27.8±1.4288). *S. aureus* and pneumococcal carriages were initially assessed by culturing nasopharyngeal swab samples by observing culturable growth on blood agar plates. Notably, culture results on blood agar showed significantly lower culture-positive rate in adults' samples as compared to the children samples (figure 3.2).

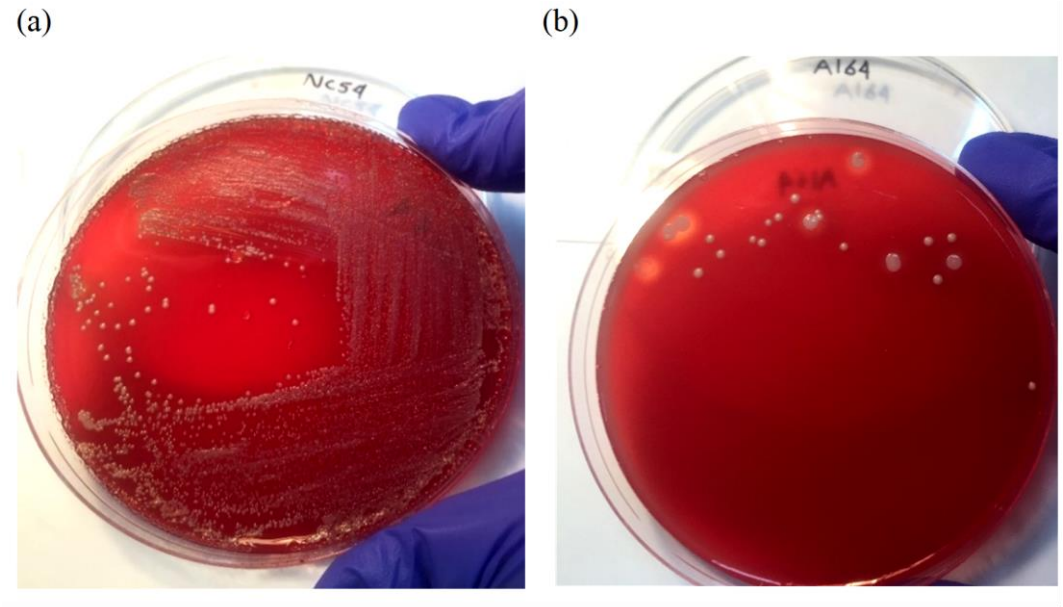


Figure 3.2: Representative culture plates showing the density of the bacterial carriage on blood agar plates. The figure represents an example of the bacterial density in the culture of nasopharyngeal swabs from children (a) and adults (b) when cultured on blood agar. The results showed a noticeable lower carriage density in adults' samples in comparison with carriage density in children.

To investigate whether there was any association between bacterial carriage in nasal swab and age, Chi-square ($\chi 2$) test was performed. Data analysis revealed another supporting evidence for our previous finding, as the results showed that there is a

correlation of decrease bacterial carriage in corresponding with age. (children n= 153, Adults n= 65, Pearson Chi-Square 11.364a, df=1. $p<0.001$) (figure 3.3).

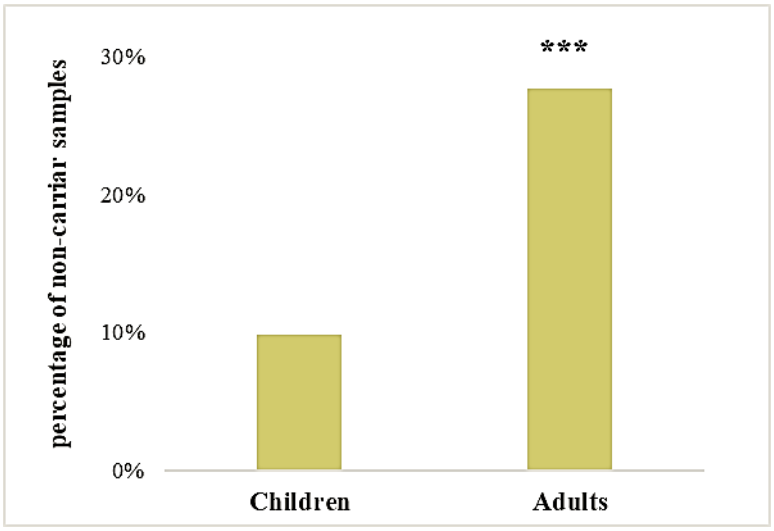


Figure 3.3: The association of bacterial non-carriers' percentage in nasal swab with age. The relationship between the absence of bacterial carriage and age was analysed by culturing nasal swab from children (n=153) and adults (n=65) on blood agar plates. Chi-square showed a remarked decrease in bacterial carriage associated with age. (Pearson Chi-Square 11.364a, df=1. ***$p<0.001$).

To study the bacterial carriage in nasal nasopharynges, Data sets were sub-grouped into several age groups. The summary of the carriage percentages per age groups was shown in table 3.1.

Table 3.1: The distribution of nasopharyngeal carriage of both *S. pneumoniae* (SP) and *S. aureus* (SA) in various age groups.

Age groups	**Nasal carriage: n (%)**				
	Carriage-ve	**SA**	**SP**	**Others**	**Total**
Young children (1-2 yrs.)	11(11%)	18 (18%)	28 (28%)	43 (43%)	100
Older children (3-16 yrs.)	3 (6%)	12 (25%)	11 (23%)	22 (46%)	48
Adults (>16 yrs.)	19 (27%)	16 (23%)	0 (0%)	35 (50%)	70
Total	33	46	39	100	218

3.3.2 *S. pneumoniae* carriage rates in nasal swabs and their association with age.

To investigate the nasopharyngeal carriage of *S. pneumoniae* in different age groups, three age groups were assigned as young children (1-2 yrs.), older children (3-16 yrs.) and adults (>16 yrs.). Results showed an age-related decrease in carriage rates and that the highest carriage ratio was observed in younger children (figure 3.4).

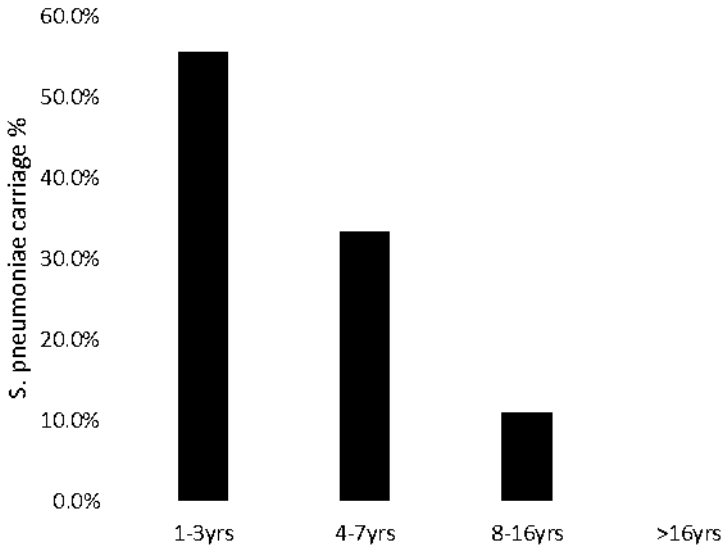


Figure 3.4: The distribution of *S. pneumoniae* carriage in nasal swabs within different age groups. The graph represents the percentages of *S. pneumoniae* carriage rates obtained from nasopharyngeal swab cultures in different age groups. In young children aged 1-3 yrs, the *S. pneumoniae* percentage was the highest and the carriage rates decreased with age (n=218).

3.3.3. *S. aureus* nasopharyngeal carriage rate in different age groups

The nasopharyngeal carriage rate of *S. aureus* was also analysed in different age groups using nasopharyngeal swab samples by both bacterial culture and RT-PCR method. Samples were considered as carriage positive when both culture and RT-PCR were shown positive.

DNA samples were extracted for nasal swabs form a total of 122 samples (children (n=85) and adults (n=37)). *S. aureus* carriage in nasal swabs was detected by RT-RT-PCR through the detection of *femA* gene amplification. Data analysed by

setting Ct values <30.9 cycles as a positive reaction. While Ct values above were considered negative. The carriage rates based on both *S. aureus* culture and RT-PCR results were presented in association with ages in figure (3.6). The age groups were defined as group 1=1-3 years old (yrs.), group2= 4-7 yrs., group 3 aged 8-16 yrs. and group 4 >16 yrs. As shown, the highest carriage rate was found in the older children group aged 8-16 yrs. (38.9%). While the lowest was in the youngest children group aged 1-3 yrs. (6.9%). $p<0.05$ (figure 3.5)

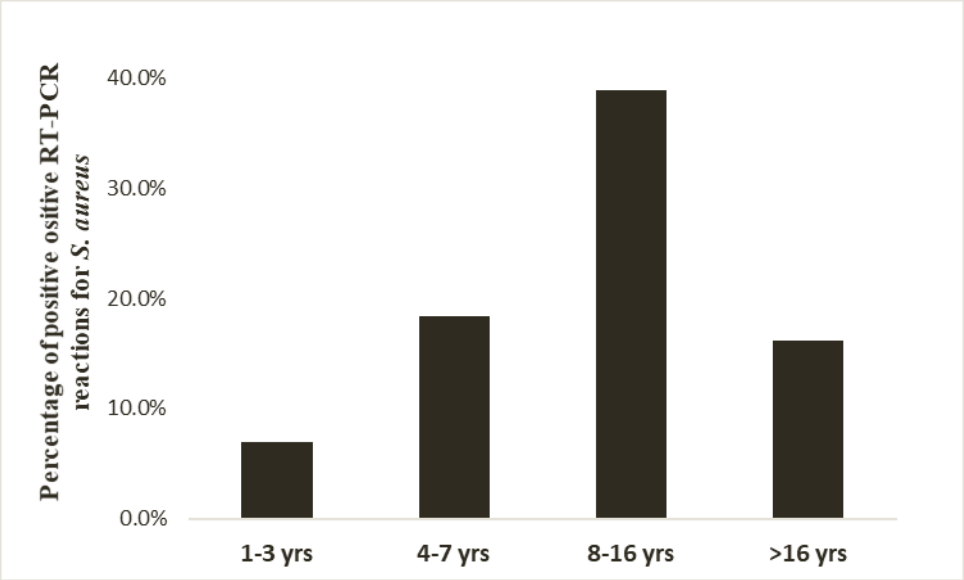


Figure 3.5: Association of *S. aureus* carriage rates in nasopharyngeal swab samples and age depending on femA RT-PCR. The bar chart shows the carriage rates of *S. aureus* in nasal swabs samples in different age groups. The result showed that the highest carriage rate in the children aged 8-16 yrs. While in younger children (aged 1-3 yrs.), the carriage rate was the lowest. (n=122, Pearson Chi-Square=7.817a, df= 3 and $p<0.05$).

3.3.4 Relationships between salivary IL-17A levels and nasopharyngeal carriages of *S. aureus* and *S. pneumoniae*.

The possible relationships between the salivary IL-17A levels and the nasopharyngeal carriages of *S. aureus* and *S. pneumoniae* were analysed in children. The carriage rate of *S. pneumoniae* in adult samples was very low, so it was not studied in this aspect. First, we measured the silvery IL-17A concentrations (pg/ml) in saliva samples from the patients underwent tonsillectomy. Then, salivary IL-17A levels were analysed in association with both *S. aureus* and *S. pneumoniae* carriage status. As can be seen in Figure 3.7, children who were *S. aureus* carriage positive had a significantly higher salivary IL-17A concentration compared to those with *S. pneumoniae* but without *S. aureus*, and those without any culturable growth (carriage negative) (figure 3.7, $p<0.001$, $p<0.001$ respectively, ANOVA following log transformation to normalise data distribution). There is a trend to show a modestly higher salivary IL-17A concentration in children who were *S. pneumoniae* positive but without *S. aureus* (9.14±2.23, n=12) than the carriage negative group (5.58±3.59, n=4), although the difference was not statistically significant (figure3.6).

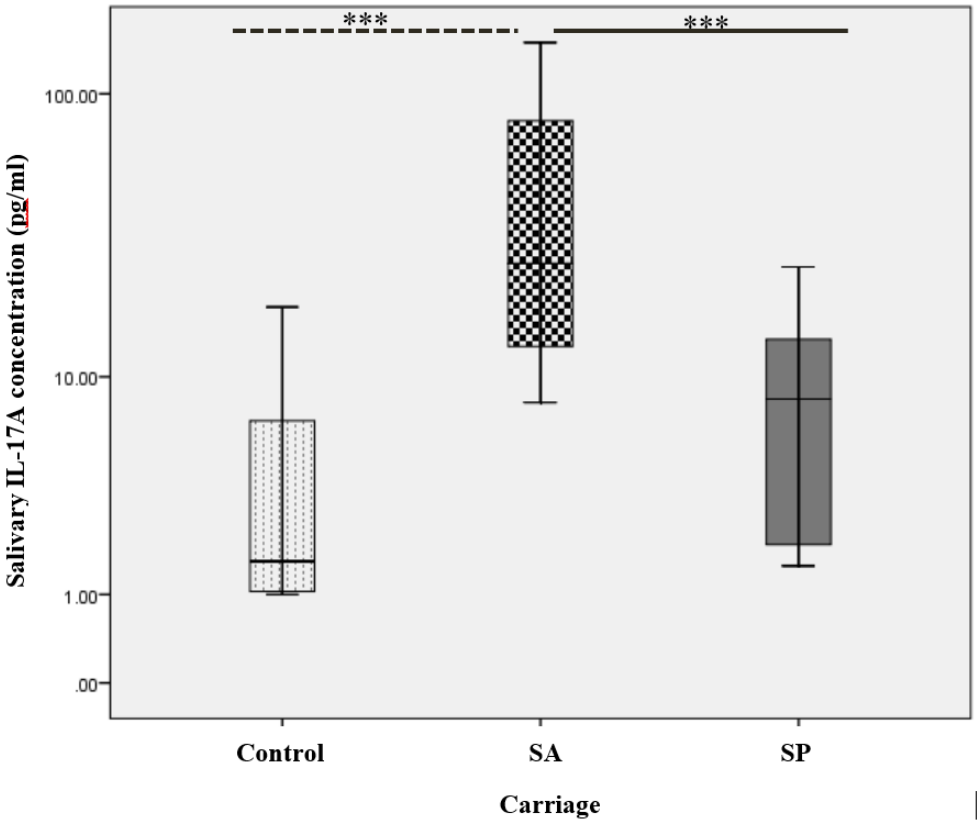


Figure 3.6: The relationship between the salivary IL-17A concentrations (pg/ml) and bacterial carriages in the nasopharynx. The figure represents salivary IL-17A concentrations and correlations with nasopharyngeal *S. aureus* (SA) and *S. pneumoniae* (SP) carriages as compared to the carriage negative (control) group. A significantly higher level in IL-17A concentration was shown in SA group (n=8) than both SP group (n=12) ($p<0.001$) and the control group (n=4) ($p<0.001$). No significant differences were found between SP and control with IL-17A salivary concentrations. (ANOVA following log transformation. Medians with interquartile range are shown).

3.3.5 *S. aureus* CCS stimulates high Th-17 response in NALT tissue.

To study the effect of *S. aureus* on Th-17 response in NALT tissue, we used *S. aureus* culture supernatant (CCS) to stimulate the NALT MNCs. Following stimulation, MNCs were stained with CD4 and IL-17A antibodies as previously described followed by FACS analysis. A representative gating strategy was shown in figure 3.7. The mean frequencies of Th-17 cells upon stimulation was analysed,

and results showed *S. aureus* CCS strain stimulate significant Th-17 response as compared with unstimulated control (n=6) (Wilcoxon signed ranks test, $p<0.05$) (figure 3.8 a and b).

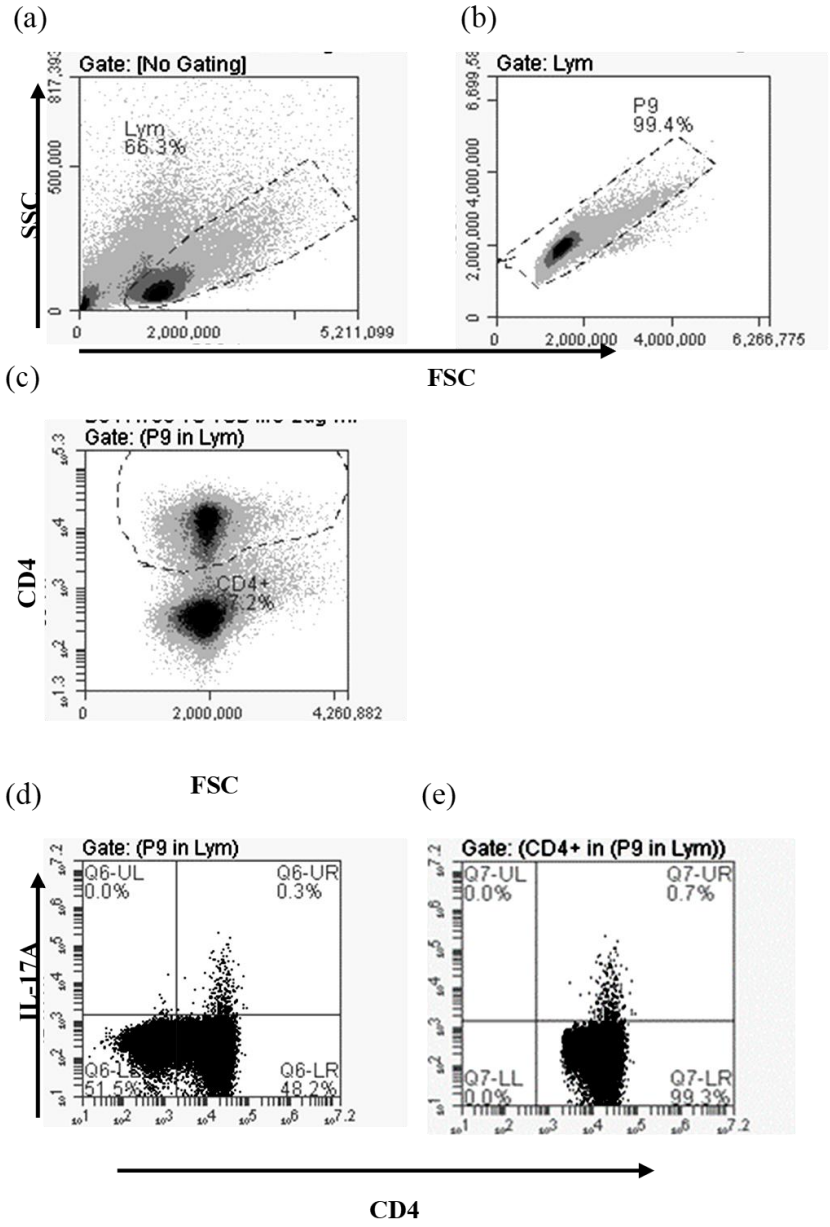
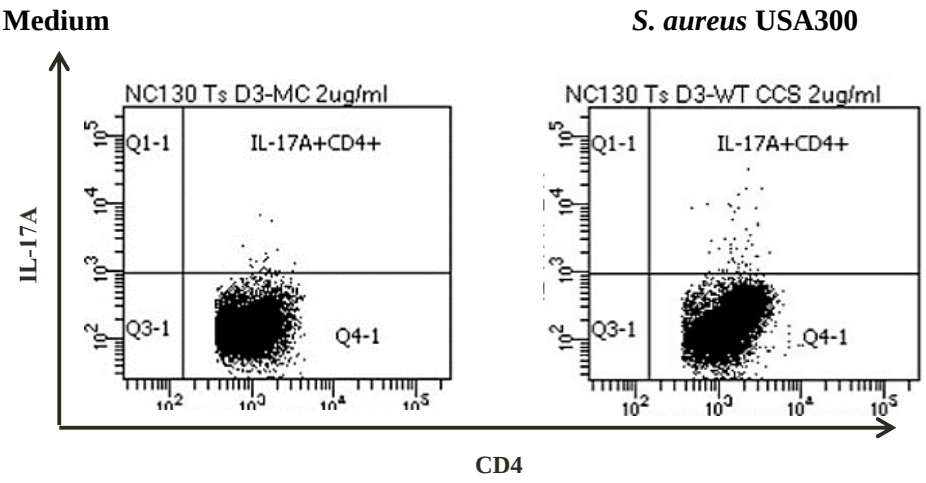


Figure 3.7: Representative FACS plots showing the gating strategy for identification of Th-17 (CD4+ IL-17A+) in tonsillar MNC. Freshly isolated tonsillar MNC were analysed and gated by typical FSC and SSC lymphocytes (a). Then, singlet cells were gated (b). CD4+ T cells were identified by gating CD4+ T cells (c). The percentage of Th-17 cells was calculated as the frequencies of IL-17A+ cells in lymphocyte (d) or CD4+ T cell population (e).

(a)



(b)

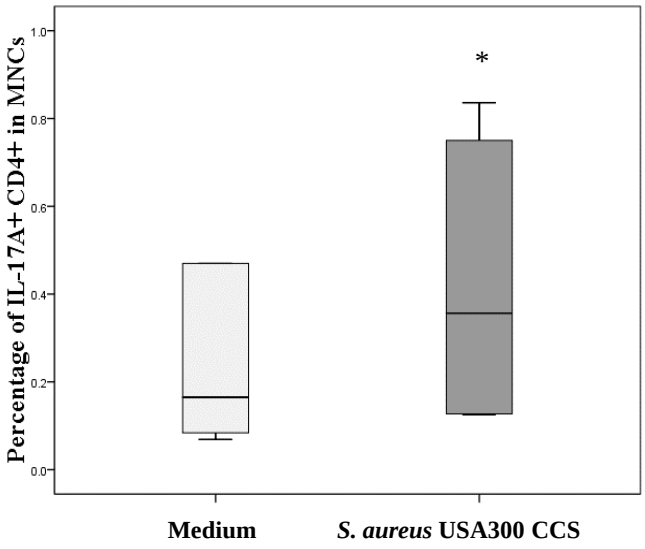


Figure 3.8: Th-17 response in tonsillar cells after *S. aureus* CCS stimulation. Freshly isolated tonsillar MNC were stimulated with *S. aureus* USA300 CCS (2µg/ml). After 3 days, cells were analysed by FACS and the increase of CD4+IL-17A+ % was calculated as compared to the medium. Th-17 cells significantly increased following USA300 CCS stimulation as shown in the representative flow cytometry plots for one sample (a) and in Wilcoxon Signed ranks analysis (b). (n=6, **P*< 0.05). Medians with interquartile range are shown.

3.4 Discussion

Nasopharyngeal colonisation with common bacteria like *S. aureus* and *S. pneumoniae* may predispose to serious illnesses such as pneumonia and meningitis. Local colonisation/infection is generally controlled by the local immune response including the CD4+T cells, such as TH-17 cells in the elimination of the colonising bacteria.

Recent studies highlighted the importance of Th-17 cells in the clearance of infection (Kolles et al, 2004; Matsuzaki, and Umemura, 2007), including both *S. pneumonia* and *S. aureus.* How the local immunity works against the bacterial carriage in human nasopharynx is poorly understood. To examine the interactions and the relationship between of the bacterial carriage and the local Th-17 cell response we first examined the carriage rate of both *S. aureus* and *S. pneumoniae* in nasopharyngeal swab samples collected from children and adults undergoing adenotonsillectomy. The carriage results were broadly in line with the previous publications (Bogaert et al., 2004; etc) In general, using blood agar culture, the total bacterial growth in the swab samples from children were significantly higher than in adults. This may reflect the gradually developed immunity with age from childhood to adulthood.

Pneumococcal culture results showed that the carriage rate was the highest in younger children aged 1-2 years, and then decreased in older children. In adults, the *S. pneumoniae* carriage rate was very low (figure 3.4). These results again were by previous studies demonstrating younger children had significantly higher carriage in comparison with older age groups (Bae, Yu *et al*. 2012, Bogaert et al., 2004, Farjo *et al.*, 2004, Grant et al, 2009, Gunnarsson et al., 1998). This age-associated decrease in *S. pneumoniae* carriage rate may be related to the development of Th-17 cell immunity.

To determine the *S. aureus* carriage status, we used both bacterial culture and RT-PCR methods to ensure the sensitivity and specificity and to minimise false positivity. Overall, the carriage rate of *S. aureus* was lowest in young children (6.9% in 1-3 years) and gradually increase with age in older children (38.9% in age 8-16 yrs.), and in adults, the carriage rate appeared to drop a little (figure 3.5 and figure 3.6). It is worth to note that that the sample size was not big enough to study in detail the carriage rate, rather find a general trend and to examine the relationship between the carriage and Th-17 response.

Our data above on *S. pneumonia and S. aureus* showed a similar pattern to that shown in previous studies in Netherland which showed *S. pneumoniae* carriage rate decreased with age and *S. aureus* carriage increased with age (Bogaert *et al*., 2004). Another study conducted by Bae *et al*, (2012) showed the comparison of the common colonisers of the nasopharynx between pre-school and elementary school children, and they found that the carriage rates of *S. pneumoniae* with *M. catarrhalis* decreased in elementary school children while the opposite was observed *S. aureus* carriage rate. These distinctive patterns of carriages or inverse relationships obtained from those two common bacteria (*S. pneumoniae* vs *S. aureus)* suggest possible interactions between the two bacterial species and/or the involvement of host immune response which helps to clear a carriage. It was thought the anti-bacterial activity of hydrogen H2O2 from *S. pneumoniae* could be involved in the inhibition of *S. aureus* (Pericone *et al*., 2000). However, neither the H2O2 secretion by *S. pneumoniae* nor the H2O2 sensitivity of *S. aureus* showed to be consistent with the co-colonisation patterns found in children (Regev-Yochay et al., 2004 and Melles et al., 2008). Therefore, the inverse relationship between these nasopharynx colonisers (*S. pneumoniae* vs *S. aureus*) could not be explained by this interaction.

The host immune responses induced by the carriage(s) may influence the association of these bacterial species in the nasopharynx. Margolis *et al*., (2009) suggested that the different colonisation pattern between *S. pneumonia* and *S. aureus* could be linked to the host immunological factors. Interestingly, the inverse association between *S. aureus and S. pneumoniae* carriage was not seen in HIV infected patients, suggesting that host CD4+ T cell immunity may contribute to the

observed negative association in the healthy population (McNally, 2006; Madhi et al., 2007).

This study focused on the investigation of local Th-17 response in correlation to bacterial colonisation. Our results on comparing the salivary IL-17A levels between *S. aureus*+, *S. pneumoniae*+, and carriage-free (culture-ve) samples showed a significantly higher level of IL-17A in *S. aureus* positive samples compared to *S. pneumoniae*+ and culture-negative samples. Furthermore, IL-17A levels in *S. pneumoniae* positive saliva samples also tended to be higher than the culture-negative group samples although it was not statistically significant (Figure 3.7), possibly due to the relatively small number of samples analysed. Our results suggest a relationship between the local IL-17A and the clearance of bacterial carriage in the nasopharynx. On mice experiment conducted by Archer *et al,* (2016), they showed a direct role of the IL-17A/IL-17F with the clearance of *S. aureus*. Mice deficient with IL-17A and IL-17F were unable to clear *S. aureus* nasopharyngeal infection. In addition, when testing the nasal secretions, they found an anti-*S. aureus* activity in the infected mice. The observed antimicrobial activity might be due to the antimicrobial peptides induced by the stimulation of IL-17A and IL-17F production upon the exposure to *S. aureus* in vivo (Zanger and Krishnea, 2012).

On the other hand, previous studies suggest *S. pneumoniae* could activate CD4+ T cell response including Th-17 cells and also T regulatory cells (Zhang *et al*, 2009). Some of *S. pneumoniae* proteins elicited IL-17A along with the stimulation of Treg in children (Mubarak *et al*, 2016). It was shown that the number of Treg in children was generally higher than in adults and the ratio of Treg/Th-17 cells was also higher in children and decreased with age (Mubarak *et al*, 2016). The results of salivary IL-17A concentrations shown in this study suggest *S. pneumoniae* carriage is associated with a small increase in IL-17A level than culture-negative samples. The carriage of *S. pneumoniae* may elicit a more modest Th-17A response as compared to *S. aureus*, and at the same time, it activates a regulatory immune response (e.g. Trg cells) which regulate the inflammatory Th-17 response. In contrast, *S. aureus* colonisation stimulates a more robust and potent Th-17 response, particularly in older children and adults.

Following the observation for our results on the salivary IL-17A levels, we further investigated the ability of *S. aureus* culture extract (CCS) to activate the Th-17 response in NALT tissue. The results demonstrated that *S. aureus* CCS stimulated a strong increase in Th-17 response analysed by flow-cytometry which was confirmed by the increase in IL-17A concentration measured by ELISA in the culture supernatants collected from the stimulated cells (figure 3.9 and figure 3.10). These results support our hypothesis that *S. aureus* activates a strong Th-17 response in NALT, which is consistent with the higher IL-17A levels in the saliva samples in *S. aureus* carriage positive subjects shown in this study.

In conclusion, nasopharyngeal carriage of *S pneumoniae* usually established in early childhood when adaptive immunity including T cell immunity is not fully mature and T regulatory cell activity helps to maintain the carriage state. Colonisation of *S. aureus* may activate a more potent T cell response including Th-17 cells whereas T regulatory cell response may be defective, and that will lead to a stronger local Th-17 response with a higher IL-17A cytokine levels in local secretions which may have a significant impact on the bacterial carriage status including *S. pneumoniae* in the nasopharynx.

Chapter 4

Activation Of Th-17 Response In Adenotonsillar Cells By *S. aureus* Proteins

Chapter four: Activation of Th-17 response in adenotonsillar cells by ***S. aureus*** proteins

4.1 Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common bacterial colonisations with a wide distribution in humans. Colonization profile that ranges from a harmless commensal bacterial residency to more serious, life-threatening tissue infections and invasive diseases (Lowy, 1998). Colonization with *S. aureus* may cause a health burden and associated with high-risk in individuals with compromised or weak immune functions (Kluytmans *et al.*, 1997), such as cases of diabetes (Tuazon *et al.*, 1975) and HIV infections (Wienke *et al.*, 1992).

S. aureus infection, particularly that caused by *MRSA* (Methicillin-resistant *S. aureus*) strains cost a substantial amount of money to public health authorities throughout the world. Despite this, there are currently no licensed vaccines against staphylococcal infection. Understanding the protective immunity against staphylococcal colonisation or carriage in humans will inform the development of novel vaccines against *S. aureus* infection.

Most of *S. aureus* infections are endogenous and believed to be sourced from nasal colonized strains of *S. aureus*, as the human mucosal tissue especially nares are a reservoir for this bacterium. In nasal tissue, *S. aureus* colonises around 20-80% of the human population (Kluytmans *et. al*, 1997, Zhang *et. al*, 2018). Surveillance studies in the USA showed that *S. aureus* nasal colonization was affecting approximately one-third of the population (Gorwitz, *et. al.* 2008). Genotyping analysis indicated that about 80% *S. aureus* bacteraemia is caused by the identical nasal recovered strains from the same individuals (von Eiff *et. al*, 2001).

Many studies demonstrated an age-associated increase in nasal carriage rate of *S. aureus* (Bogaert *et. al*, 2004). Young children in pre-school and elementary schools were shown to have lower carriage rate of *S. aureus* compared to older children (Bae *et. al*, 2012).

The mechanisms by which the host clear *S. aureus* nasal carriage or colonisation is still not well defined. Cellular immunity including Th-17, a subset of CD4+ T cells has been implicated to be important in clearance of infection. The role of Th-17 in bacterial infections usually involve the secretion of effector cytokines IL-17A and

IL-17F and in their turn, this response directed the other cells of the immune system to respond by attracting neutrophils to the site of infection (Matsuzaki, and Umemura, 2007) and the stimulation of antibacterial mediators production by local epithelial cell (Kolls and Linden 2004; Weaver *et al.* 2007). Studies in murine models showed that in the absence of IL-17A and IL-17F, mice lost their ability to clear nasal colonisation (Archer *et al,* 2013, Archer *et al,* 2016). The same study found that in humans, the clearance of *S. aureus* carriage was associated with the antimicrobial peptides in nasal secretions. These peptides were produced due to the local *S. aureus* colonisations as they were found to be absent in the non-colonised individuals (Archer *et al.,* 2016).

Invasiveness of *S. aureus* infections is mainly related to its secretion of a wide range of virulence factors (Lowy, 1998). Those include extracellular toxins, such as toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins, haemolysins (α-, β-, γ-, δ-toxins) and tissue lytic enzymes as proteinases, etc. In addition to the extracellular secretions, *S. aureus* possesses various cell wall proteins with broad effects on different types of human cells. These factors elicit both innate and adaptive immune responses in the affected host (Zhang *et. al*, 2018). Although, some of these virulence factors has been studied previously along with their potential protective properties in murine models against *S. aureus* carriage by both active and passive immunisation (Narita *et. al,* 2010; Frank, *et. al,* 2012, Brady *et al,* 2013 and Mocca, *et al,* 2014), their role in eliciting Th-17 response in human nasopharynx-associated lymphoid tissue (NALT) was not fully explored.

One of these interesting proteins is Hla toxin, a pore-forming protein of *S. aureus*, is an essential virulence factor in both the invasiveness and adherence to host tissue (Popov *et al*. 2015). Several studies investigated the immune response induced by Hla toxin and its' derivative HlaH35L, a recombinant non-toxic mutant. It has been shown that immunization with HlaH35L elicited a protective antibody response in mouse-model (Mocca, *et al*, 2014). In addition to the antibody-mediated response, T cells response was also investigated in mouse models. In these studies, a comparison between wild type strain and its Hla mutant (Hla-) showed a stronger Th-17 response by the wild type in lung infection compared to its mutant (Frank, et al., 2012).

The immune mechanism for the clearance of the nosocomial microbial infection has been suggested to be orchestrated by Th-17 cells. Antigen-specific CD4+ Th-17 is derived from naïve Th cells upon the exposure to some specific antigens through independent pathways distinct from Th-1 and Th-2 and depending on Th-17polarzing cytokines (Dong, 2011).

The mechanisms by Th-17 cells against infection is orchestrated by the production of proinflammatory cytokines- including IL-17A which mediates activation of neutrophil-killing of bacteria and the stimulating of antibacterial peptides production from the epithelial cells (Kolls and Linden 2004; Matsuzaki, and Umemura, 2007; Weaver et al., 2007). Th-17 also produce IL-22, a cytokine which may play an important role to regulate the production of the antibacterial peptides and control *S. aureus* nosocomial infection. The participation of IL-22 against *S. aureus* infection has been supported in IL-22 deficient mice that showed an extended lesion of infection in the absence of IL-22 (Mulcahy *et al.*, 2016).

Considering the critical role of Th-17/IL-17A in the nasopharyngeal clearance of bacterial carriage, we have studied the capacity of *S. aureus* protein antigens to activate Th-17 response in nasopharynx-associated lymphoid tissue. The results from the study will provide valuable information for a novel protection approach against *S. aureus* infection.

4.1.1 Aim of the study

In this chapter, we aimed to investigate the capacity of Th-17 cells activation by several *S. aureus* recombinant proteins antigens in NALT of children and adults, including:

- Screening the effect of *S. aureus* antigens on Th-17 stimulation and whether they elicit Th-17 cytokine production in NALT.
- Following screening and selection of Th-17 activators, cytokines profile stimulated by certain *S. aureus* antigens (HlaH35L) will be studied.
- The effect of Hla on Th-17 cell activation by comparing *S. aureus* CCS from a wild-type (Hla-producing strain) with a non-Hla-producing mutant strain.

4.2. Experimental design

To study whether *S. aureus* protein antigens activate CD4+ T cell proliferation and Th-17 response, freshly isolated tonsillar MNC were pre-labelled with CFSE and stimulated with *S. aureus* antigens for 5-6 days followed by flow cytometry for T cell proliferation. Th-17 cell activation following antigen stimulation was examined by intracellular IL-17 staining and flow cytometry. Culture supernatants of the antigen-stimulated MNC were collected according to the experiment requirements at 24, 48 as well as 72 hr post-incubation and analysed for cytokines concentrations by ELISA and CBA array.

4.2.1 Samples and Human subjects

For this study, tonsillar samples were collected from immune-competent subjects undergoing tonsillectomy from children and adults. Patients who were on antibiotics within 3 weeks of the operation or had severely inflamed tonsillar tissues excluded from the study. Informed consent was obtained in each case before being enrolled in the study.

4.2.2 Purified recombinant *S. aureus* Proteins

Nine recombinant proteins (IsdA, ClfB, SdrC, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A) of both conservative structural and secreted proteins were used as antigen stimulants for Adenotonsillar cells (obtained from Novartis vaccine, Sienna, Italy).

4.2.3 Stimulation of MNCs with the purified *S. aureus* proteins antigens

Nine recombinant proteins (IsdA, ClfB, SdrC, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A) were used to stimulate adenotonsillar cells to screen their potency of activating CD4+ Th-cell proliferation and Th-17 cell activation. For dose optimization of stimulation, several concentrations were used for stimulation of the cells (0.5-10 µg/ml) of each protein. Optimum concentrations for cell stimulation were 10 µg/ml. Adenotonsillar cells were stimulated by recombinant proteins and activation of Th-17 was detected by flow cytometry along with cytokine detection by ELISA.

4.2.4 Concentrated culture supernatant (CCS) of *S. aureus*.

S. aureus strains CCS were prepared from three strains included the MRSA strain USA300 LAC (Wt) (Bagnoli *et al*., 2015) and its isogenic mutant strain hla: ermB (Hla-) (Popov *et al.*, 2015).
S. aureus CCS from the above three strains were produced following the method described previously (Bagnoli *et al*., 2015). At the first step, serial dilutions of CCS preparation of each strain (1 – 100 ng/ml) were used to optimize the CCS concentration for adenotonsillar cell stimulation and 20ng/ml was used as the concentration to be used as a stimulant for studying the effect of CCS on Th-17 cells.

4.2.5 Adenotonsillar cells CFSE labelling for monitoring cell proliferation

CFSE labelling of adenotonsillar cells was performed to stain 5-6x107 of adenotonsillar cells. Cells were washed twice with sterile PBS followed by centrifugation. Cell pellets were then re-suspended with PBS and incubated with CFSE solution for 8 min. The reaction was quenched using 10ml of ice-cold RPMI-1640 and the cells were centrifuged, re-suspended then adjusted into 4x106 cells/ml to be ready for stimulation.

4.2.5.1 Detection of CD4+ T cells proliferation

Pre-labelled adenotonsillar cells with CFSE were stimulated with 10µg/ml *S. aureus* recombinant proteins and incubated for 5 days at 37ºC in the presence of CO2 (5%). After harvesting, cells were stained with mouse anti-human CD3-APC, and mouse anti-human phycoerythrin CD4 (PE) antibodies then T-helper cell proliferation was analysed by flow cytometry (BD FACS Calibre, BD).

4.2.6 Stimulation of adenotonsillar MNC with *S. aureus* CCS and purified proteins

Adenotonsillar cells were isolated by Ficoll and then adjusted to 4*106 cell/ml by RPMI-1640. After the cells being prepared, the cells were stimulated with different stimulants as detailed below and according to the experimental requirements. For the CCS experiment, tonsillar MNC were stimulated with the prepared *S.*

aureus CCSs (20ng/ml) or purified protein antigens (10µg/ml). Cells were incubated for 3 days at 37ºC in the presence of CO2 (5%). After stimulation, the culture supernatants were collected and stored at -70 ºC for measuring the cytokines production by ELISA. Cells were re-suspended with RPMI-1640 and incubated for 6hr with the addition of BFA to immobilize the intracellular cytokines inside the cells. Finally, cells were collected with 0.05% BSA-PBS to be stained for intracellular cytokines.

4.2.6.1 Intracellular staining for detection of Th-17 cells.

Following cells harvesting, cells were first stained for the surface markers as described previously and followed by intracellular staining. Cells were fixed and/ permeabilized using fixation/ Permeabilization buffer (eBiosciences, UK) then stained with mouse anti-human IL-17A-PE or APC conjugated (BD Biosciences, UK) or mouse anti-human- IL-22-APC (eBiosciences, UK) antibodies, incubated for 60min in a dark place at RT, washed and then re-suspended with 0.5 ml of FACS buffer. Cells were kept in 4°C until analysed by flow cytometry (FACS Calibur, BD Celesta or BD ACCURI; BD Biosciences).
During cell acquisition, lymphocytes were first gated from CD3+/CD4+ T-lymphocytes and then the percentage of Th-17 was determined as the number of the IL-17A in the CD4+ T cell gate. Flow cytometry data were analysed using WinMDI, BD Accuri and/or FACS Diva software depending on the experimental setting at the time of the experiments.

4.2.6.2 Measurement of Th-17 cytokines (IL-17A, and IL-22) production in adenotonsillar MNC by ELISA

In vitro production of Th-17 cytokines (IL-17A, IL-17F and IL-22) in adenotonsillar MNC were measured by human IL-17A, and IL-22 Ready-Set-Go® sets (eBiosciences, UK). First, ELISA plates were coated with capture antibody solution and incubated overnight at 4°C. Then, plates were washed 5 times with PBS-T and blocked for 1hr. Following blocking, both samples and standards were added to the plate, then the plate was incubated overnight at 4°C to maximize the sensitivity. After washing the plate, the detection antibody solution was added to the plate and incubated for 60 min at RT. Plates were washed and incubated with

the HRP-conjugated antibodies for 15 min before the reaction being stopped by 50µl/well of stop solution (2N H_2SO_4). ODs were measured at 450nm (Opsys MR, Thermolabsystems, UK) and cytokines concentrations (pg/ml) were calculated against the standard curve. Data were analysed by microplate software Deltasoft PC (Biometallics, Inc. USA).

4.2.6.3 Measurement of Th-1/Th-2 and Th-17 cytokines profile from adenotonsillar MNC by CBA.

T helper subsets cytokines profiles were analysed by measuring the level of 13 different signature cytokines including Th-1, Th-2, Th-17 subsets by using Legendplex Human Th Cytokine Panel (13-plex) (Biolegend, UK). Culture supernatants at 72hr of culture were used for this purpose. Test mixture was prepared by adding 25 µl of assay buffer to all tubes, an equal volume of each of the standards and samples were added separately to each tube. Thoroughly mixed beads and detection antibodies were added to all tubes. Mixture incubated in the dark on a plate shaker for 2hrs at RT. Next, without washing, streptavidin-PE conjugated antibodies were added, and the tubes were incubated at the same conditions for 30 min. At the end of the incubation, the beads were spanned down, washed and re-suspended with 400µl of the washing buffer to be analysed by flow cytometry.

4.2.7 Statistical analysis

Normality test of data was performed first and then the differences between two groups were analysed accordingly. Normally distributed data were analysed by paired t (parametric) test. Non-normally distributed data were analysed by Wilcoxon signed-rank test. The results were considered statistically significant with a *p-value* of < 0.05.

4.3 Results

4.3.1 CD4+ T cell proliferative response following stimulation by *S. aureus* protein antigens

To determine if *S. aureus* derived protein antigens (IsdA, ClfB, SdrC, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A) do activate CD4+ T cell response, CFSE labelled adenotonsillar MNC were stimulated with the nine recombinant proteins respectively (10µg/ml) for 5 days. We used anti-CD3 antibodies or *S. aureus* CCS (5µg/ml) as a potent CD4+ Th cells activator (positive control). Cells were collected and analysed by flow cytometry. Fifty thousand events were collected from each sample. The gating strategy for the CD4+Th cell proliferation analysis is indicated in figure 4.1.

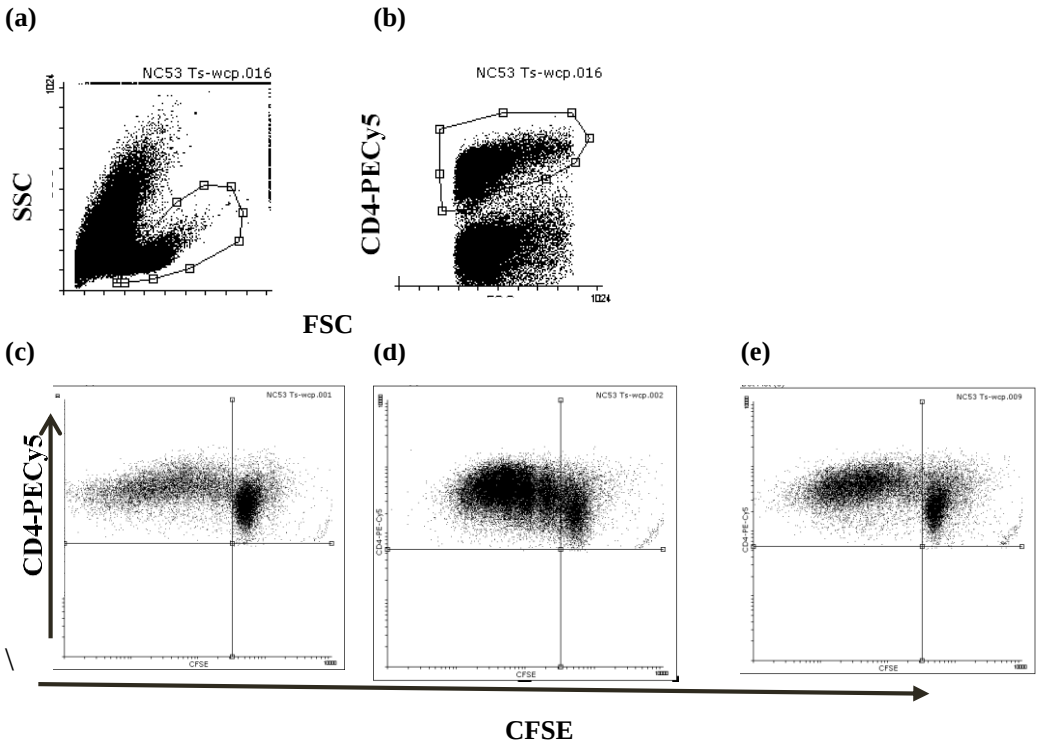


Figure 4.1: The gating strategy for analysis of CD4+ T cell proliferation.

CFSE-labelled MNC were stimulated with *S. aureus* recombinant proteins for five days. Lymphocytes gated first based on forward (FSC) and side scatter (SSC) characteristics (a). Within lymphocytes, CD4+ Th cells were then gated (b). The CD4+ T cell proliferation index was calculated as the percentage of the proliferating cells ($CFSE^{dim}$) in total CD4+ T cells in MNCs. The figure shows the differences between unstimulated (c), stimulated adenotonsillar MNC with *S. aureus* CCS (as positive control) (d) and HlaH35L (10µg/ml) (e).

Following analysis of variance and paired t-test, results showed that there is a significant and consistent CD4+ T cell proliferative response after five days of stimulation with HlaH35L (12.12±0.68) among all other recombinant proteins used compared to control group (8.64±0.30) (Figure 4.2, n=7, $p<0.01$). Among other antigens, ClfB also showed a significant increase in CD4+ T cell proliferative response (10.19±0.50) (Figure 4.2, n=7, $p<0.05$). There was no significant CD4+ T cell proliferative response following stimulation by the other protein antigens tested. As HlaH35L antigen-induced the highest response, we analysed this protein antigen in more details in subsequent experiments.

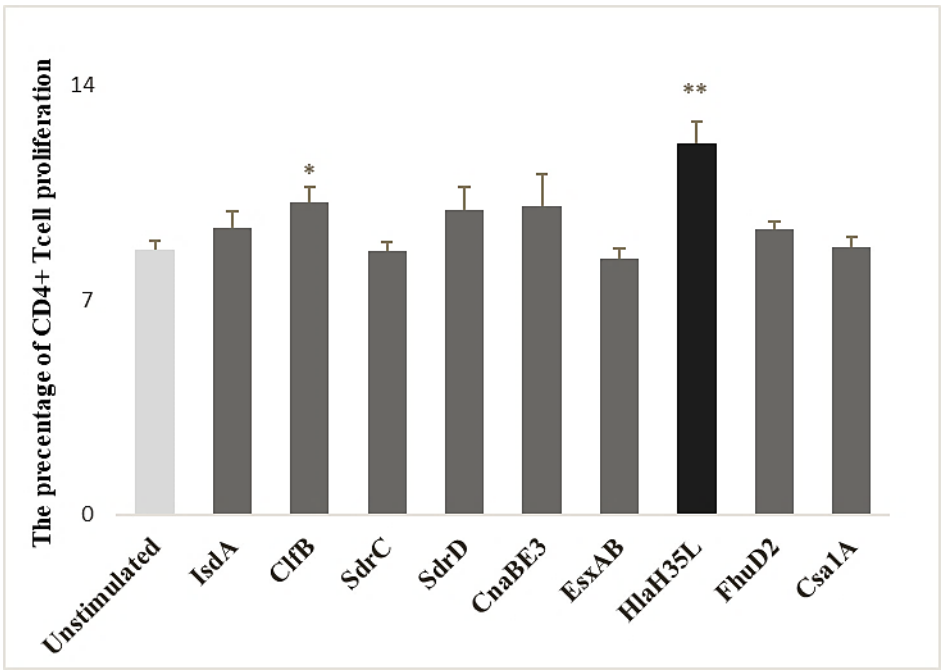


Figure 4.2: CD4+ T cell proliferative response in adenotonsillar MNC after stimulation with recombinant *S. aureus* proteins antigens. The figure shows the means of the CD4+ T cell proliferation index % after stimulation of tonsillar MNCs with *S. aureus* proteins (IsdA, ClfB, SdrC, SdrD, CnaBE3, EsxAB, HlaH35L, FhuD2 and Csa1A respectively) compared to unstimulated control (n=7). Mean±SEM shown. *= $p<0.05$, ** $p<0.01$

4.3.2 HlaH35L recombinant protein induce CD4+ T cell proliferation in adenotonsillar MNC

To determine whether HlaH35L induce naive T cell proliferation, adenotonsillar MNC were first depleted of memory (CD45RO+) T cells which contain Th-17. Then, CD45RO- MNC were labelled with CFSE and adjusted at 4x106 cell/ml then stimulated with HlaH35L and incubated for 5 days in 37Cº.

Results showed that HlaH35L induced a modest proliferation of naive CD4+ T cells population compared to the control group (Figure 4.3, n=5, $p<0.05$).

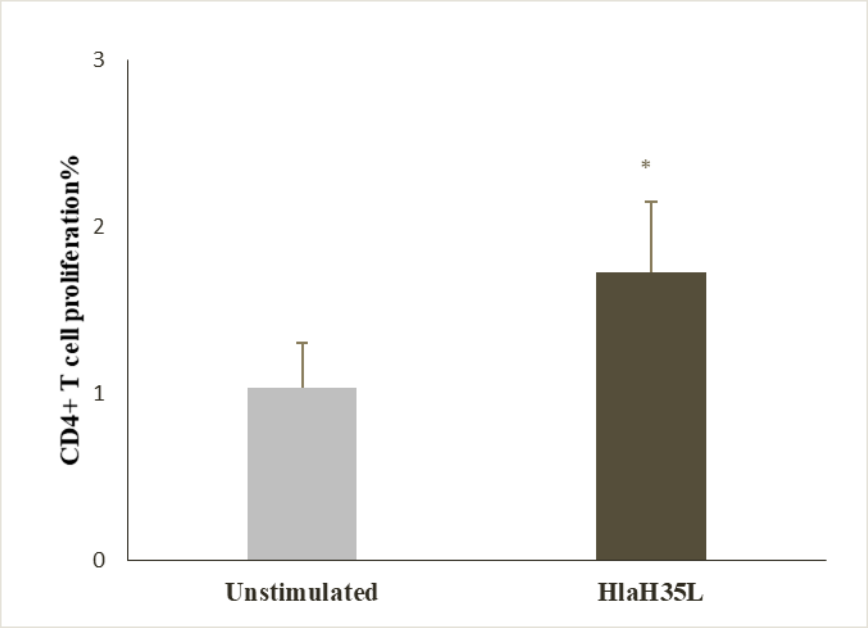


Figure 4.3: Naïve CD4+ T cells (CD45RO-) proliferation response in adenotonsillar MNC after stimulation with HlaH35L. In the figure, the proliferation index of CD4+ CD45RO- Th cells from HlaH35L stimulated cells compared with unstimulated control and it shows HlaH35L induced a proliferative CD4+ T cell response in CD45RO- MNC compared to the control group (n=5, *= p<0.05). Mean±SEM shown.

4.3.3 Activation of Th-17 cells by *S. aureus* protein antigens

Having shown the CD4+ T cell response, we further determined whether *S. aureus* protein antigens activate Th-17 cells. Adenotonsillar MNCs were stimulated with purified *S. aureus* recombinant proteins for 3 days. At the last 6 hours of incubation BFA was added to all cells along with PMA/Ionomycin. At the end of the incubation, cells were collected and stained for CD4, then fixed and permeabilized for IL-17A staining. As shown in figure 4.5, HlaH35L stimulation activated a marked Th-17 response in adenotonsillar MNC (n=18 * $p<0.01$). There is no significant increase in CD4+ IL-17A+ response after stimulation by other protein antigens (data not shown). Figure 4.4 showed a representative experiment with the gating strategy. The mean frequencies of Th-17 cells ±SEM after HlaH35L activation were shown in figurer 4.5.

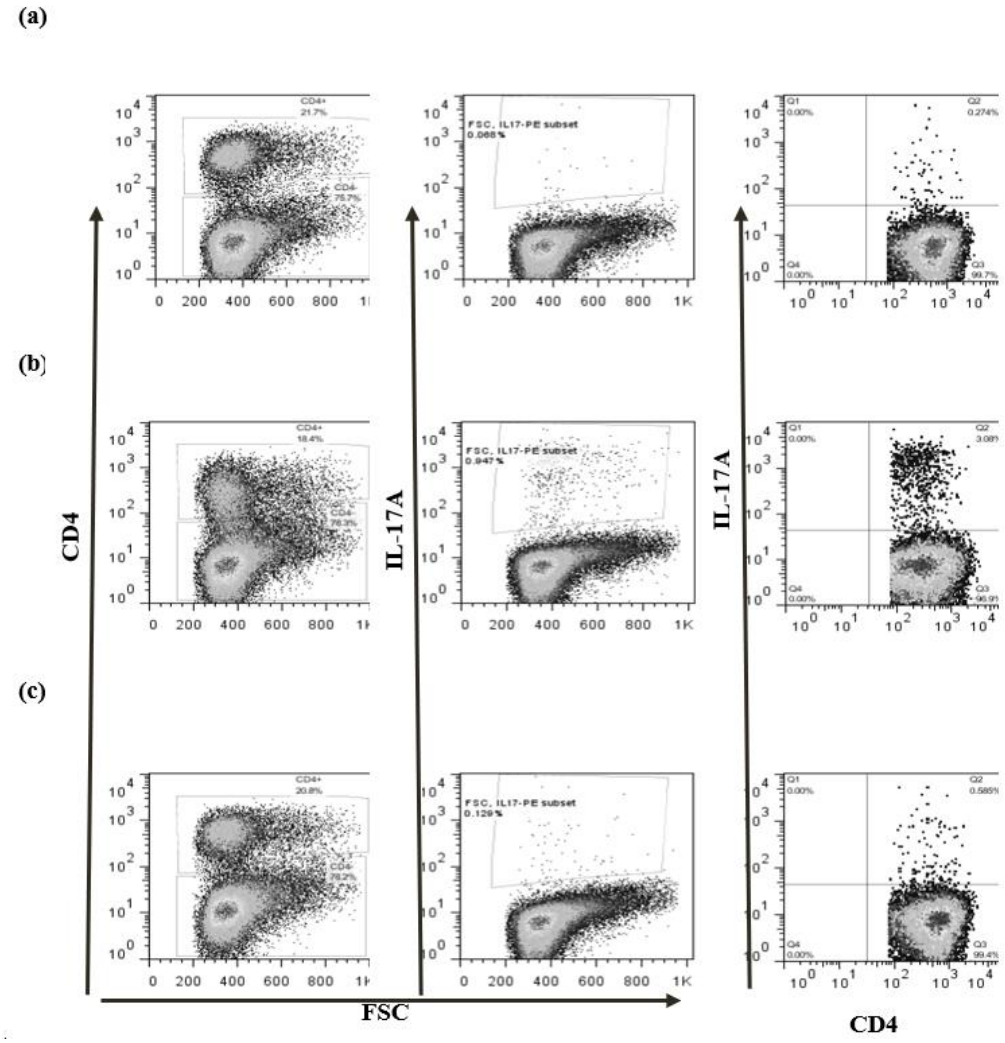


Figure 4.4: The gating strategy for analysing Th-17 cell response in MNC after the stimulation with *S. aureus* recombinant proteins. Lymphocytes were gated first as showed previously. Within the lymphocyte gate, CD4+T cells were gated. Then, the percentage of IL-17A+ cells in CD4+ T cells was calculated. The figure shows unstimulated medium control (a), PMA/Ionomycin positive control (b) and stimulated adenotonsillar MNC by HlaH35L (c).

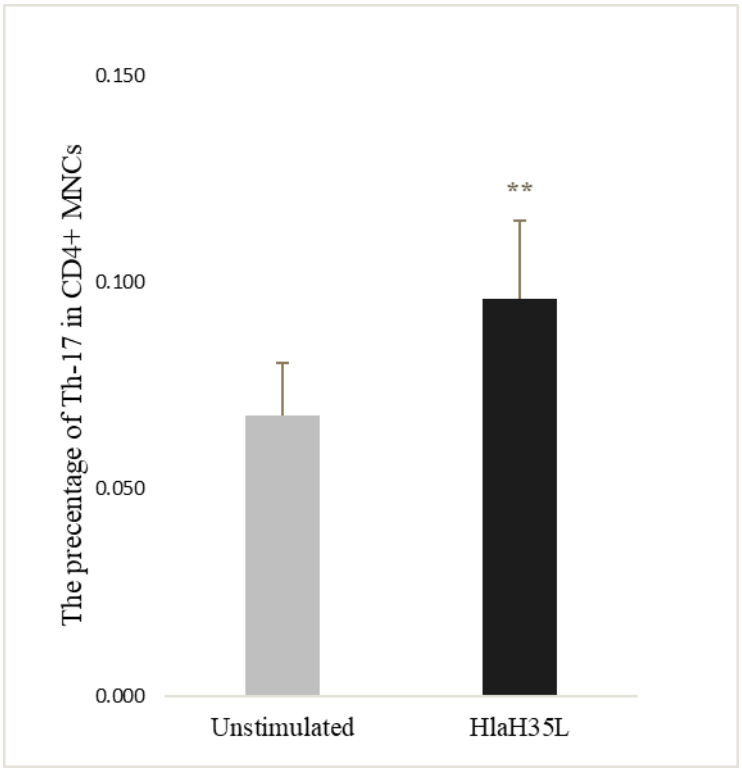


Figure 4.5: Th-17 response in adenotonsillar MNCs to the stimulation with *S. aureus* proteins. The screening of *S. aureus* proteins ability in activating Th-17 cells in MNC. The percentage of CD4+ IL-17+ cells was detected by FACS after 72 hr of stimulation with HlaH35L (10µg/ml) respectively. HlaH35L stimulation activated a marked increase in IL-17A+ Th-17 cells (n=18, $**p<0.01$). Mean±SEM is shown.

4.3.4 HlaH35L activated IL-17A production in tonsillar MNC

To study the ability of *S. aureus* recombinant proteins to stimulate IL-17A production, adenotonsillar MNCs were stimulated with nine purified *S. aureus* recombinant proteins for 3 days and then the cell culture supernatants were collected and analysed for IL-17A production by ELISA. As shown in table 4.3, HlaH35L stimulation elicited a significant production of IL-17A in adenotonsillar MNC (figure 4.6, n=11, ** $p<0.01$). All other proteins appeared to have no significant effect on IL-17A production according to Wilcoxon rank test (Table 4.3).

Table 4.1: Wilcoxon rank test statistical analysis of IL-17A ELISA showing the IL-17A (pg/ml) in tonsillar MNC after 72hrs of stimulation with recombinant *S. aureus* proteins (n=11).

	Unstimulated	**IsdA**	**ClfB**	**SdrC**	**SdrD**	**CnaBE3**	**EsxAB**	**HlaH35L**	**FhuD2**	**Csa1A**
Mean (pg/ml)	801.46	796.36	810.73	790.18	858.18	813.91	800.73	1086.36	766	829.36
SEM	199.58	187.60	202.30	249.48	220.47	227.40	226.30	218.25	207.44	227.95
P-value		0.929	0.756	0.534	0.05 *	0.594	0.722	0.003 **	0.056	0.929

n=11

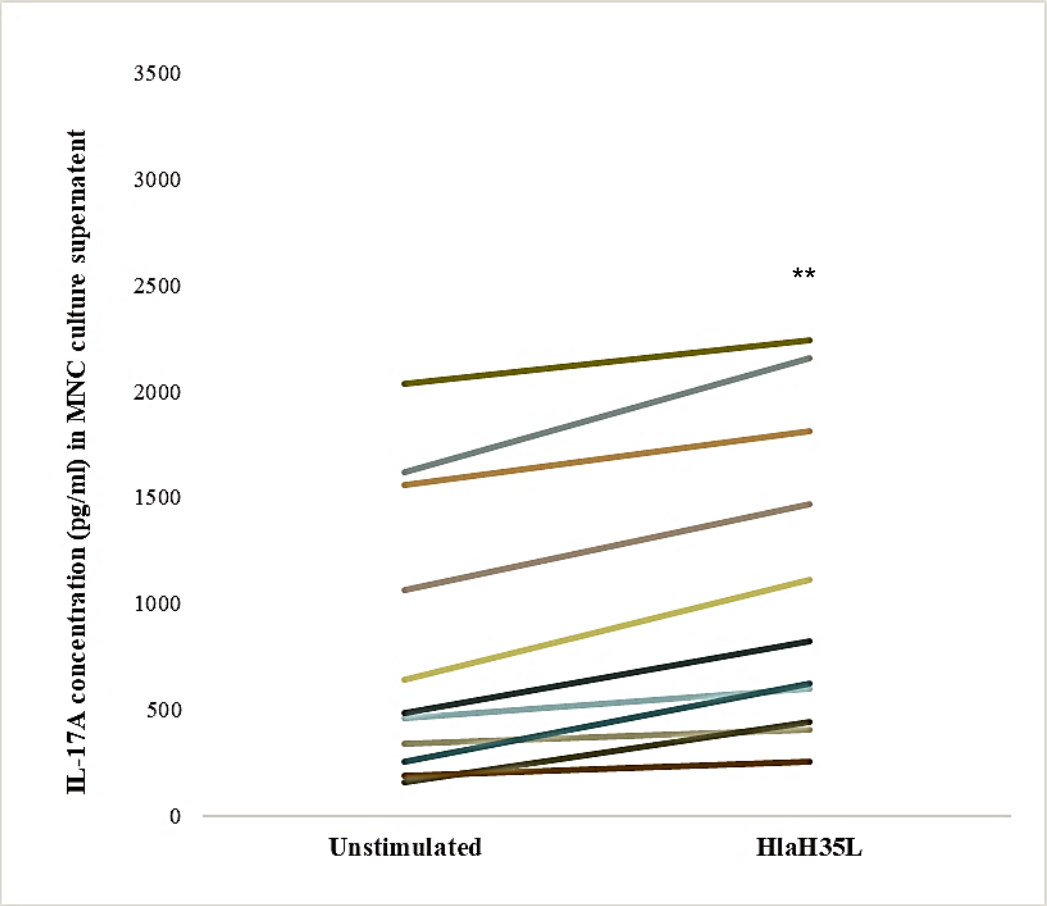


Figure 4.6: IL-17A concentration in adenotonsillar culture supernatant after the stimulation with *S. aureus* proteins. IL-17A concentration (pg/ml) measured by ELISA after 72 hr of stimulation with HlaH35L compared to unstimulated MNC. HlaH35L stimulation significantly stimulated the production of IL-17A. (n=11, ** $p<0.01$)

4.3.5 EsxAB elicited IL-22 production in tonsillar MNC

Tonsillar MNCs were stimulated with nine *S. aureus* recombinant proteins and then IL-22 levels in culture supernatants were analysed following cell stimulation for 3 days. Interestingly, results analysed with paired t-test showed that among all recombinant proteins, EsxAB stimulation elicited a significant IL-22 production in adenotonsillar MNC culture supernatant after 72 hr of cell culture. Figure 4.7 shows the concentration of IL-22 in tonsillar MNC culture supernatants following EsxAB stimulation compared to the unstimulated control (n=13, $p<0.05$).

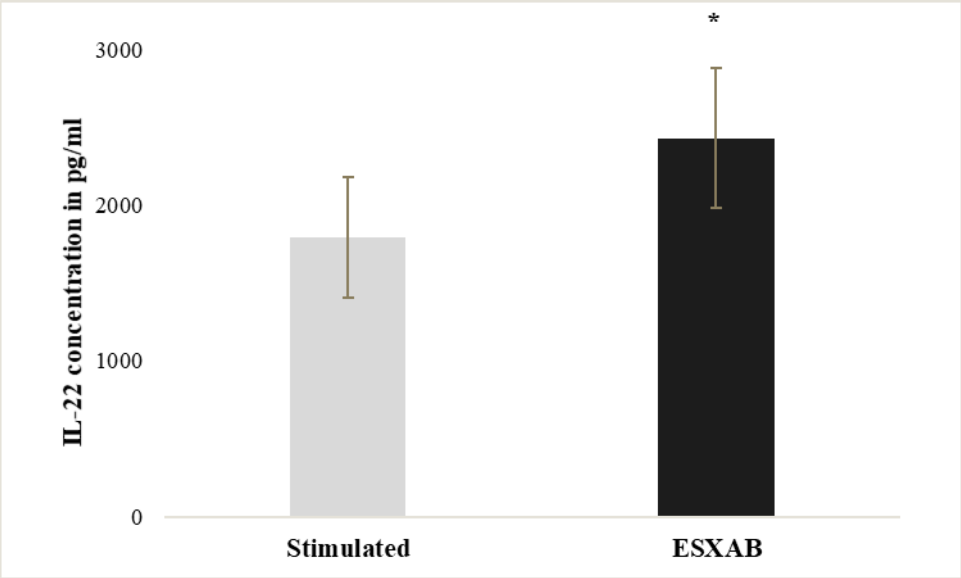


Figure 4.7: The concentrations of IL-22 in MNCs following the stimulation with *S. aureus* proteins. Adenotonsillar culture supernatant collected and analysed for IL-22 concentration by ELISA after 72 hr of stimulation with EsxAB(10µg/ml) (n=8). The figure represented IL-22 concentrations pre and post-stimulation with EsxAB and statistical analysis showed that EsxAB elicited significant production of IL-22 in MNCs. (n=13, *p<0.05). Mean±SEM is shown.

4.3.6 HlaH35L activate a higher Th-17 response in adults than in children

Further analysis was done to investigate the difference in HlaH35L-activated Th-17 tonsillar MNCs between children and adults. Adenotonsillar MNC from both children (n=17) and adults (n=7) were stimulated with HlaH35L and analysed by flow cytometry. Results were analysed by independent samples t-test. As seen in figure 4.8, a higher Th-17 response in tonsillar MNC was detected in adults than in children.

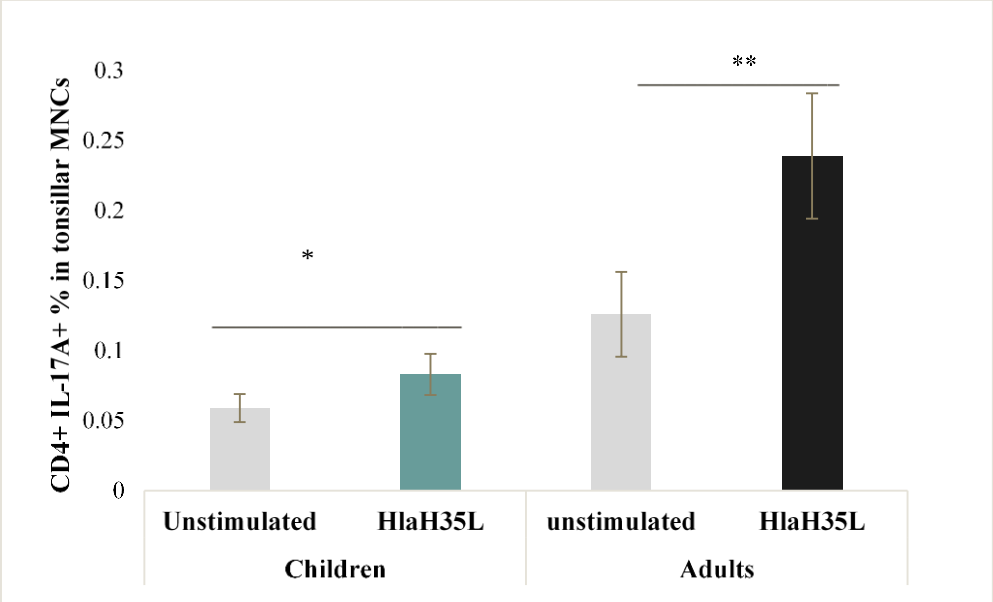


Figure 4.8: Comparison of Th-17 percentage in adenotonsillar MNCs between children and adults. Th-17response in adenotonsillar cells were detected after the stimulation with HlaH35L for 72 hr in children and adults' subjects. Statistical analysis of the means shows a significantly higher response of Th-17 were detected in adults than in children following HlaH35L stimulation (children n=17 and adults n=7, *p<0.05, **p<0.01). Mean±SEM are shown.

4.3.7 Cytokine response following stimulation with HlaH35L.

Following the previous results that stimulation with HlaH35L elicited a marked Th-17 response, we further analysed the cytokine profile in tonsillar MNC after the stimulation with HlaH35L by using CBA array kit. For this experiment, tonsillar MNC were stimulated with HlaH35L, and culture supernatant collected after 72hr and analysed for IL-5, IL-13, IL-6, IL-9, IL-10, IFNγ, TNFα, IL-17A, IL-17F, IL-21, IL-22 by flow cytometry.

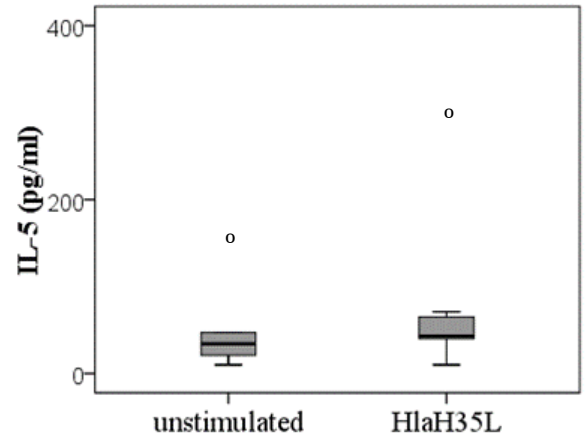
Analysed results of nine paired samples confirm results as above and show a significant increase of IL-17A concentration following the stimulation with HlaH35L. In addition to IL-17A (p<0.01), IL-17F (p<0.01), IL-10 (p<0.05) and IL-6 (p<0.05) levels were increased in MNCs culture supernatants in response to the stimulation with HlaH35L compared to the unstimulated control. Results were analysed with Wilcoxon ranked test represented in figure 4.9 (table 4.2)

Table 4.2: The effect of HlaH35L stimulation the production of several cytokines in MNCs supernatants. results were analysed after 72 hr by CBA array and analysed with Wilcoxon ranked test.

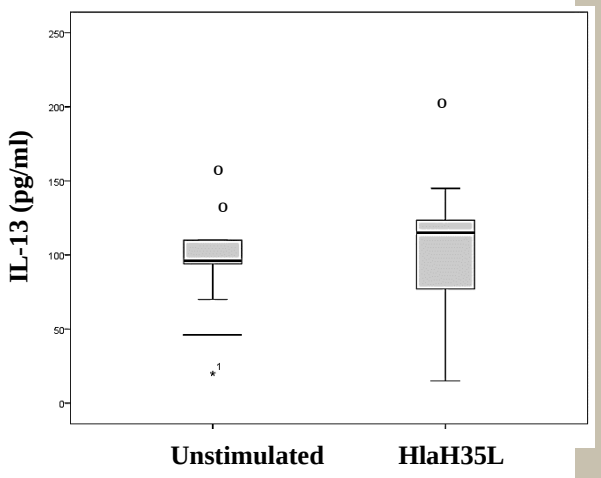
	IL-5	IL13	IL-2	IL-6	IL-9	IL10	IFNγ	TNFα	IL17A	IL-17F	IL-21	IL-22
Z	-2.10	-1.72	-0.92	-2.09	-1.84	-2.4	-1.58	-1.48	-2.70	-2.70	-1.24	-1.07
Sig.	0.035	0.086	0.358	0.037	0.066	0.017	0.114	0.139	0.007	0.007	0.214	0.285
***p*<0.05**				*		*			**	**		

Wilcoxon Signed Ranks Test

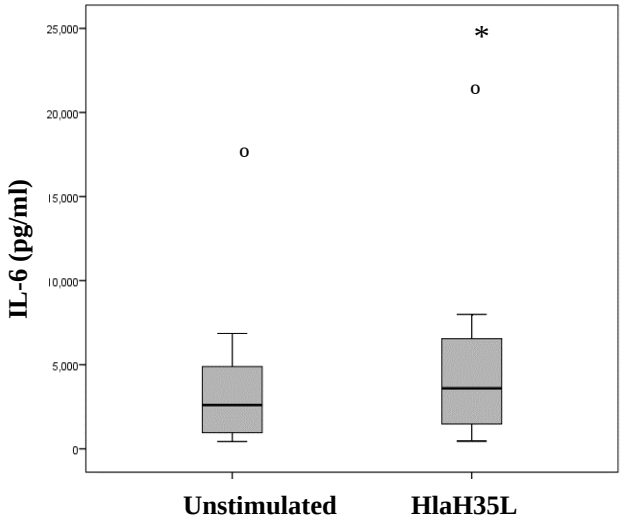
(a)



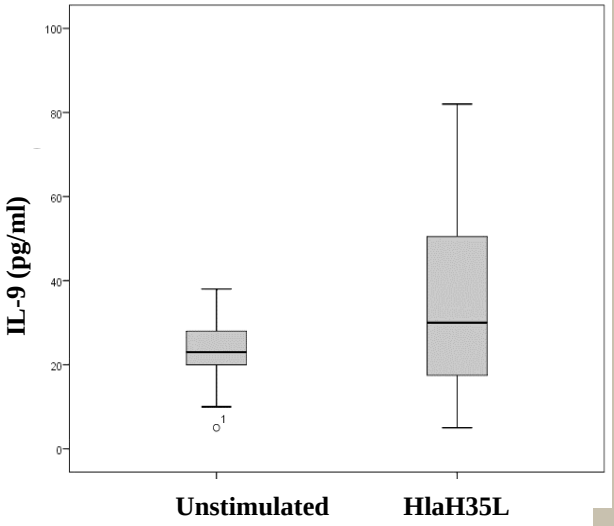
(b)



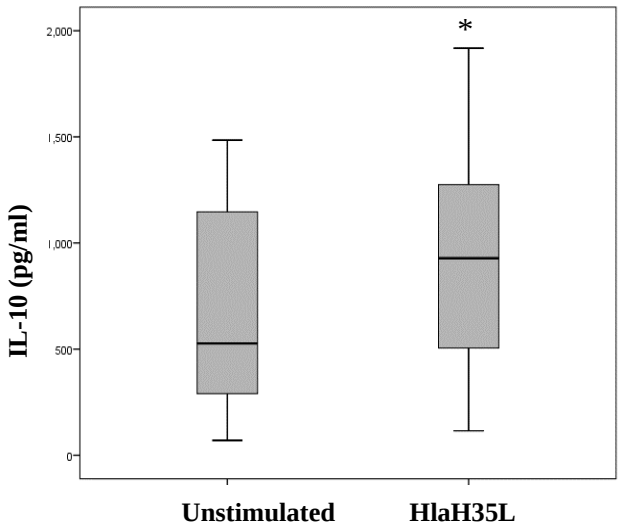
(c)



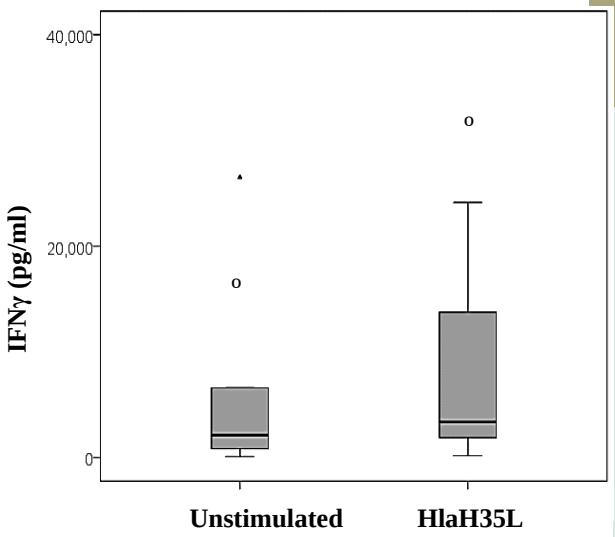
(b)



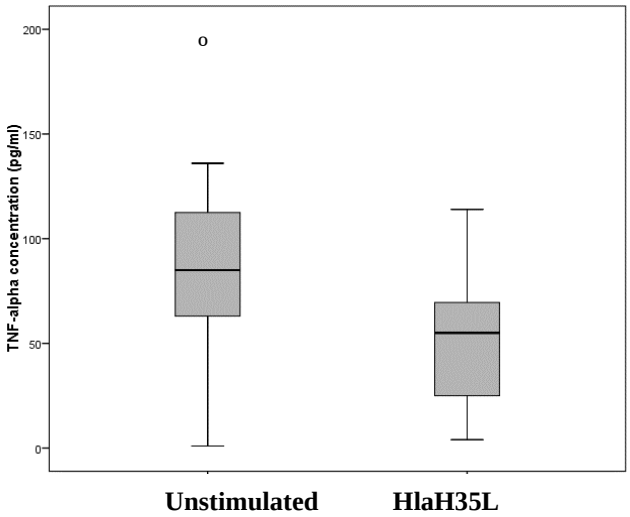
(e)


IL-10 (pg/ml)
2,000
1,500
1,000
500
0
*
Unstimulated
HlaH35L

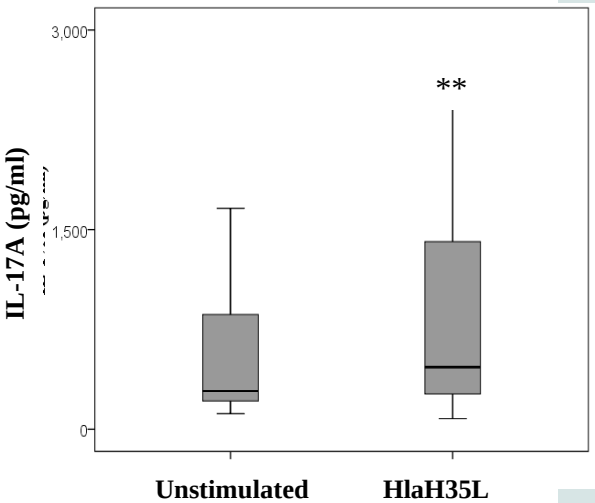
(f)


IFNγ (pg/ml)
40,000
20,000
0
o
o
Unstimulated
HlaH35L

(g)


TNF-alpha concentration (pg/ml)
200
150
100
50
0
o
Unstimulated
HlaH35L

(h)


IL-17A (pg/ml)
3,000
1,500
0
**
Unstimulated
HlaH35L

(i)

(j)

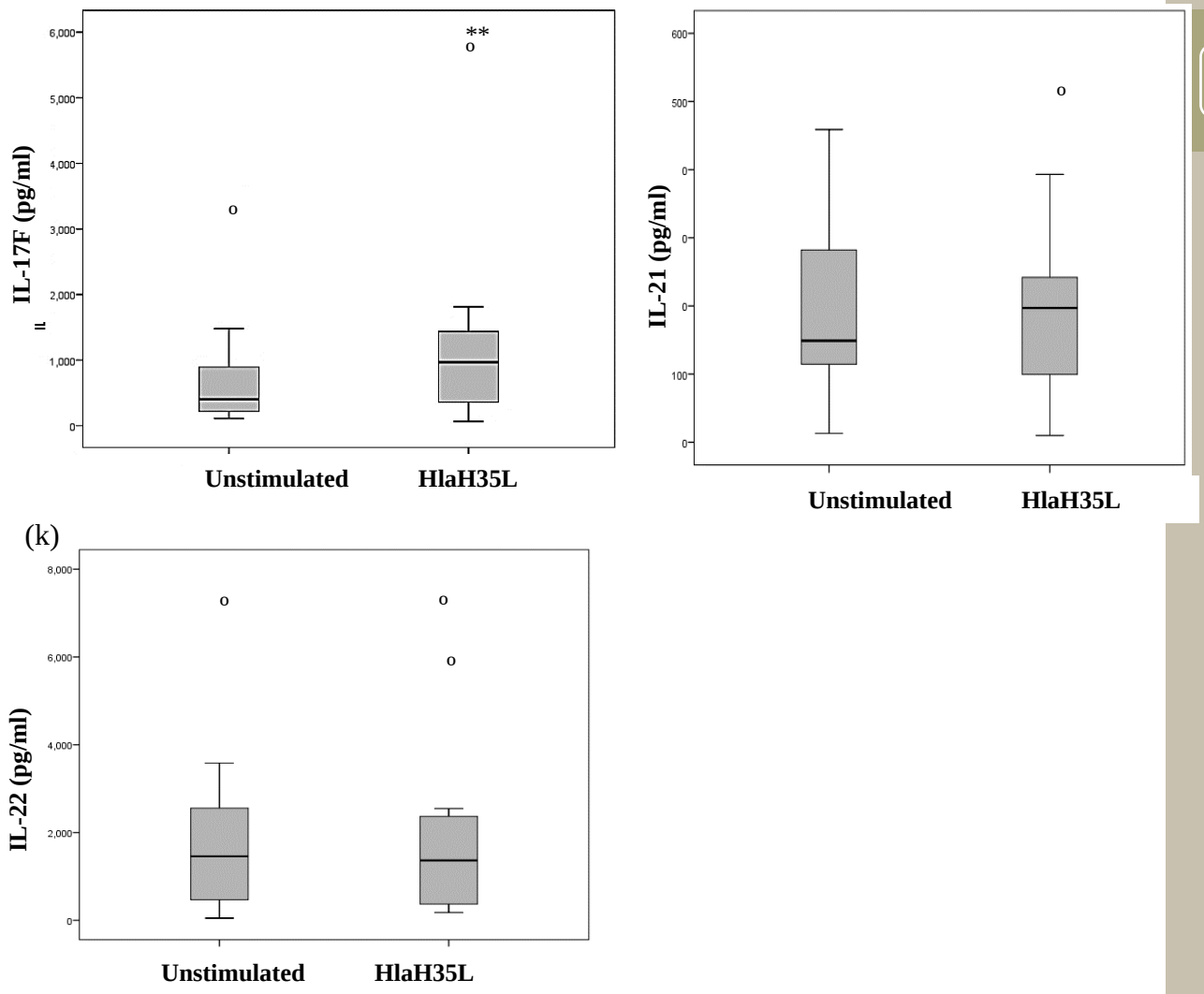
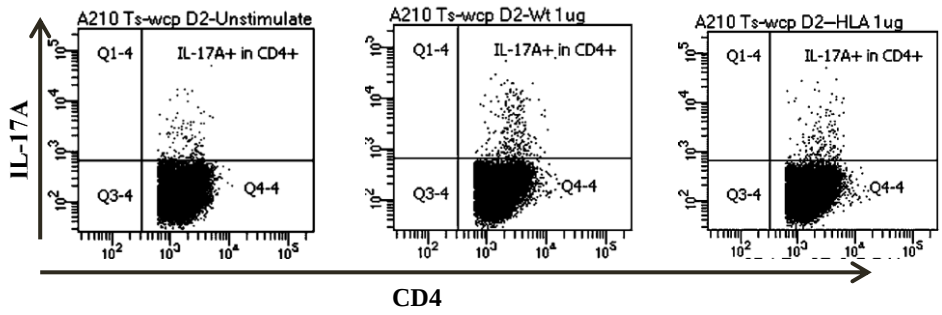


Figure 4.9: The concentrations of IL-5, IL-13, IL-6, IL-9, IL-10, IFNγ, TNFα, IL-17A, IL-17F, IL-21, IL-22 cytokines (pg/ml) in 72 hr culture supernatants after the stimulation with HlaH35L. Cytokines were measured by CBA then results analysed and showed that. HlaH35L stimulated IL-17A (h), IL-17F (i), IL-6 (c), and IL-10 (e) cytokine production. In contrast, no significant effect on the levels of IL-5 (a), IL-13 (b), IL-9 (d), IFNγ (f) , TNF-α (g) IL-21(j)and IL-22 (k) cytokines (pg/ml) after stimulation with HlaH35L. Cytokines were measured with CBA and results were shown as medians and interquartile range (n=9, *p<0.05 and **p<0.01). (º outliers)

4.3.8 A comparison between Hla+ *S. aureus* strain and Hla- mutant strain on the activation of Th-17 in adenotonsillar MNC.

To further investigate the effect of Hla protein in Th-17 activation, a wild-type of *S. aureus* strain expressing Hla toxin (WT) and its' isogenic Hla-/- mutant strain was used to stimulate tonsillar MNC. The concentrated culture supernatants (CCS) were prepared by culturing the strains in broth for overnight and then their culture supernatants were collected and concentrated. These CCS were used to stimulate adenotonsillar MNCs. MNCs were stimulated with the CCS for 72hr followed by flow-cytometry. Results represented in figure 4.10 (b) display the Mean±SEM of Th-17 frequencies upon stimulation. It was shown that CCS derived from the mutant Hla- strain stimulated significantly lower Th-17 response when compared to the WT strain ($*p<0.05$) although both WT($**p<0.01$) and Hla- ($*p<0.05$) CCS appeared to induce higher Th-17 cells than unstimulated cells.

(a)



(b)

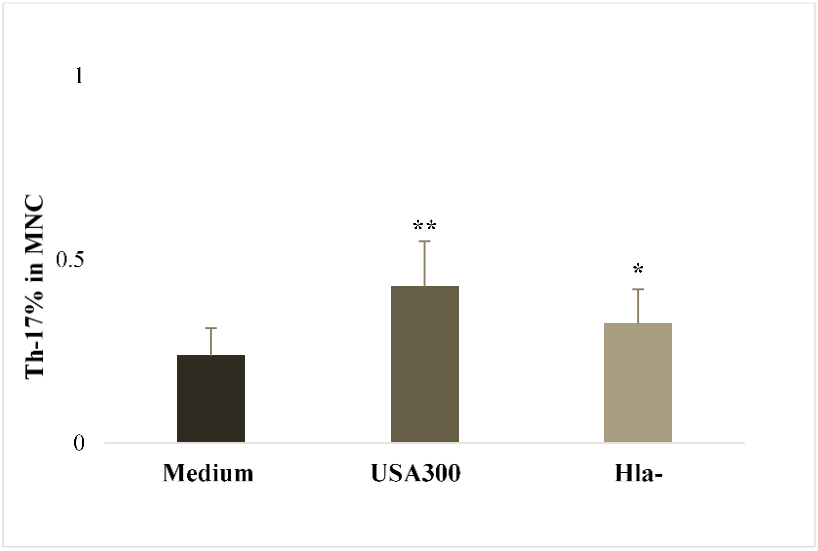


Figure 4.10: Activation of Th-17 cells by *S. aureus* USA300 Lac CCS (WT), isogenic Hla mutant CCS (Hla-) in adenotonsillar MNCs. Representative FACS plots on Th-17 cells (a). Comparison between the WT strain and the Hla- mutant strain on Th-17 activation (b). (n=6). (*$p<0.05$, **$p<0.01$, paired T-test). Mean±SEM is shown.

4.4 Discussion

In this study, I first studied whether some recombinant staphylococcal protein antigens activate a CD4+ T cell response. Using CFSE cell tracing, I have analysed the capacity of nine *S. aureus* proteins antigens to stimulate a T cell proliferative response, and I have shown that HlaH35L, a non-toxic mutant of *S. aureus* Hla toxin, activated a marked CD4+ T cell proliferative response in adenotonsillar MNCs. In addition, clumping factor β (Clfβ) was also shown to stimulate a CD4+ T cell response. In a murine model of nasal colonisation with Clfβ- mutant strain showed reduced colonisation ability and passive immunisation with anti-Clfβ antibodies protected against *S. aureus* colonisation (Schaffer *et. al*, 2006).

Since that HlaH35L was shown to activate a marked CD4+ T cell response, the next step was aimed to evaluate the Th-17 responses in adenotonsillar MNC after the stimulation with *S. aureus* proteins. In this study, I found that the HlaH35L activated a significant Th-17 response in adenotonsillar MNC detected by flow cytometry (Figure 4.5). This result was further confirmed by measuring the IL-17A concentrations in the cell culture supernatants by ELISA following stimulation of tonsillar MNC (Figure 4.6).

Hla toxin, a pore-forming extracellular toxin produced by *S. aureus*. Hla has a crucial role in both the invasiveness and adherence to host tissue (Popov *et al*. 2015). One of the recombinant non-toxic derivatives of Hla toxin is HlaH35L in which histidine was replaced with Lysin (Menzies and Kernodle, 1994). Immunization with HlaH35L has elicited a protective antibody response in previous mouse-model (Mocca, *et al*, 2014). In addition to the antibody-mediated response, the protective role of Hla by eliciting T cells response was also investigated in previous pneumonia mouse-model. Through comparison between wild type *S. aureus* strain and its Hla mutant (Hla-), a robust Th-17 response was detected after the challenge with wild type *S. aureus* compared to its Hla mutant infection (Frank, 2012). In this study, I showed that stimulation of tonsillar MNCs by HlaH35L alone was able to elicit Th-17 response. Several recent studies using murine models have evaluated the use of *S. aureus* recombinant purified proteins, either as an individual antigen or using antigen combinations (Banogli *et al*, 2014, Frank *et. al*, 2012) and shown that some *S. aureus* proteins were able to induce Th-17 response in mice

models. Furthermore, immunisation with a combination of five conservative recombinant proteins (including HlaH35L) was necessary to elicit both B-cell mediated antibody response and Th-17 protective response in a mice model of *S. aureus* challenge (Banogli *et. al*. 2014, Frank *et. al,* 2012).

I further analysed the cytokine production profile in the cell culture supernatants following HlaH35L stimulation of tonsillar MNC. It was shown that HlaH35L significantly elicited IL-6 along with IL-17F/IL-17A in MNCs in NALT (figure 4.9).

The importance of Th-17 in the clearance of bacterial carriage is mainly due to their role in neutrophil recruitment to the site of infection and the production of antimicrobial peptides. The main mediators of Th-17 cells are IL-17A along with IL-17F and IL-22 cytokines. IL-17A is the major cytokine of Th-17 cells and it has a main role in neutrophil recruitment (Matsuzaki, and Umemura, 2007). IL-17F, on the other hand, is also important for the anti-pathogenic properties and anti-bacterial protein production by epithelial cells (Kolls and Linden 2004; Weaver *et al.* 2007). IL-6 is a proinflammatory cytokine shown to be important to Th-17 induction (Camporeale and Poli. 2012) and the protection against *S. aureus* infection (Gauguet *et al.* 2015). IL-6 was shown to be involved in signalling activation of STAT3, one of the main regulators of Th-17 cell differentiation (Camporeale and Poli. 2012). HlaH35L was shown to activate IL-6 production which may play a role in the Th-17 induction from naïve T cells.

In this study, the Th-17 response by HlaH35L was compared between children and adults. The results showed a significant difference between children and adults. HlaH35L was able to activate Th-17 response in both Children (n=17, $p<0.05$) and in adults (n=7, $p<0.01$) (figure 4.8). The increase in Th-17 response with age might be related to the accumulated exposure to bacterial infections and the accumulation of memory Th-17. Previous studies suggest an age-associated increase in Th-17 response in human NALT (Mubarak, A *et al.*, 2016).

Another finding in this chapter was that when tonsillar MNCs were stimulated with EsxAB protein, it elicited the production of IL-22 cytokine (figure 4.7). EsxAB consist of two small protein A and B and secreted by ESTAT-6 secreting system

(ESS) (Burts, *et al.*, 2005). Esx A and B showed a role in regulating Th-1/Th-17 by stimulating of DC (Cruciani *et al.* 2017). In addition, Korea *et. al*, 2014 showed that ESX AB has an essential role of *S. aureus* pathogenesis through apoptosis. The production source of IL-22 is not exclusive only to Th-17 but also produced by epithelial cells and by some ILC3 cells. The importance of IL-22 in maintaining homeostasis (Vivier, *et al*. 2009) of nasal microbial residency as well as restoring of epithelial cells integrity and the production of AMPs has been demonstrated in previous studies (Mulcahy *et al*, 2016).

IL-22 was found to be responsible for the regulation of pathogenic burden through the regulation of the antimicrobial peptides (Nikoopour, *et al*, 2015). A study by Mulcahy *et. al.*, (2016) found that in a murine model of *S. aureus* nasal colonisation, IL-22 deficient mice showed higher carriage rate with decreased expression of *S. aureus* ligands and antibacterial protein.

In summary, this chapter studied the capacity of several recombinant *S. aureus* proteins to activate CD4+ T cell response and Th-17 response in particular in tonsillar MNC. I showed that HlaH35L, a non-toxic derivative of Hla toxin, activated a marked Th-17 response in tonsillar MNC. The results on cell stimulation with Hla-expression wild-type *S. aureus* CCS compared with a Hla-/- mutant CCS also support the importance of Hla in Th-17 cell activation. The finding from this study support that Hla derived non-toxic protein molecule such as HlaH35L may be a potential candidate as to provide immunity to bacterial carriage in the nasopharynx.

Chapter 5

The Relationship Between Type 3 Innate Lymphoid Cells (Ilc3) And Th-17 Cells In Human Nalt And Pneumococcal Carriage

Chapter 5: The relationship between type 3 innate lymphoid cells (ILC3) and Th-17 cells in human NALT and pneumococcal carriage.

5.1 Introduction

ILC3 is a subpopulation of innate lymphoid cells (ILC) that also contain ILC1, ILC2 and NK cells. Apart from NK cells, innate lymphoid cells are distinguished with the expression of CD127+ (IL-7Rα), (Satoh-Takayama, 2008). ILCs were first classified into three subgroups according to their expression of nuclear factors and cytokines profile (Spits, *et. al*., 2013; Bjorklund *et. al.,* 2016). Among ILCs, ILC3 are identified phenotypically by lineage markers (Lin) -ve, CD127+, c-kit+ (Hazenburg and Spits, 2014). Along with cellular expressed markers, ILC3 could be distinguished genotypically by the expression of the nuclear factor Retinoid-related orphan receptor γt (RORγt). In addition to markers expression, ILC3 subpopulation identified by the capacity of production of Th-17 cells effector cytokines, IL-17A and/or IL-22 (Zook and Kee 2016). Thus, ILC3 were considered as the innate resemblance to Th-17 subset of helper T cells. ILC3 are heterogeneous lymphoid population that abundantly distributed in mucosal sites. According to their surface expression of the natural cytotoxicity receptor (NCR) (Luci *et al.*, 2009), ILC3 were further divided into NCR- and NCR+ subsets. Part of NCR- cells that involve lymphoid tissue-inducer (LTi) sub-group. LTi

are distinguished by the production of IL-17A (Cupedo *et al.,* 2009). On the other hand, NCR+ recognized by IL-22 production and this population plays a pivotal role in the balancing microbiota-pathogen interactions (Hepworth *et al.*, 2013; Hepworth *et al.*, 2015).

Apart from secondary lymphoid tissues, ILC3 were found in peripheral blood circulation, umbilical cord with steady distribution in both children and adults (Vely, *et al.*, 2016). Bjoklund *et al.,* (2016) tried to define the heterogeneity of ILC3 in human tonsils by using single cells mRNA sequencing (scmRNA seq.) and it showed that the ILC3 was the main population among other ILC groups. According to their data, they were able to address three different heterogenetic subtypes depending on the transcriptional factors: the first population, IL-22 producing NCR+ (NKp44 encoding) representing the activated ILC3 cells. The

second subtypes were the smallest group among the rest and expressed both CD62L and CD45RA, so they represented naïve ILC3. While the third population was less restrictive in transcriptional factors profile, expressing both NKp44- and NKp44+ cells and co-express HLA-DR, produce IL-17A (but not IL-22) in low levels, and in contrast to other ILC3 groups, HLA-DR+ ILC3 seemed to be responsive to IL-23 and IL-1β.

According to their role in the tissue as early-activated cells that produce several immunological mediators, their functions extend and vary depending on the expressed CD markers and the preclinical statues of the human tissue. ILC3 population plays a crucial role in maintaining mucosal homeostasis, regulation of the commensal microbiota and the regulation of other T helper cells especially, IL-17 producing Th cells in cases of human autoimmune disease and inflammation (Hepworth *et al.*, 2013; Vivier *et al.*, 2018).

During recent years, researchers were focusing on ILC3 immunoregulatory capacities and homeostasis maintenance properties. Although it has been suggested as early activated cells against infection, the role of ILC3 during bacterial infection has not been fully defined. A murine-pneumonia model was used to evaluate the distribution and the function of ILC3 during bacterial infection. The outcomes showed that ILC3 was the main earliest source of IL-22 production in response to IL-23. Furthermore, IL-22 producing ILC3 population significantly increased after the *S. pneumoniae* infection. Nevertheless, the increase in ILC3 did not seem to be associated with the elimination of the established infection (Van Maele *et al.*, 2014).

Two mechanisms have been proposed to underline the role of ILC3 in maintaining homeostasis and regulating expansion of Th-17 cells. The first scenario focused on their expression of IL-22. NCR+ IL-22 producing ILC3 were found to preserve a healthy gut environment through the nourishment of the commensal bacteria and elimination of pathogenic bacterial spreading once occurred. The other proposed mechanism was through their contribution to regulating Th-17. Th-17 co-regulation by ILC3 was through the expression of Aryl Hydrocarbon Receptor (AHR). In a murine study conducted by Qiu *et al.*, (2013), AHR-/- mice were producing lower IL-22 than normal mice. This reduction of IL-22 level was co-combined with an expansion in Th-17 frequency. The same study refers to the role of IL-22+ ILC3 in

passively regulation Th-17 response. As the deficiency in IL-22+ ILC3 led to the breakdown of the gut microbiota balance. The disproportion was emphasised by the over-growth of gut-inhabited filamentous bacteria and that promoted a Th-17 mediated response.

In addition to their role in the maintenance of homeostasis, ILC3 were shown to directly regulating Th-17 response via their ability of processing and presenting antigens to CD4+ T lymphocytes. It was discovered that a group of ILC3 were expressing HLA-DR (Hepworth *et al.*, 2013). The expression of MHC-II molecules by ILC3 appeared to play a pivotal role in both the regulation of activated commensal bacteria-specific T-cells and induction of cell apoptosis during inflammation. During human inflammatory bowel disease (IBD), the total ILC3 percentage was not affected in IBD paediatric patients. In contrast, a substantial reduction of colonic HLA-DR+ ILC3 but not of CD4+ T cells was observed. This reduction was inversely correlated with the expansion of Th-17 (Hepworth *et al.*, 2015). Th-17 regulation by ILC3 has not yet been defined. on the other hand, it has also been shown that ILC3 has a significant contribution against infections (as previously mentioned). Besides, the studies highlighted the role of both ILC3 with Th-17 in homeostasis and during inflammation need to be more explored. So far, the depletion of ILC3 alone was associated with lower levels of IL-22 and the susceptibility to infection and inflammation which led to systematic inflammatory condition or chronic disease. These outcomes were significantly magnified when both the ILC3 and Th-17 responses were impaired. During depletion of Rag (Recombination activating gene) in mice, a gene depletion impaired the maturation in adaptive cellular immunity. When combining ILC3 and Rag depletion the retaining of gut microbiota in mice intestine failed (Cella *et al.*, 2009; Sonnenberg *et al.*, 2012).

Even though both Th-17 and ILC3 exhibited similar transcriptional and effector cytokine profiles upon stimulation, the effector responses generated from the two populations differed in terms of intensity and functionality. Aggressive Th-17 response upon infection or inflammation could promote undesirable or overpowering immune response including tissue damage and autoimmune disease. It is therefore critical to understand the regulatory mechanisms required to regulate the over-reactive responses mediated by Th-17 cells and to demonstrate an effective

approach for tuning these undesirable immune responses especially in case of chronic inflammation or autoimmune disease. One of these approaches is the use of Th-17 inhibitors molecules for therapeutic purposes and must go under extensive research to select an effective and safe formula. Previous evaluation of using blocking IL-17 antibodies showed several adverse health conditions (Hueber *et al*, 2012). Recently, selective-targeting pharmacological chemical compounds have been studied as a targeted therapeutic agent for controlling Th-17 response (Xiao *et al*, 2014). One of these agents is GSK805, a small molecular-weight RORγt transient inhibitor that appeared to inhibit Th-17 response but not ILC3 response in animal studies. It was demonstrated that GSK805 possesses a specific Th-17 inhibitory effect in both mice models of gut inflammation and Crohn's disease in paediatric patients. These results encouraged us to do further investigations on these RORγt inhibitors (Withers *et al.*, 2015).

5.1.1 Aim of the study

The relationship between Th-17 and ILC3 cells and their interactions with bacterial carriage in human nasopharynx are not well-defined and need further research. Therefore, this chapter aimed to study:

- The frequency of ILC3 and relationship with Th-17 in NALT.
- The association of IL-22-producing ILC3 and pneumococcal carriage in children.
- Effect of *S. aureus* and *S. pneumoniae* on ILC.
- The effect of GSK805 RORγt inhibitor on both Th-17 and ILC3 in NALT.

5.2 Experimental design:

The main objectives for this chapter were to study the relationship between ILC3 and Th-17 cells in NALT and their association with pneumococcal carriage in children. Due to the relatively low number of *S. aureus* carriage I could not study the relationship of ILC3/ Th-17 in relation with *S. aureus* carriage First step was to characterize the ILC3 in NALT (tonsillar tissue) from children and adults. Next step was the assessment of the frequencies of IL-17A and IL-22 producing ILC3 and Th-17 cells in the same tissue samples. Then, the correlation between ILC3 and Th-17, followed by analysis of the relationship between ILC3 *and S. pneumoniae* carriage. Concerning *S. aureus* effect on ILC population, it was studied by the studying ILC upon the stimulation of MNC with *S. aureus* CCS and their effect on HLA-DR expression. Finally, the effect of a RORγt inhibitor (GSK805) was studied on *S. aureus*-activated ILC3 and Th-17 responses.

5.2.1 Human subjects

For this study, tonsillar samples were collected from patients conducting adenotonsillectomy from children and adults. Patients how were given antibiotics within 3 weeks of the operation or these tonsillar tissues showing overt inflammation were excluded from the study. All individuals or their guardians were asked to sign consents before enrolling in the study.

5.2.2 Detection of ILC3 in NALT by flow-cytometry

Freshly isolated adenoidal and tonsillar cells were washed with staining buffer (0.2% BSA-PBS) and then stained with surface markers required for ILC3 identification. Markers used are: Lineage cocktail no: 1 (Lin-1) (which included CD3, CD14, CD16, CD19, CD20, and CD56), CD127, c-Kit (CD117), and NKp44. After staining, cells were washed and re-suspended with staining buffer. As soon as the cells were ready for acquisition, data were acquired and analysed by BD Accuri (BD, UK). The gating strategy used for ILC3 identification can be shown in figure (5.1).

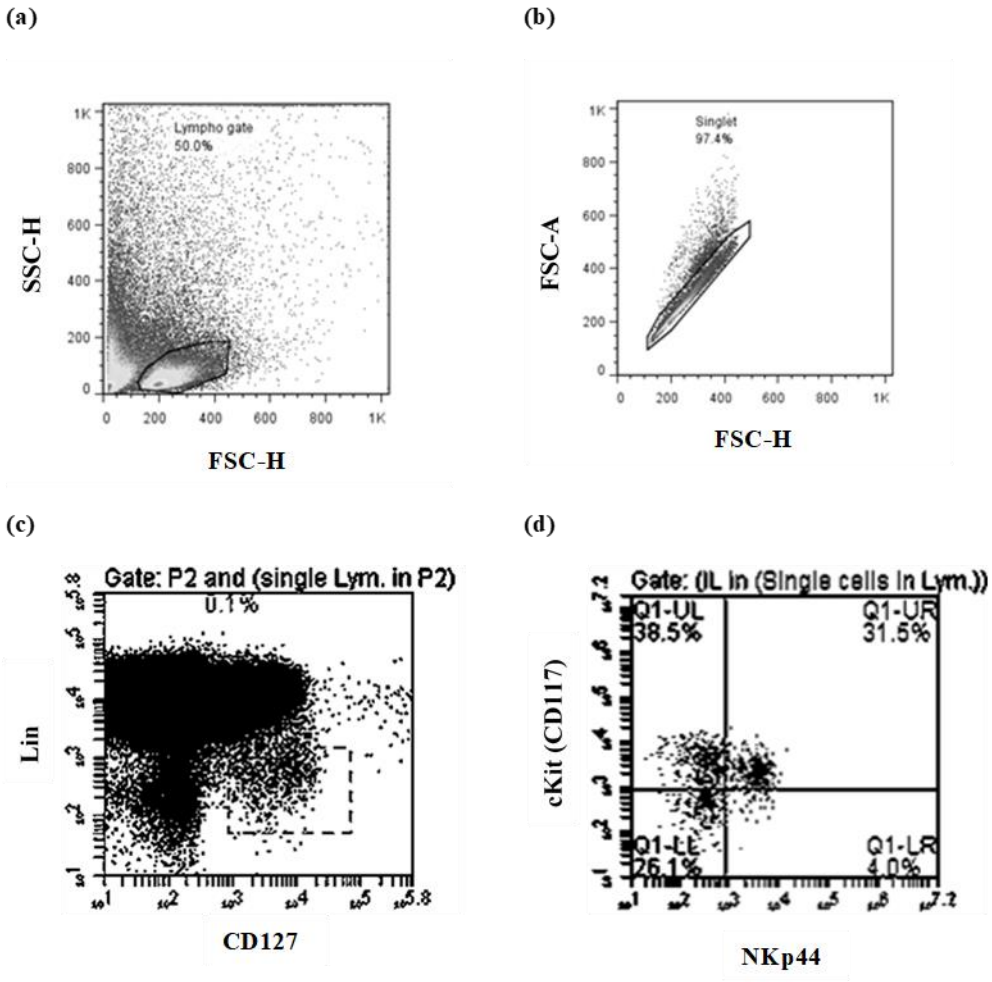


Figure 5.1: The gating strategy of innate lymphocytes 3 population in tonsillar and adenoidal tissue. MNCs stained for Lineage, CD127, cKit and NKp44 markers. First, the "lymphocyte" population was gated according to the typical forward (FSC) and side scattered characteristics (SSC) (a). Singlet cells then were gated by using FSC-H and FSC-A (b). Next, Innate lymphoid cells (ILC) were gated as Lin-/ CD127+ population (c). Finally, ILC3 cells population were sub-divided by using cKit and NKp44 markers into cKit+/NKp44+ and cKit+/NKp44- ILC3 cells (d).

5.2.2.1 Detection of IL-17A- and IL-22-producing ILC3 in NALT of adults and children

To assess IL-17A and IL-22 producing ILC3 population in tonsillar tissue, flow-cytometry was used with staining a panel of surface and cellular markers. For this experiment, freshly isolated MNC were stimulated with PMA/Ion, along with BFA for 6 hrs at 37Cº. At the end of the incubation period, cells were collected and stained with surface antibodies. The ILC3 staining panel included Lineage cocktail no: 1 (Lin-1) (CD3, CD14, CD16, CD19, CD20, and CD56), CD127, cKit (CD117), NKp44, and HLA-DR. Cells were washed twice with staining buffer and fixed/permeabilized by eBiosciences fixation/ permeabilization buffer. Finally, Data were acquired by BD FACS Celesta and analysed using BD FACS Diva software (BD, UK) The gating strategy for the staining panel is shown in figure (5.2)

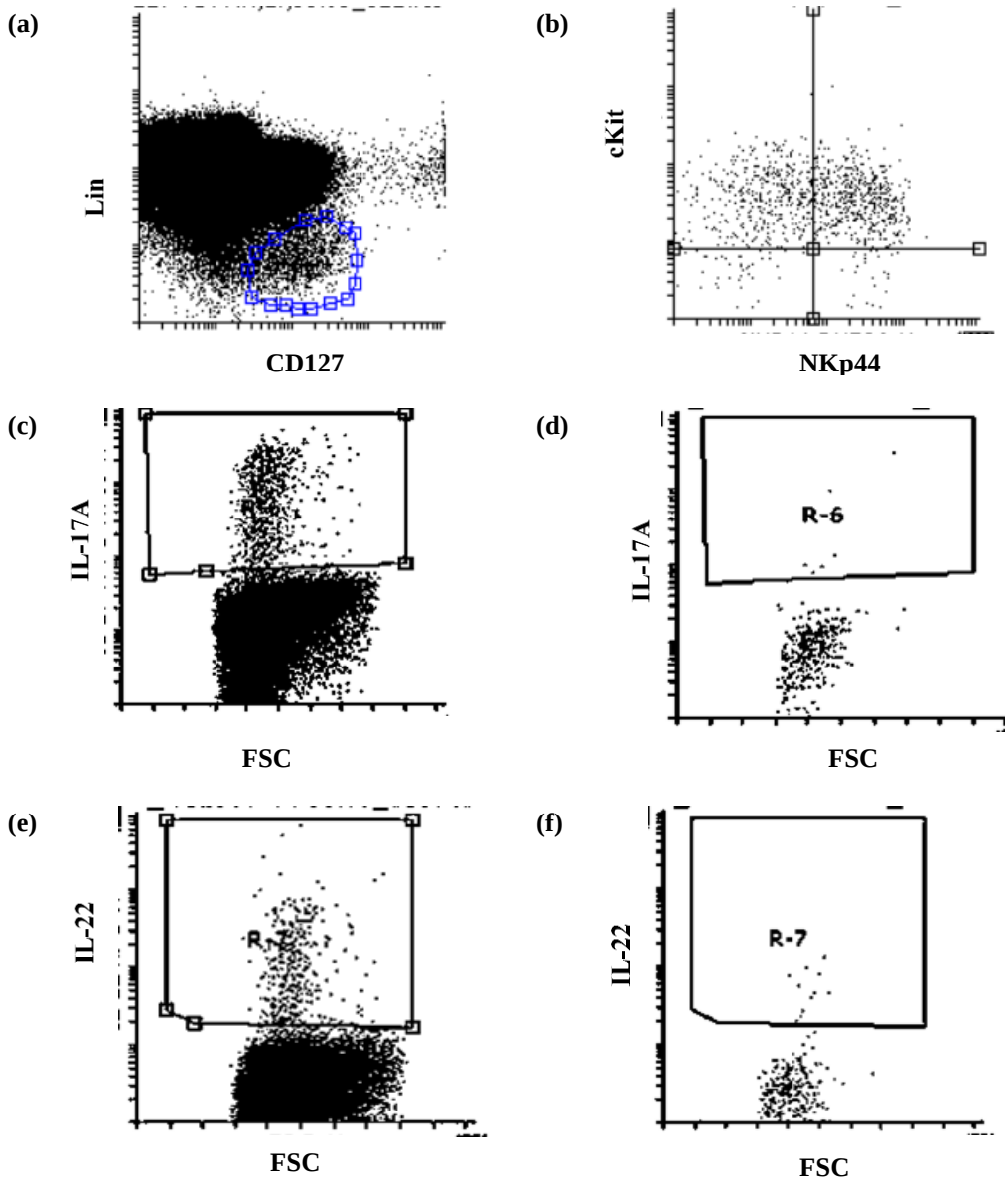


Figure 5.2: The gating strategy for detecting IL-17A-/IL-22-producing ILC3 in tonsillar MNC. First, Innate lymphoid cells (ILC) were gated as Lin-/ CD127+ population (a). ILC3 cells population were gate by plotting the ILC population into cKit+/NKp44+ and cKit+/NKp44- ILC3 cells (b). % of IL-17A+ and IL-22+ cells in whole MNC were recorded (c and e accordingly). For the detection of IL-17A+ and IL-22 + cells in ILC population, the percentage of IL-17A+ ILC3 and IL-22+ in ILC3 were recorded (d and f respectively).

5.2.3 Determination of the effect of *S. aureus* and *S. pneumoniae* on ILC3 cells

To determine whether there is any effect of *S. aureus* and *S. pneumoniae on ILC3 cells*, *S. aureus* and *S. pneumoniae* CCS was used to stimulate tonsillar MNC, followed by analysis of ILC3 population. For this experiment, tonsillar MNC were first adjusted to 4*106 cell/ml with supplemented RPMI-1640 and stimulated with *S. aureus or S. pneumoniae* CCS at 37Co for three days. In the last 6 hrs of the incubation, (1x) brefeldin A (BFA) was added to the cell cultures and incubated at 37Co. At the end of culture, the cells were harvested, stained with surface markers, fixed and permeabilized (eBiosciences, UK), followed by staining intracellularly with anti-cytokine antibodies. Data were acquired by BD FACS Celesta and analysed with FACS Diva software.

5.2.4 Analysis of the effect of RORγt inhibition on Th-17 response activated by *S. aureus* CCS stimulation.

To determine if there is any effect of RORγt inhibition on ILC3 or Th-17 response activated by *S. aureus* stimulation, tonsillar MNC were adjusted to 4x106 cell/ml in RPMI-1640 and incubated with 0.5µM of RORγt antagonist II (Merch Millipore, UK), a RORγt inhibitor, which was reported to suppresses T helper 17 (Th-17) responses. The concentration was first optimised in preliminary experiments and the final concentration for blocking was 0.5 µM. Then, tonsillar MNC were incubated with the inhibitor at two-time points, 2 hrs and 8hrs respectively, followed by stimulation with *S. aureus* CCS for overnight. In the last 6 hrs of the incubation period, brefeldin A (BFA) was added. At the end of cell culture, culture supernatants were collected and kept in -20Cº before cytokine analysis by ELISA. The cells were then harvested, stained with surface markers, fixed and permeabilized (eBiosciences, UK) followed by intracellular cytokine staining and flow-cytometry by BD Celesta and analysed by FACS Diva software.

5.2.4.1 Measurement of IL-17A

For the measurement of IL-17A production from adenotonsillar MNC and PBMC culture supernatant following stimulation, IL-17A ELISA Ready-Set-Go® set (eBiosciences, UK) was used and the procedure was conducted according to the manufacturer's instructions. The plates were ready for OD measurement at 450nm, and the concentration (pg/ml) was determined for each sample against the standard curve with Deltasoft PC software (Biometallics).

5.2.5 Statistical analysis

Normality of data distribution was checked and once confirmed the differences between two groups were analysed by Student t-test or analysed by paired t-test. Data were not normally distributed analysed by Wilcoxon signed-rank test. Correlation of two related parameters was analysed by Pearson's correlation coefficient. Results were considered statistically significant with a $p\text{-}value < 0.05$.

5.3 Results

5.3.1 The frequency of ILC3 in adenotonsillar MNC

To determine the frequencies of ILC3 within NALT tissue, adenotonsillar tissue from 34 children aged between 2-16 years old were analysed in this experiment. Freshly isolated tonsillar MNC were stained with ILC3 markers (Lineage-1, CD127, cKit and NKp44). The median frequencies of ILC (Lin-, CD127+) and ILC3 (Lin-, CD127+, cKit+) in tonsillar MNC were 0.12%, 0.065% respectively (n=34) (figure 5.3). In general, the ILC3 frequency in adenoidal tissue was shown to be higher than in the tonsillar tissue in the paired samples according to Wilcoxon Signed Ranks Test (n=7; $p<0.001$) (figure 5.4).

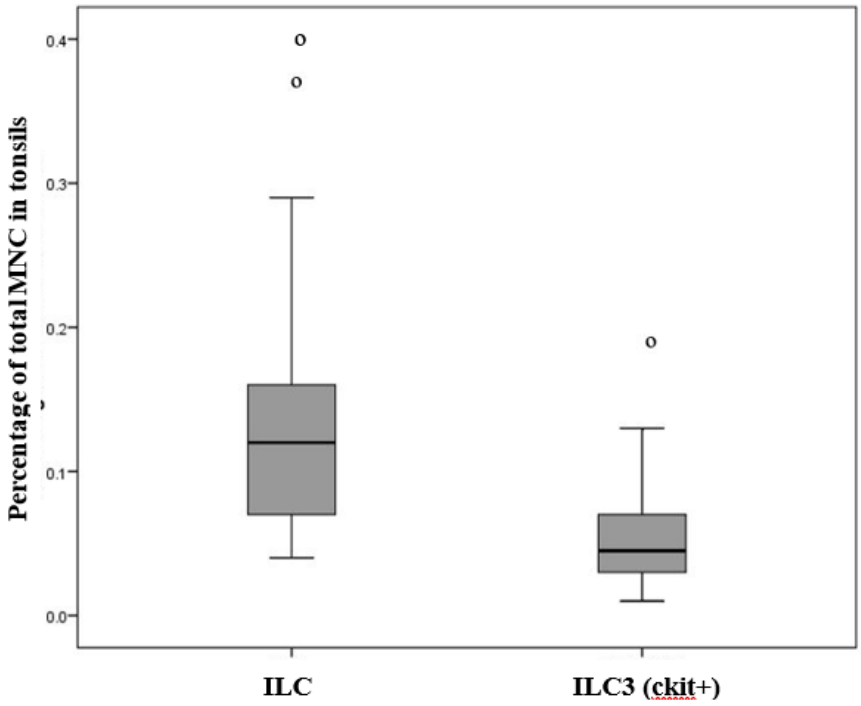


Figure 5.3: The frequencies of ILC, ILC3 in tonsillar cells. The proportions of ILC and ILC3 within the total tonsillar MNC population were shown. Data represent the medians and interquartile range of both ILC and ILC3 percentages in tonsillar MNCs of children (n=34). (º=Outlier). The median and interquartile range are shown.

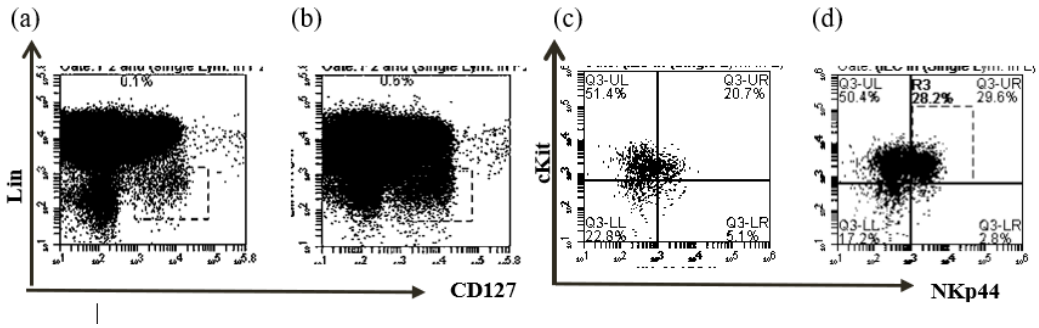


Figure 5.4: The percentages of ILC3 population in children's tonsils and Adenoids. Freshly isolated tonsillar and adenoidal MNC were labelled with Lin-1, CD127, cKit and NKp44. ILC population was gated using Lin-1, CD127 markers and the ratio was calculated in tonsils and adenoids (a and b respectively) and the ILC3 was calculated using cKit+ population ratio in MNC (c and d respectively). It is noticeable that adenoidal MNC having higher ILC3% (b) and higher NKp44+ ILC3% (d) than tonsil MNC.

5.3.2 Comparison of frequencies of IL-17A+ and IL-22+ ILC3 in tonsillar MNC between children and adults

To examine the frequencies of IL-17A+ and IL-22+ ILC3 population in tonsillar MNCs, freshly isolated tonsillar MNCs from adults and children were analysed by staining for ILC3 markers and intracellular cytokine staining for IL-17A and IL-22 after PMA/Ion stimulation.

5.3.2.1 The frequency of IL-17A+ ILC3 is lower in children than adults

IL-17A-producing cells within the ILC3 population were analysed and expressed as the % of IL-17A+ cells in Lin -ve, CD127+, cKit+ population. As shown in figure (5.5). the frequency of IL-17A+ ILC3 cells in children was significantly lower than in adults (children =6, adults=6, $p < 0.05$ Student's t-test).

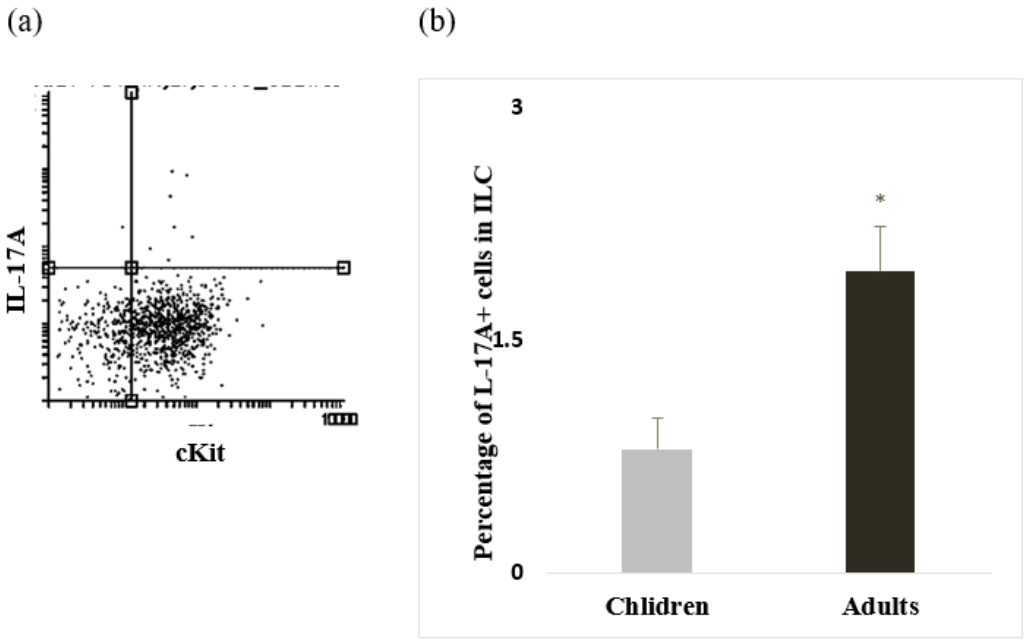


Figure 5.5: Comparison of the frequencies of IL-17A+ ILC cells in tonsillar MNC between children and adults. Representative FACS dot plot showing the frequency of IL-17A+cells in ILC population and IL-17A-producing cells were mainly from cKit+ ILC3 population (a). Comparison of the IL-17A+ ILC cells in tonsillar MNC between children (n=6) and adults (n=6). Results showing that IL-17A+ ILC% is higher in adults than children (*p<0.05) (b). Mean±SEM are shown.

5.3.2.2. The frequency of IL-22+ ILC3 is higher in children than in adults

The frequency of IL-22 producing ILC3 within the ILC population in tonsillar MNC of adults and children were analysed and expressed as the % of IL-22-producing ILC3 in ILC cells. As shown in figure (5.6a), IL-22 producing ILC3 were predominantly NKp44+ ILC cells. Furthermore, the frequency of IL-22+ ILC3 cells in tonsillar MNC of children was significantly higher than in adults (children =6, adults=6, p<0.05) (figure 5.6).

a b

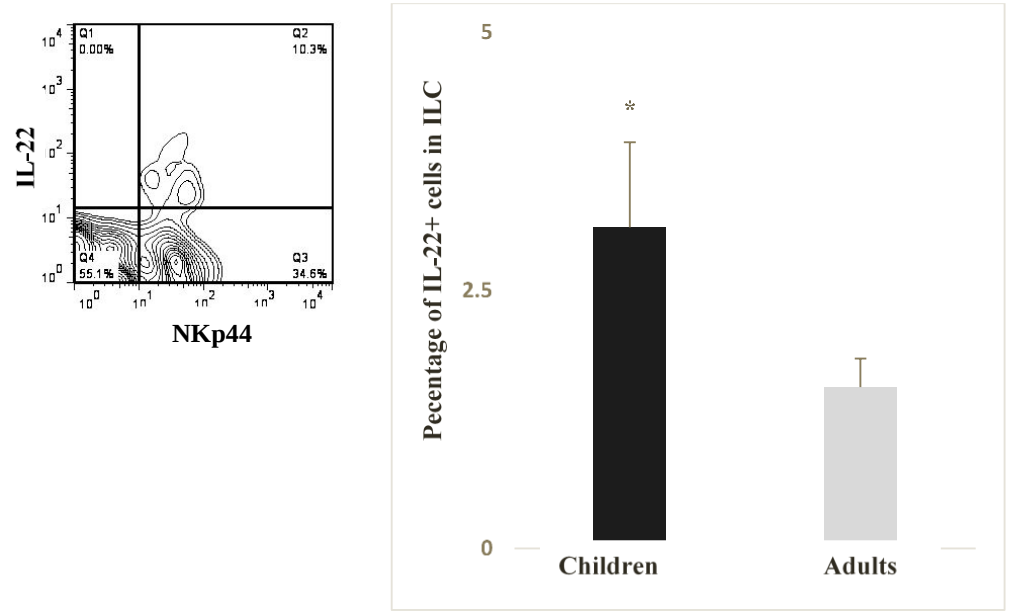


Figure 5.6: Detection of IL-22-producing ILC3 and Comparison of the frequency of IL-22+ ILC population in the tonsils of children and adults. A representative dot plot displays that IL-22+ ILC3 cells were predominantly from NKp44+ ILC3 population (a). Comparison of the IL-122+ ILC cells between the tonsils of children (n=6) and adults (n=6) samples. Results showed that IL-22+ ILC % is higher in children than adults (* $p<0.05$) (b). Mean±SEM is shown.

5.3.2.3 The ratio of IL-22+/IL-17+ ILC3 in children is markedly higher than in adults and associated with a lower Th-17 frequency

To determine whether there is any difference in IL-22 and IL-17A producing ILC3 frequencies between children and adults, and their potential relationship to Th-17 response, the ratio of IL-22+ to IL-17A+ ILC in tonsillar MNC was compared between children and adults.

The ratio was expressed as the percentage of IL-22+ ILC to IL-17A+ ILC cells. As shown in figure 5.8, the ratio of IL-22+/IL-17A+ ILC was significantly higher in children (4.0983±1.10763, n=6) than in adults (1.10763±0.11735, n=5) ($p<0.05$).

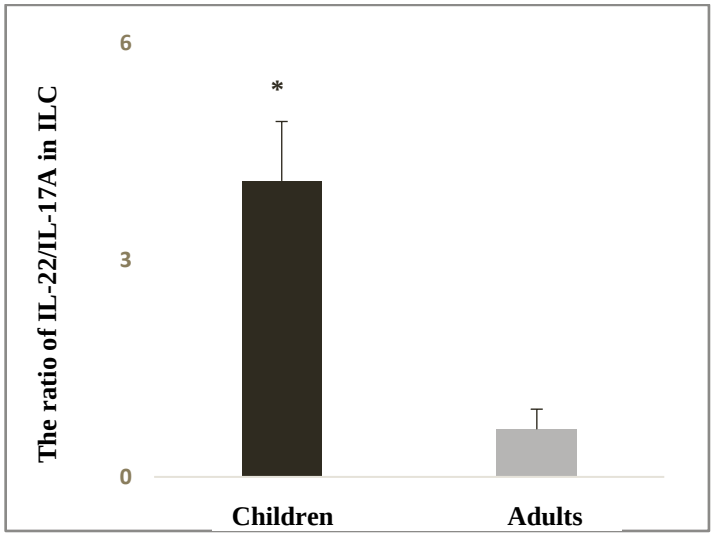


Figure 5.7: Comparison of the ratio of IL-22+/IL-17A+ ILC in tonsillar MNC between children and adults. The comparison of the ratio of IL-22+ ILC to IL-17A+ ILC population in tonsils of children and adults. Results analysed show a significantly higher ratio of IL-22+ to IL-17A/+ ILC3 population in children than in adults' samples. Children (n=6) and adults (n=5) (*p<0.05). Mean±SEM is shown. Figure b: comparison of Th-17 frequency between children and adults.

The marked difference between children and adults ILC3 in their capacity to produce IL-22 and IL-17 may suggest a potential functional difference of ILC3 between children and adults. So, we next analysed whether there is any relationship between the ILC3 capacity to produce IL-22 and Th-17 frequency in tonsillar MNC of children and adults. To measure the frequencies of Th-17 in tonsillar MNC, freshly isolated MNCs were analysed by staining for surface CD4 marker and intracellular cytokine staining for IL-17A after stimulation with PMA/Ion for 6 hours, and the frequency was expressed as the % of IL-17A+ CD4+ cells. A marked difference in Th-17 frequencies in tonsillar MNC between adults and children was shown (figure 5.8). The Mean±SEM frequency of IL-17A+CD4+ T cells in children tonsillar MNC was 0.8533±0.06179 compared to 2.75±0.30794 in adults. Showing that Th-17 was remarkably low in children samples (*p-value* < 0.0001).

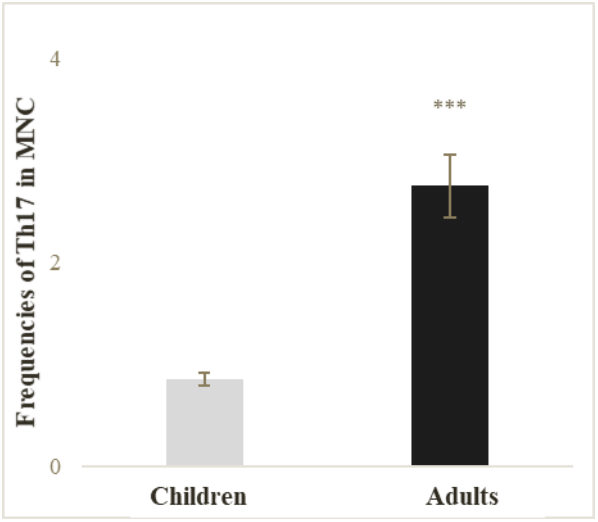


Figure 5.8: The frequency of Th-17 in tonsillar MNC in children and adults. The results of IL-17A CD4+ producing cells in freshly isolated MNCs showed significantly higher Th-17 cells in adults compared children (***=p<0.0001).

5.3.3 A higher ILC3 frequency in children is associated with *S. pneumoniae* carriage

We previously showed Th-17 frequency is low in children and increases with age, and that correlated with the reduction of *S. pneumoniae* carriage (Mubarak, 2014). As a higher IL-22+ ILC3 frequency appeared to be associated with a low Th-17 frequency in children as shown in this study, we further determined whether a higher ILC3 frequency was associated with a higher pneumococcal carriage rate in children. As NKp44+ ILC3 is associated with IL-22 expression, we also determined the relationship between NKp44+ILC3 frequency and pneumococcal carriage.

To study the association between *S. pneumoniae* carriage and ILC3 frequency in NALT, ILC3 frequency in adenotonsillar MNC was analysed by flow cytometry using Lin-ve, CD127+ and cKit+ and expressed as the percentage of tonsillar MNC. Pneumococcal carriage was defined by positive *S. pneumoniae* culture+.

As shown in figure (5.9), the carriage positive subjects had higher ILC3 frequency in tonsillar MNCs than in carriage negative children. (n=10 for culture+, and n=22 culture-, **=p<0.01).

When the ILC3 frequency in adenoidal MNC was compared, a more marked difference was shown between pneumococcal carriage+ and carriage- children (n=4 for culture+, and n=5 culture-, **=$p<0.01$) (figure 5.10).

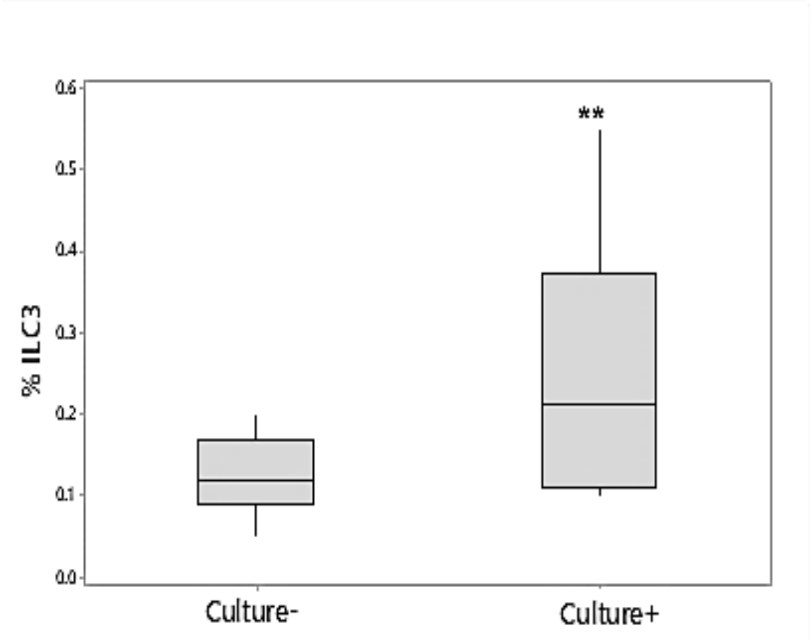


Figure 5.9: ILC3 frequency in tonsillar MNC with *S. pneumoniae* carriage in children. The relationship of ILC3 frequency in children tonsillar MNCs with the *S. pneumoniae* carriage. Data represent the medians and interquartile range of tonsillar ILC3 ratio in both *S. pneumoniae* carriage + and carriage -. Results showed that children's tonsils with *S. pneumoniae* carriage had a significant higher percentage of ILC3 than in *S. pneumoniae* children (n=10 for culture+, and n=22 culture-, **$p<0.01$).

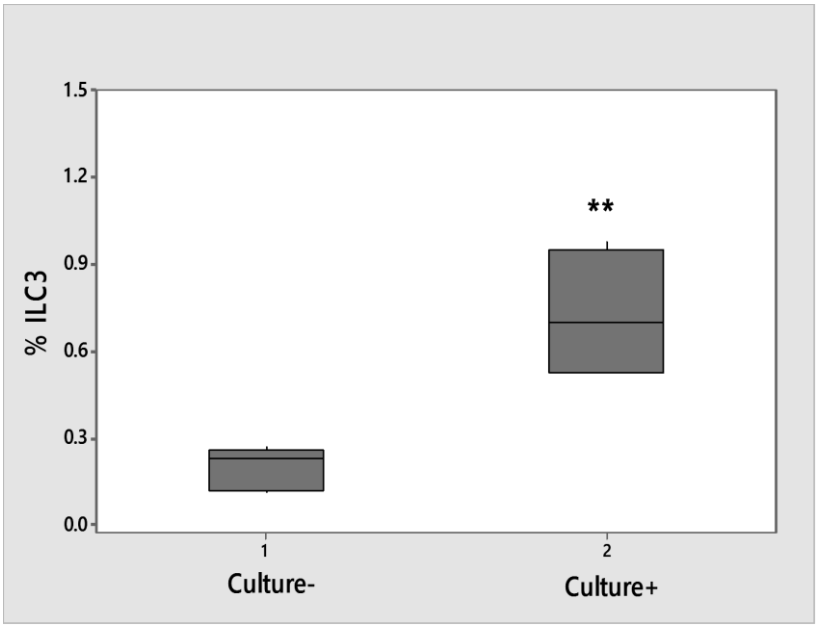


Figure 5.10: The relationship between the frequency of ILC3 in adenoidal MNC and *S. pneumoniae* carriage in children. Results represented by the medians and interquartile range of adenoidal ILC3 ratio in both *S. pneumoniae* carriage + and carriage -. The analysis showed that ILC3 ratio in children's adenoid with *S. pneumoniae* carriage is significantly higher than in carriage negative. (**=$p<0.01$). (n=4 for culture+, and n=5 culture-).

We further examined the relationship between the frequency of NKp44+ILC3 or NKp44-ILC3 in tonsillar MNC and *S. pneumoniae* carriage in the nasopharynx of children. As shown in figure 5.11, there was a significantly higher frequency of NKp44+ILC3 in children with *S. pneumoniae* carriage than in those carriage-negative children (figure 5.11a, $p<0.01$). However, there was no difference in NKp44- ILC3 frequency between carriage + and carriage – children (figure 5.11b, n=10 and 20 respectively for culture+ and culture-, $p<0.05$).

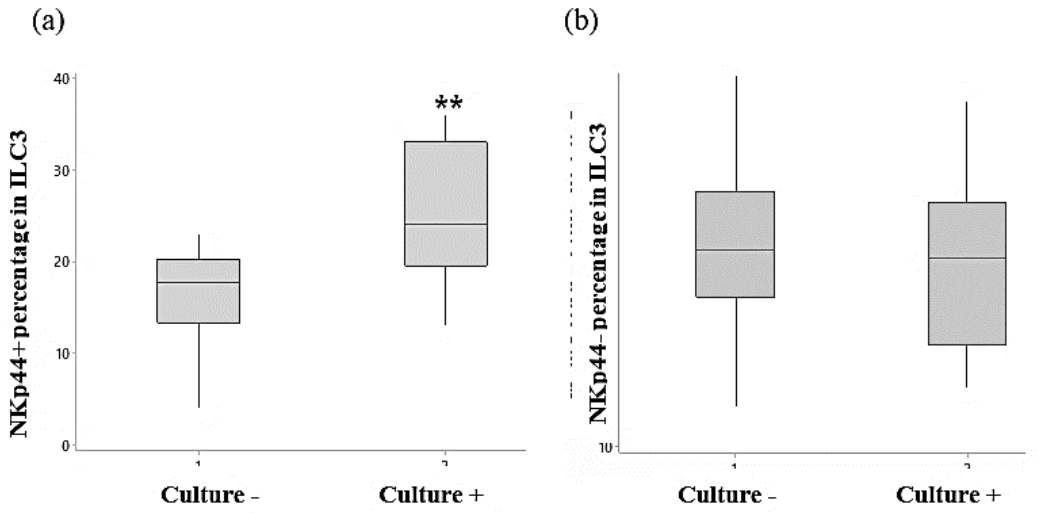


Figure 5.11: Comparison of the frequency between NKp44+ILC3 and NKp44-ILC3 in tonsillar MNC in relation with *S. pneumoniae* carriage in the nasopharynx of children. The analysis showed that the frequency of NKp44+ILC3 in children with *S. pneumoniae* carriage positive was significantly higher than in those carriage negative children (a). In contrast, there was no significant difference in NKp44- ILC3 frequency between carriage + and carriage – children (b). (n=10 and 20 respectively for culture+ and culture-, $**p<0.01$).

5.3.4. The effect of *S. aureus* and *S. pneumoniae* CCS on ILC population

It would be interesting to know the interactions between the bacterial carriage and ILC in the human nasopharynx. It has been well reported that local microbiome interacts and influences ILC in the gastrointestinal tract (Hepworth *et al*, 2013; Hepworth *et al.*, 2015; Li, *et al.*, 2017). To study the effect of *S. aureus* and *S. pneumoniae* on ILC population, tonsillar MNCs from three samples (S1: CD45RO-Naïve MNCs from child samples, S2: Whole MNCs cells from child samples and S3: Whole-cell population MNCs obtained from adult tonsils). MNCs were co-cultured with two *S. aureus* CCS or *S. pneumoniae* CCS (Spn) for 72hr at 37ºC and in the last six hours with the co-incubation of BFA. Two *S. aureus* CCS used were generated from two different strains one was superantigenic (Sau-Sag+) and the second was non-superantigenic (Sau-SAg-) strain, respectively. The cells were collected and stained for ILC by surface markers, and intracellular markers,

followed by flow-cytometry. Changes in the percentages of total ILC population were recorded following *S. aureus* or *S. pneumoniae* CCS stimulation compared with medium control (M).

As shown in figure (5.12). ILC number was reduced following both *S. aureus* CCS stimulation (Sau-Sag+ and SauSAg-), but increased after Spn CCS stimulation, all compared to the control group (M). When statistically analysed, the effect of *Sau* CCS on ILC for samples for nine samples caused a significant reduction in ILC frequency (n=9, $p<0.001$, figure 5.13).

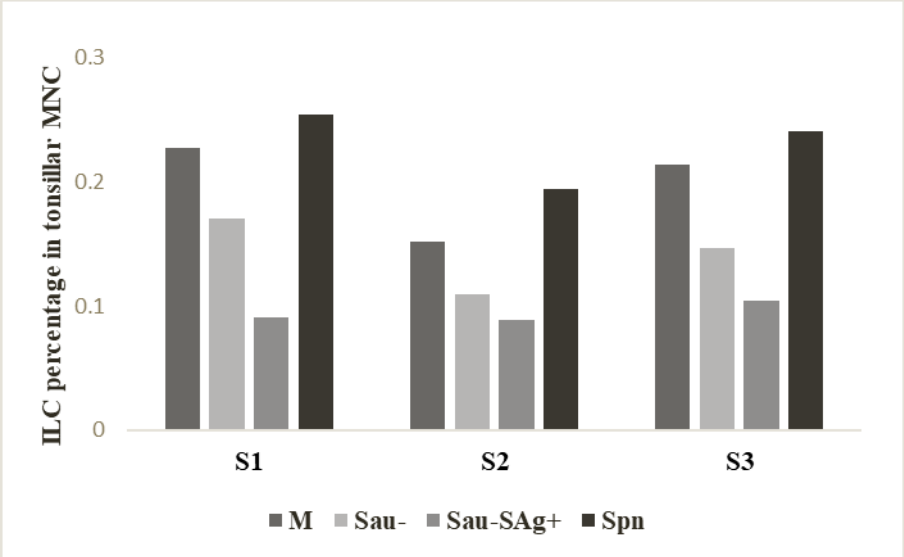


Figure 5.12: The effect of *S. aureus* and *S. pneumoniae* CCS stimulation on ILC in tonsillar MNCs. The stimulation of three tonsillar MNC samples (from 3 subjects) by superantigenic *S. aureus* (Sau-SAg+), non-superantigenic *S. aureus* (SauSAg-) and *S. pneumoniae* (Spn) showed adverse effects on ILC cells. After both *S. aureus*, CCS stimulation (SAg+ and SAg-) ILC population reduced. In contrast, an increase in ILC number was noticed following *S. pneumoniae* CCS stimulation in comparison to the control group (M). ILC frequencies (% in MNC) are shown.

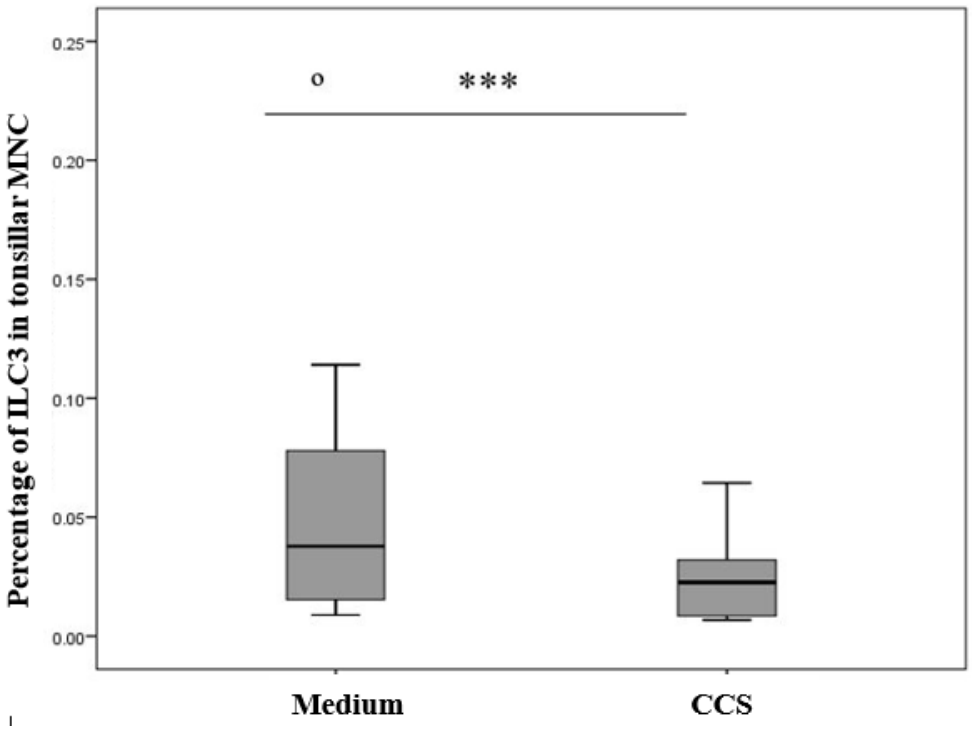


Figure 5.13: The effect of *S. aureus* CCS on ILC3 population in MNCs. comparisons showed a significant reduction in ILC3 population in tonsillar MNCs upon stimulation with *S. aureus* CCS compared to medium control. Results were expressed as Median and interquartile range (n=9, ***p<0.001). (^{o}=outlier).

5.3.4.1 HLA-DR expression in ILC3 population reduced following *S. aureus* stimulation

In addition to the previous findings of the reduction in ILC population, HLA-DR expression was also analysed in ILC3 upon the stimulation with *S. aureus* CCS. Wilcoxon ranked test showed that there is a significant reduction in HLA-DR+ ILC3 frequency after Sau SAg- CCS stimulation (figure 5.14, n=9, p<0.001).

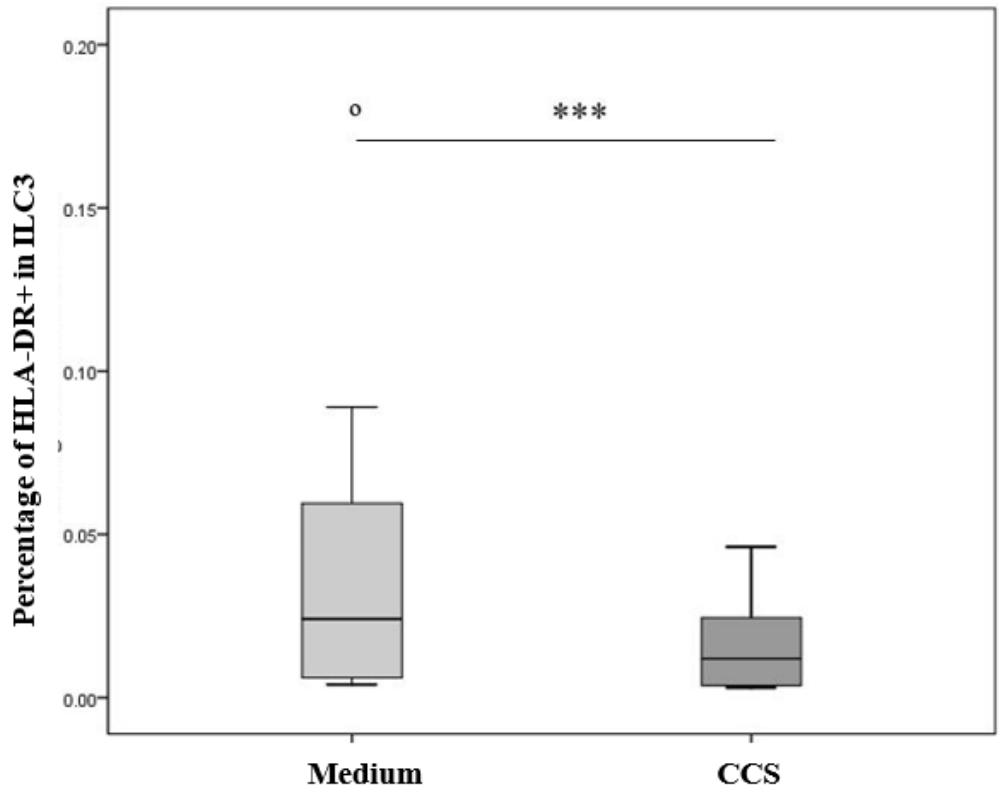


Figure 5.14: The effect of *S. aureus* CCS (SAg-) on HLA-DR expression in ILC3 population. results showed a pattern of reduction HLA-DR + ILC3% upon the stimulation with *S. aureus* CCS. Results are expressed as Median and interquartile range (n=9, ***, $p<0.001$). (º=outlier).

5.3.5 The effect of RORγt inhibitor (GSK 805) on Th-17 and ILC3 responses.

As RORγt has been reported to be a transcription factor for both Th-17 and ILC3 differentiation, we examined if RORγt inhibition would have any effect on *S. aureus* activated Th-17 and ILC3 response. GSK805, RORγt Inverse Agonist II, was used for blocking IL-17A from Th-17 cells in an optimum concentration of 0.5µM. MNCs were co-incubated with GSK 805 for 8 hrs at 37Cº and then one group of cells was stimulated with *S. aureus CCS* and incubated for overnight at 37Cº. Then, cells were collected and stained, and data were analysed by flow cytometry.

Results showed a significant reduction in the proportion of IL-17A+ cells in tonsillar MNCs ($p<0.01$) and in the proportion of IL-17A+ cells (Th-17) in CD4+ T cell population ($p<0.001$) in tonsillar MNC pre-incubated with RORγt inhibitor (GSK805) when compared to that stimulated with *S. aureus* CCS alone as control (n=14). (figure 5.15. a and b respectively).

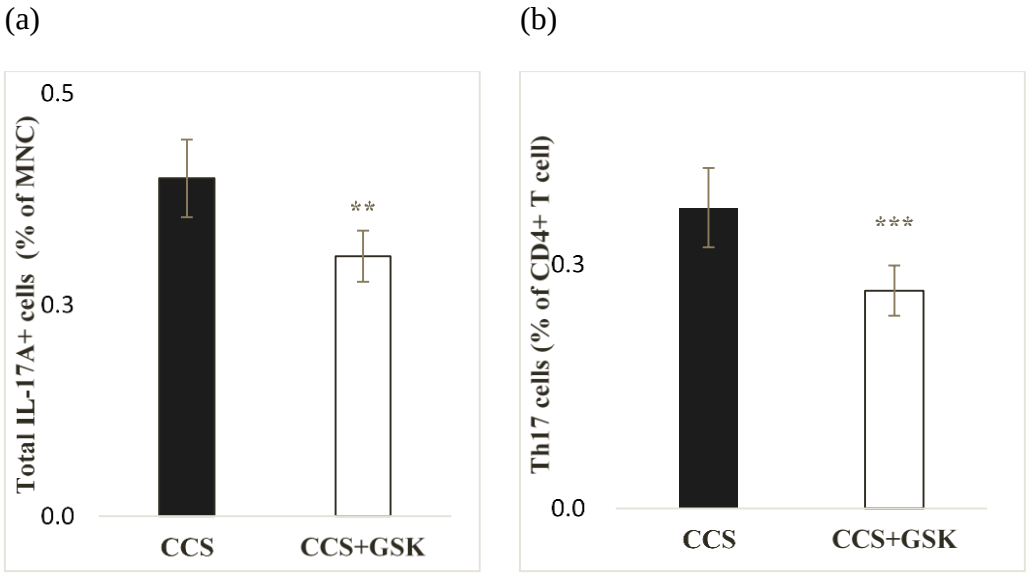


Figure 5.15: The effect of RORγt invertase inhibitor on the expression of IL-17A in tonsillar MNCs and CD4+ T cells. After stimulation with *S. aureus* CCS, Paired T-test analysis showed that blocking with GSK805 (0.5µM) inhibited *S. aureus* CCS-activated IL-17A responses in total IL-17A+ cells of tonsillar MNC(a), and CD4+Th cells (Th-17) in tonsillar MNCs (b). (**$p<0.01$, ***$p<0.001$, n=14). Mean±SEM is shown.

Interestingly, when the effect of GSK805 on *S. aureus* CCS stimulated ILC3 was analysed, the significant decrease in ILC3 number caused by *S. aureus* CCS stimulation appeared to be moderated by the addition of GSK805. (figure 5.16, n=6, $p<0.05$).

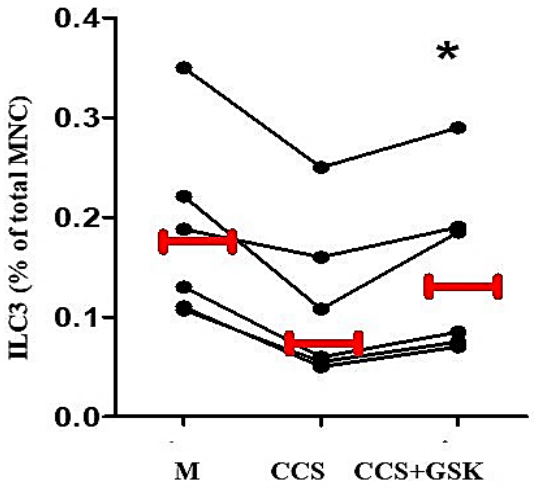


Figure 5.16: Effect of GSK805 on *S. aureus* CCS-induced reduction in ILC3 number in tonsillar MNC. Comparison in ILC3 percentage between MNC treated by *S. aureus* CCS and that by *S. aureus* CCS with the addition of GSK805 (0.5µM). A significant decrease in ILC3 % upon CCS treatment. While when GSK805 was added, ILC3 % was moderately decreased compared to CCS-treated. (n=6, *$p<0.05$). Medians (red lines) are shown.

The inhibitory effect of GSK805 on Th-17 response in tonsillar MNC was further confirmed by the reduction of IL-17A production form MNCs. IL-17A concentration (measured by ELISA) in tonsillar cell culture supernatant following co-culturing with GSK805 was analysed by paired t-test. The results showed a significant reduction in IL-17A concentration when MNCs were co-cultured with GSK805 and CCS in comparison with *S. aureus* CCS stimulation alone (figure 5.17, n=8, $p<0.01$).

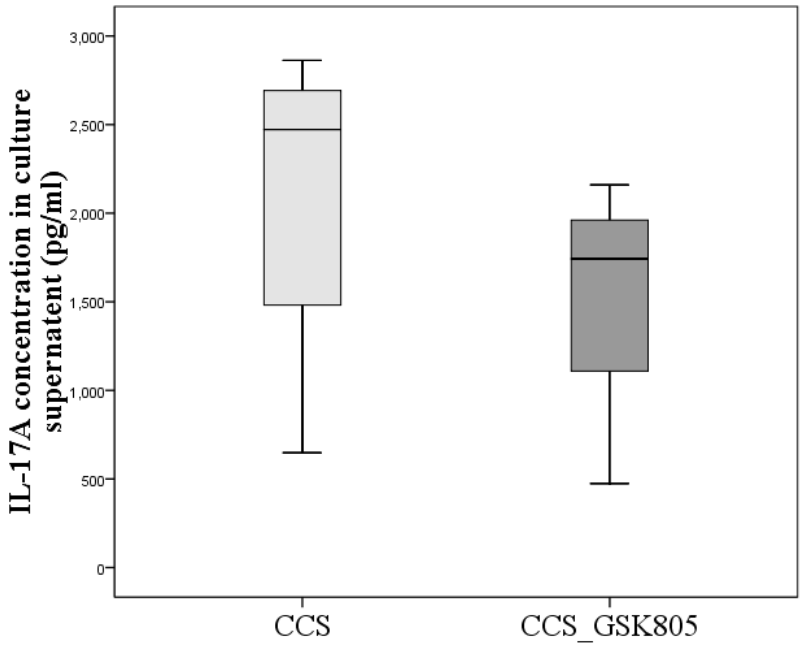


Figure 5.17: The effect of GSK805 on the production of IL-17A in tonsillar MNC after stimulation with *S. aureus* CCS. Paired T-test analysis showed that after 72 hr of CCS stimulation with GSK805 (0.5µM) co-incubation in the MNC culture, GSK805 significantly reduced IL-17A production in tonsillar MNC culture supernatants compared to the *S. aureus* stimulation alone (n=8,**$p<0.01$). Mean±SEM is shown.

5.4 Discussion

In this chapter, I focused on studying the relationship and functional diversity between ILC3 and Th-17 in both children and adults, besides, on ILC3 association with *S. pneumoniae* carriages in the nasopharynx. I also studied the effect of RORγt inhibitor on both Th-17 and ILC3 activation in NALT.

Firstly, I identified ILC and ILC3 population using a number of phenotypic markers including Lin-1, CD127, cKit and NKp44. Lin-ve and CD127+ cells were identified as total ILC and among these ILC, cKit+ cells were identified as ILC3 (figure 5.3). Within the total ILC, ILC3 were the predominant cell population. These data were consistent with previously published data (Bjorklund *et. al*, 2016 and Bar-Ephraim *et. al*, 2017). In a study conducted by Bjorklund *et. al*, (2016), ILC3 were analysed within three tonsillar samples using RNA expression analyses and showed ILC3 were the predominant cell population among ILC cells. NKp44+ ILC3, the predominant IL-22 producing ILC3 population, may play a critical role in keeping local mucosal homeostasis and immune regulation in the nasopharynx.

Next, I investigated the ability of cytokine expression including IL-17A and IL-22 in the ILC3 cells and whether there is any difference between adults and children. Also, I studied if these potential differences will have any association with bacterial carriage status in nasopharyngeal tissue from childhood towards adulthood. Several previous publications reported the functional properties of ILC3 cells including their cytokine production, contribution to CD4+ T cell regulation, the regulatory role during bacterial infection and inflammatory responses (Cella et al., 2009; Sonnenberg et al., 2012; Hepworth et al., 2013; Spits, et al., 2013; Hepworth et al., 2015; Bjorklund et al., 2016 and Bar-Ephraim et al., 2017). Most of these data were collected from murine models and involved ILC3 cells in the gastrointestinal tract. There is limited data available regarding the interactions between ILC3 with bacterial carriages in the nasopharynx of children and adults.

After studying ILC3 frequencies in adenotonsillar MNCs, I compared the ability of IL-22 and IL-17A production between tonsillar ILC3 in children and adults, and the results show that there is a distinct difference in their frequencies between adults and children. The frequency of IL-22+ ILC in children was significantly higher than in adults (Figure 5.6 a and b). In contrast, the frequency of IL-17A+ ILC3 in adults

was higher than in children (Figure 5.5 a and b). These findings would support the hypothesis that in children the predominance of IL-22 producing ILC3 may mediate a stronger regulation/suppression of pro-inflammatory Th-17 response than in adults. Subsequently, I showed that this pre-dominance of IL-22 producing ILC3 over IL-17 producing ILC3 in children is associated with a markedly lower Th-17 frequency in tonsillar MNC in children than in adults (figure 5.7). The results also showed that Th-17 (IL-17A+CD4+ T cells) were significantly higher in adults than in children (Figure 5.8 a, b), which are consistent with our group's previously published work on the relationship of Th-17 cell frequency in NALT and age and a stronger Th-17 response in adults was associated with a reduction of pneumococcal carriage (Mubarak, 2014).

Considering the important role of IL-22 and ILC3 in mucosal homeostasis and immune regulation, this study, hypothesized that the predominance of IL-22 producing ILC3 in NALT of children may promote local homeostasis by inhibiting Th-17 activation and thereby favour pneumococcal carriage. Earlier studies have shown a critical role for Th-17 cells in bacterial clearance including *S. pneumoniae* (Lu et al., 2008) which is one of the most common colonisers in the nasopharynx of children. therefore, the relationship between ILC3 frequency and *S. pneumoniae* carriage was examined in the nasopharynx of children. Results showed that the ILC3 frequency in adenotonsillar MNC was significantly higher in *S. pneumoniae* carriage positive children than in carriage negative children (figure 5.9 and 5.10 respectively). Furthermore, that demonstrated it was the higher frequency of NKp44+ILC3, but not of NKp44- ILC3 that was associated with *S. pneumoniae* carriage in children. These results suggest ILC3 may play an important role in mediating pneumococcal carriage in children (figure 5.11 a and b), likely through the IL-22 producing NKp44+ ILC3 which may inhibit Th-17 response in NALT.

It has been reported in a mouse model that there was a significant increase in IL-22+ ILC3 numbers during *S. pneumoniae* infection. Nevertheless, this increase in the ILC3 frequency did not aid the clearance of this infection (Van Maele *et. al,* 2014). The results are consistent with the result of the study mentioned above as it demonstrated the increase in ILC3 population in carriage positive children. A higher NKp44+ ILC3 frequency (but not NKp44- ILC3) was associated with *S.*

pneumoniae nasopharyngeal carriage in children (Figure 5.11 and figure 5.12 a and b) would support the notion of this ILC3 cell subset might be crucial in immune regulation and homeostasis and promoting pneumococcal carriage status in children.

Next, the effects of both *S. aureus* and *S. pneumoniae* CCS stimulation on ILC including ILC3 population in tonsillar MNC was investigated. There was a significant reduction of ILC population upon the stimulation by *S. aureus* CCS (Figure 5.13). On the effect of *S. pneumoniae* CCS stimulation on ILC population, it was interesting to find there was an increase in the ILC population following *S. pneumoniae* stimulation, which was in contrast to the reduction found in ILC upon the stimulation by *S. aureus*, either by a super-antigenic (Sau-SAg+) strain or by a non-superantigenic strain (sua SAg-) (Figure 5.12). These results suggest a differential effect of *S. aureus* and *S. pneumoniae* on ILC3 population and propose a differential interaction between the two bacteria and ILC population. As *S. aureus* stimulation also elicited a potent response of Th-17 cells in NALT, it would be interesting to study whether the reduction of ILC3 following *S. aureus* stimulation is associated with the potent Th-17 response. Hepworth *et. al.,* (2015) reported that in paediatric patients with colonic inflammation, there was an ILC3 reduction in the affected patients and that was accompanied by a Th-17 expansion at the same time.

To further examine the possible effect of *S. aureus* on ILC3 function, tonsillar MNC were stimulated by *S. aureus* CCS and analysis for HLA-DR expression within ILC3 was performed. Interestingly, there was a significant reduction of HLA-DR expression in ILC3 population following *S. aureus* CCS stimulation (Figure 5.14). Previously, it has been proposed that HLA-DR+ (MHC-II) ILC3 were an important cell population that has a role in controlling the tissue homeostasis by presenting antigens to antigen-dependent CD4+ T lymphocytes (Bjorklun*d et. al,* 2016). The results here in this study suggest a possible effect of *S. aureus* on HLA-DR + ILC3 which has been reported as a Th-17 regulator to inhibit the Th-17 response. Such effect of *S. aureus* CCS on the reduction of ILC3 population in NALT tissue may contribute to the compromise of immune-modulatory properties in the mucosal immune tissue and leads to a potent Th-17 activation upon *S. aureus* infection.

In the last part of this study, I investigated the possible differential effects of RORγt inhibitor (GSK805) on the Th-17 and ILC responses activated by *S. aureus* stimulation in tonsillar tissue. The results indicate that there was a significant inhibitory effect of GSK805 on the Th-17 activation by *S. aureus* CCS (figure 5.15). By contrast, GSK805 pre-incubation appeared to prevent or reduce the *S. aureus* CCS effect on ILC3 cells (figure 5.16). IL-17A levels in tonsillar MNC culture supernatants were measured and the result are consistent with the earlier results showing inhibition of Th-17 response by GSK805 (figure5.17), as most of the IL-17A production were from Th-17 cells. These results suggest that a pro-inflammatory Th-17 over-activation during infection or prolonged tissue inflammation, which could potentially cause autoimmune conditions, may be regulated by temporarily inhibiting RORγt transcription without affecting the ILC3 population (Wither *et al*., 2015).

5.4.1 the limitation that interpreted our outcome in this chapter:

During the lab work of this chapter there were some limitations that need to address.

1. The experiment was first local (saliva) and serum IL-17A levels but due to low levels of IL-17A in serum samples, we collected data from local salivary samples.
2. The optimisation process took several attempts and due to the relatively low volumes collected from each subject, the experiments was limited to small sample size.
3. Three groups of samples were chosen depending on their carriage status. The value of this experiment was to investigate the relationship between S. aureus carriage and the local IL-17A in NALT.

Despite the fact that the sampling size was low especially when was compared to the samples size that used for the nasal swabs culture, our results showed a pattern of increase in local IL-17A combined with *S. aureus* carriage. Therefore, these results encourage future detailed cohort-study to find the association between the salivary-IL-17A with nasopharyngeal carriage.

Chapter 6

CONCLUSION

Chapter six: Conclusion

6.1 Conclusion

In this project, I have observed an age-related inverse relationship in nasopharyngeal carriage rates between *S. pneumoniae* and *S. aureus* in our studied children and adults. *S. pneumoniae* carriage rate was highest in younger children then decrease with age, while *S. aureus* carriage rate was low in young children and increased in older children to adults. When the relationship of the local salivary IL-17A levels (measured by ELISA) and the nasopharyngeal carriages was examined, I found *S. aureus* carriage was associated with the highest IL-17A levels in saliva samples, as compared to *S. aureus* carriage negative samples There was a trend to show a modest increase in salivary IL-17A concentration in *S. pneumoniae* carriage positive subjects as compared to those who were culture negatives, although the increase was not reaching significance, possibly due to the small sample size. These results indicate a strong local Th-17 response activated by *S. aureus* colonisation. This hypothesis was supported by ex-vivo stimulation of adenotonsillar cells (MNC) with culture extract (CCS) generated from *S. aureus* which revealed a marked CD4+IL-17A+(Th-17) cell response by this stimulation. These results support the hypothesis that *S. aureus* carriage in the nasopharynx is associated with activation of local Th-17 response in human NALT tissues.

A genetically inactivated *S. aureus* α-haemolysin (HlaH35L) protein antigen has been studied in detail and shown to be a candidate for the Th-17 activation in NALT. This result was supported by screening the CD4+ IL-17A+ response in tonsillar MNC using several conserved recombinant proteins from *S. aureus*. HlaH35L activated significant CD4+ proliferation and Th-17 response in tonsillar MNC. These findings were also supported by measuring cytokine concentrations of different CD4+ Th subsets in response to HlaH35L stimulation (cytokines concentration produced by Th-1, Th-2, Th-17, and Tfh cells were detected by CBA). HlaH35L elicited Th-17 effector cytokines (IL-17A/IL-17F) and IL-6 in tonsillar MNC. As IL-6 known to be involved in Th-17 induction from previous studies, the activation of IL-6 production by HlaH35L suggest that HlaH35L may not only activates memory Th-17 cells but also involved in the induction of Th-17 cells from naïve CD4+ T cells during *S. aureus* colonisation in the nasopharynx.

The effect of the native form of α-haemolysin was also examined subsequently. When comparing between CCS from Hla producing USA300 *S. aureus* strain (wild type: wt.) and CCS generated from isogenic mutant strain (Hla-), the former (wt CCS) stimulated significantly higher Th-17 response in tonsillar MNC than that by Hla- CCS. Therefore, HlaH35L, as a non-toxic protein antigen that activates a marked Th-17 response, maybe a candidate antigen with the potential to induce protection against *S. aureus* infections.

Innate lymphoid cells (ILC3) may be particularly important in maintaining local mucosal homeostasis for young children. Nevertheless, they may promote *S. pneumoniae* carriage by maintaining homeostasis in the nasopharynx. The analysis showed that numbers of IL-22+ ILC3 cells in children were higher than in adults. Besides, NKp44+ ILC3 cells were shown to be higher in both tonsillar and adenoidal MNC in *S. pneumoniae* carriage positive than in carriage negative children. These findings support the hypothesis that ILC3 cells in NALT may play an important role in pneumococcal carriage.

By contrast, IL-17A+ ILC cells were shown to be more dominant in adults than in children. This is consistent with the finding that the Th-17 frequency was significantly higher in adults compared to children. These findings may suggest a regulatory role for ILC population on Th-17 cell response, particularly during childhood, to prevent possible Th-17 mediated inflammatory pathology due to the continuous exposure to bacterial pathogens in the nasopharynx.

The results from this study suggest that *S. pneumoniae* colonisation in the nasopharynx may promote IL-22+ ILC3 cells, which in turn would provide a favourable environment for *S. pneumoniae* carriage. On the other hand, *S. aureus* colonisation may result in a reduction of IL-22+ILC3 cells, and that would promote an enhanced Th-17 response.

S. aureus appeared to negatively affect the ILC3 population, as it was shown to be associated with a reduction in ILC cells when adenotonsillar MNC were stimulated with *S. aureus* CCS. Furthermore, when analysing the effect of *S. aureus* CCS on HLA-DR+ expression on ILC population, a reduction in HLA-DR+ ILC cells was observed. This may suggest a significant role of HLA-DR+ ILC3 in the regulation of Th-17 response, and the absence/defect of HLA-DR+ ILC3 cells may activate an enhanced Th-17 response upon the exposure to Th-17-activating pathogens.

Finally, GSK-805 (a RORγt Inverse Agonist) was shown to selectively inhibit the Th-17 response in tonsillar MNC activated by *S. aureus* stimulation, but not inhibit ILC3 cells. GSK-805 pre-incubation with tonsillar MNC appeared to inhibit the reduction in ILC number caused by *S. aureus* stimulation. These results may have therapeutic implications. A proinflammatory Th-17 over-activation caused by infection, which could potentially cause autoimmune conditions, may be regulated by temporarily inhibiting RORγt transcription, as this inhibition will reduce the over-active Th-17 response without affecting ILC3 cells.

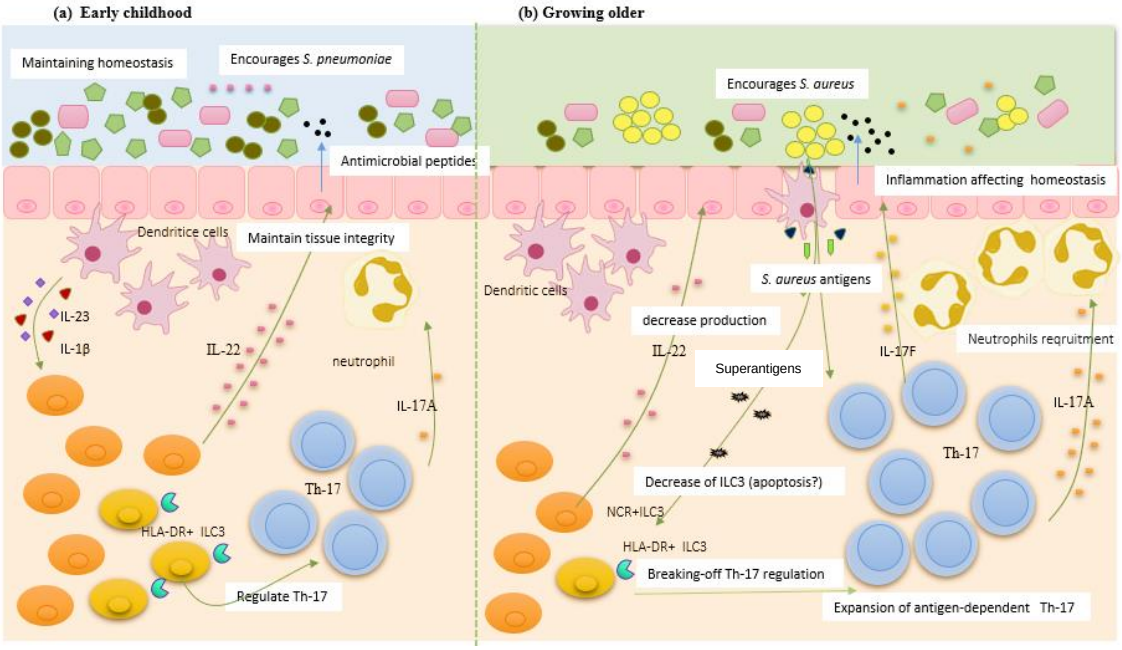


Figure 6.1: The relationship between Innate lymphoid cells 3 (ILC3) and Th-17 and their effect on nasopharyngeal colonisation.

The figure shows the proposed relationship between ILC3 and Th-17 cells in both early childhood and during aging process and their effect of nasopharyngeal colonisation. In early childhood (a), the abundancy of NCR+ ILC3 in the tissue produces substantial IL-22 that maintains the tissue integrity. In addition to HLA-DR+ ILC3 that regulates Th-17 proliferation. So, IL-22/IL-17A ratio in the tissue is kept high. Thus, S. *pneumoniae* colonisation in nasopharynx nourishes. During the aging process and with continuous exposure to pathogenic bacteria, Th-17 cells increase (specially antigen-dependent Th-17). When the introduction of *S. aureus* occurs, both NCR+ILC3 and HLA-DR expression reduced. Although the mechanism of reduction is not clear yet, but the effect of *S. aureus* antigens like superantigens and Hla toxin is clearly proved to massively stimulates Th-17 expiation and IL-17A production. Therefore, the balanced environment of IL-22/IL-17A is no longer maintained neither *S. pneumonia* residence. That eventually, favours the establishment of *S. aureus* colonisation (b).

6.2 Future studies

The results in the study add important information to our understanding about the immune regulation of nasopharyngeal carriage of common bacterial carriages including *S. pneumoniae* and *S. aureus* and their interactions. *Ex*tensive studies in this area will be important to improve our understanding. These may include the following:

- Studying the role and the mechanisms of NKp44+ ILC3 in mediating *S. pneumoniae* carriage in the nasopharynx.
- Investigating the molecular basis of HLA-DR+ ILC3 regulation to Th-17 in human NALT.
- Study of the effect of HlaH35L on B-cell antibody response in human NALT
- Identification of the components in *S. aureus* CCS that activate a strong Th-17 response in human NALT.

A more detailed study on the possible therapeutic inhibitory effect of GSK-805 on over-active Th-17 response in adenotonsillar MNCs activated by pathogenic bacteria such as superantigenic *S. aureus*.

Chapter 7

REFERENCES

1. Acosta-Rodriguez, E. V., Napolitani, G., Lanzavecchia, A., & Sallusto, F. 2007. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17–producing human T helper cells. Nature Immunology, 8(9), 942-949.

2. Archer, N. K., Adappa, N. D., Palmer, J. N., Cohen, N. A., Harro, J. M., Lee, S. K., . . . Shirtliff, M. E. 2016. Interleukin-17A (IL-17A) and IL-17F Are Critical for Antimicrobial Peptide Production and Clearance of Staphylococcus aureus Nasal Colonization. Infection and Immunity, 84(12), 3575-3583.

3. Archer, N. K; Harro, J. M, and Shirtliff, M. E. 2013. Clearance of Staphylococcus aureus nasal carriage is T cell dependent and mediated through interleukin-17A expression and neutrophil influx. Infect Immun 81:2070–2075.

4. Arlian, B. M., & Tinker, J. K. 2011. Mucosal Immunization with a Staphylococcus aureus IsdA-Cholera Toxin A2/B Chimera Induces Antigen-Specific Th2-Type Responses in Mice. Clinical and Vaccine Immunology, 18(9), 1543-1551.

5. Askarian, F., Ajayi, C., Hanssen, A.-M., van Sorge, N. M., Pettersen, I., Diep, D. B., . . . Johannessen, M. 2016. The interaction between Staphylococcus aureus SdrD and desmoglein 1 is important for adhesion to host cells. Scientific Reports, 6, 22134.

6. Askarian, F., Uchiyama, S., Valderrama, J. A., Ajayi, C., Sollid, J. U., Sorge, N. M. Johannessen, M. 2016. Serine-Aspartate Repeat Protein D Increases Staphylococcus aureus Virulence and Survival in Blood. Infection and Immunity, 85(1), 330.

7. Bae, S., Yu, J. Y., Lee, K., Lee, S., Park, B., & Kang, Y. 2012. Nasal colonization by four potential respiratory bacteria in healthy children

attending kindergarten or elementary school in Seoul, Korea. Journal of Medical Microbiology, 61(5), 678-685.

8. Bagnoli, F., Fontana, M. R., Soldaini, E., Mishra, R. P., Fiaschi, L., Cartocci, E., . . . Grandi, G. 2015. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection againstStaphylococcus aureus. Proceedings of the National Academy of Sciences, 112(12), 3680-3685.

9. Bar-Ephraim, YE, Konijn, T,Den Haan, JMM, Reijmers, RM, Mebius, RE, Cornelissen, F,Papazian, N, Hoogenboezem, RM, Sanders, MA, Cupedo, T, Westerman, BA, Gönültas, M, and Kwekkeboom, J. 2017. 'Cross-Tissue Transcriptomic Analysis of Human Secondary Lymphoid Organ-Residing ILC3s Reveals a Quiescent State in the Absence of Inflammation', Cell Reports, vol. 21, no. 3, pp. 823–833.

10. Becherelli, M., Prachi, P., Viciani, E., Biagini, M., Fiaschi, L., Chiarot, E., . . . Manetti, A. G. (2013). Protective Activity of the CnaBE3 Domain Conserved among Staphylococcus aureus Sdr Proteins. PLoS ONE, 8(9).

11. Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. & Kuchroo, V. K. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH-17 and regulatory T cells. Nature, 441, 235–238.

12. Bhakdi, S., & Tranum-Jensen, J. 1991. Alpha-toxin of Staphylococcus aureus. Microbiological Reviews, 55(4), 733-751.

13. Bjorklund, A, Forkel, M., Picelli, S., Konya, V., Theorell, J., Friberg, D., Sandberg, R., & Mjosberg, J. n.d. 2016.'The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing', Nature Immunology, 17 (4), 451-460.

14. Bogaert, D, A van Belkum, M Sluijter, A Luijendijk, R de Groot, H C Rümke, H A Verbrugh, and P W M Hermans. 2004. "Colonisation by Streptococcus Pneumoniae and Staphylococcus Aureus in Healthy Children." Lancet (London, England) 363 (9424): 1871–72.

15. Bogaert, D, A van Belkum, M Sluijter, A Luijendijk, R de Groot, H C Rümke, H A Verbrugh, and P W M Hermans. 2004. “Colonisation by Streptococcus Pneumoniae and Staphylococcus Aureus in Healthy Children.” Lancet (London, England) 363 (9424): 1871–72.

16. Bogaert, D., De Groot, R. & Hermans, P. W. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet. Infect. Dis. 4, 144–154.

17. Brady, R.A., Mocca, C. P., Prabhakara, R., Plaut, R. D., Shirtliff, M. E. Merkel, T. J., Burns, D. L. 2013. Evaluation of Genetically Inactivated Alpha Toxin for Protection In Multiple Mouse Models Of Staphylococcus aureus Infection. Plos ONE 8 (4).

18. Brandtzaeg, P. 2015. Chapter 103: Immunobiology of the Tonsils and Adenoids. Mucosal Immunology, 1985–201.

19. Broere, F., Apasov, S. G., Sitkovsky, M. V., & Eden, W. V. 2011. A2 T cell subsets and T cell-mediated immunity. Principles of Immunopharmacology, 15-27.

20. Bronner, S. M., Zbieg, J. R., & Crawford, J. J. 2016. RORγ antagonists and inverse agonists: A patent review. Expert Opinion on Therapeutic Patents, 27(1), 101-112.

21. Brooks, G. F. and Carroll, K. C. 2007. The Streptococci. in Brooks GF, C. K., Butel JS and Morse SA, ed. Jawetz, Melnick, and Adelberg's Medical Microbiology 24th Edition ed., Blacklick, OH, USA: McGraw-Hill Medical Publishing Division, 233-248.

22. Bubeck Wardenburg, J., Patel, R. J., & Schneewind, O. 2007. Surface proteins and exotoxins are required for the pathogenesis of Staphylococcus aureus pneumonia. Infect. Immun, 75, 1040–1044

23. Buonocore, S., Ahern, P. P., Uhlig, H. H., Ivanov, I. I., Littman, D. R., Maloy, K. J., & Powrie, F. 2010. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature, 464(7293), 1371-1375.

24. Burts, M. L., Williams, W. A., DeBord, K. & Missiakas, D. M. 2005. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of Staphylococcus aureus infections. Proc Natl Acad Sci USA 102, 1169–1174.

25. Camporeale, A., & Poli, V. 2012. IL-6, IL-17 and STAT3: a holy trinity in auto-immunity? Front Biosci 17, 2306–2326.

26. Carvalho, M. D., Tondella, M. L., Mccaustland, K., Weidlich, L., Mcgee, L., Mayer, L. W., . . . Sampson, J. S. 2007. Evaluation and Improvement of Real-Time PCR Assays Targeting lytA, ply, and psaA Genes for Detection of Pneumococcal DNA. Journal of Clinical Microbiology, 45(8), 2460-2466.

27. Cassat, J. E., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, and M. S. Smeltzer. 2005. Comparative genomics of Staphylococcus aureus musculoskeletal isolates. J. Bacteriol. 187:576–592.

28. Cella, M, Fuchs, A, Vermi, W, Facchetti, F, Otero, K, Lennerz, J, Doherty, J, Mills, J, & Colonna, M. 2009. A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity, Nature, 457(7230), 722-725.

29. Cella, M, Fuchs, A, Vermi, W, Facchetti, F, Otero, K, Lennerz, J, Doherty, J, Mills, J, and Colonna, M. 2009, 'A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity', Nature, 457, 7230, pp. 722-725.

30. Chandler, L. J., Reisner, B. S., Woods, G. L., & Jafri, A. K. 2000. Comparison of four methods for identifying Streptococcus pneumoniae. Diagnostic Microbiology and Infectious Disease, 37(4), 285-287.

31. Chen K, McAleer Jeremy P, Lin Y, et al. Th-17 Cells Mediate Clade-Specific, Serotype-Independent Mucosal Immunity. Immunity 2011; 35:997-1009.

32. Clarke, S. R., Mohamed, R., Bian, L., Routh, A. F., Kokai-Kun, J. F., Mond, J. J., . . . Foster, S. J. 2007. The Staphylococcus aureus Surface Protein IsdA Mediates Resistance to Innate Defenses of Human Skin. Cell Host & Microbe, 1(3), 199-212.

33. Clarke, S., Brummell, K., Horsburgh, M., Mcdowell, P., Mohamad, S., Stapleton, M., . . . Foster, S. 2006. Identification of In Vivo–Expressed Antigens of Staphylococcus aureus and Their Use in Vaccinations for Protection against Nasal Carriage. The Journal of Infectious Diseases, 193(8), 1098-1108.

34. Crellin, N. K., Trifari, S., Kaplan, C. D., Cupedo, T., & Spits, H. 2010. Human NKp44 IL-22 cells and LTi-like cells constitute a stable RORC lineage distinct from conventional natural killer cells. The Journal of Experimental Medicine, 207(2), 281-290.

35. Crellin, N. K., Trifari, S., Kaplan, C. D., Satoh-Takayama, N., Santo, J. P., & Spits, H. 2010. Regulation of Cytokine Secretion in Human CD127 LTi-like Innate Lymphoid Cells by Toll-like Receptor 2. Immunity, 33(5), 752-764.

36. Cruciani, Melania, Marilena P. Etna, Romina Camilli, Elena Giacomini, Zulema A. Percario, Martina Severa, and Silvia Sandini et al. 2017. "Staphylococcus Aureus Esx Factors Control Human Dendritic Cell Functions Conditioning Th1/Th17 Response". Frontiers In Cellular And Infection Microbiology 7.

37. Cupedo, T, Crellin, N, Papazian, N, Rombouts, E, Weijer, K, Grogan, J, Fibbe, W, Cornelissen, J, and Spits, H., 2009, 'Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells', Nature Immunology, 10, 1, pp. 66-74.

38. Curtis MM, Way SS. Interleukin-17 in host defence against bacterial, mycobacterial, and fungal pathogens. Immunology 2009; 126:177-85.

39. Dambacher, J., Beigel, F., Zitzmann, K., Toni, E. N., Goke, B., Diepolder, H. M., . . . Brand, S. 2008. The role of the novel Th-17 cytokine IL-26 in intestinal inflammation. Gut, 58(9), 1207-1217.

40. Dey, I and Bishayi, B. 2017. Role of Th-17 and Treg cells in septic arthritis and the impact of the Th-17/Treg -derived cytokines in the pathogenesis of *S. aureus* induced septic arthritis in mice, Microbial Pathogenesis, Volume 113, Pages 248-264.

41. Dhingra, p. L. 2010. Diseases of ear, nose and throat. 5th edition. Place of publication not identified: elsevier india.

42. Dong, C. 2011. Genetic controls of Th-17 cell differentiation and plasticity. Experimental & Molecular Medicine: EMM, (1), 1.

43. Dubin PJaK, J. K. 2008. Th-17 cytokines and mucosal immunity. Immunological Reviews; 226:160-71.

44. Dukers-Muijrers, N. H., Stobberingh, E., Beisser, P., Boesten, R. C., Jacobs, P., & Hoebe, C. J. 2012. Nasal carriage of Streptococcus pneumoniae serotypes and Staphylococcus aureus in Streptococcus pneumoniae-vaccinated and non-vaccinated young children. Epidemiology and Infection, 141(03), 631-638.

45. Eberl, G., & Littman, D. R. 2004. Thymic Origin of Intestinal T Cells Revealed by Fate Mapping of ROR t Cells. Science, 305(5681), 248-251.

46. Eberl, G., Marmon, S., Sunshine, M., Rennert, P. D., Choi, Y., & Littman, D. R. 2003. An essential function for the nuclear receptor RORγt in the generation of fetal lymphoid tissue inducer cells. Nature Immunology, 5(1), 64-73.

47. Feuillie, C., Formosa-Dague, C., Hays, L. M. C., Vervaeck, O., Derclaye, S., Brennan, M. P., . . . Dufrêne, Y. F. 2017. Molecular interactions and inhibition of the staphylococcal biofilm-forming protein SdrC. Proceedings of the National Academy of Sciences, 114(14), 3738.

48. Flasche S, Le Polain de Waroux O, Prieto-Merino D, Edmunds WJ. 2014. Age-Dependent Prevalence of Nasopharyngeal Carriage of Streptococcus pneumoniae before Conjugate Vaccine Introduction: A Prediction Model Based on a Meta-Analysis. PLoS ONE 9(1).

49. Foster, T. J. and Hoeoek, M. 1998. Surface protein adhesins of Staphylococcus aureus. Trends In Microbiology, n:12. p. 484.

50. Foster, T.J. 2005. Immune evasion by staphylococci. Nat. Rev. Microbiol.

51. Frank, K. M., Zhou, T., Moreno-Vinasco, L., Hollett, B., Garcia, J. G., & Wardenburg, J. B. 2012. Host Response Signature to Staphylococcus aureus Alpha-Hemolysin Implicates Pulmonary Th-17 Response. Infection and Immunity, 80(9), 3161-3169.

52. Galdiero S, & Gouaux E. 2004. High resolution crystallographic studies of α-hemolysin–phospholipid complexes define heptamer–lipid head group interactions: Implication for understanding protein–lipid interactions. Protein Science: A Publication of the Protein Society;13(6):1503-1511.

53. Garcia-Rodriguez, J. A., & F., M. J., Martínez. 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. Journal of Antimicrobial Chemotherapy, 50(90003), 59-74.

54. Gauguet, Stefanie, Samantha D'Ortona, Kathryn Ahnger-Pier, Biyan Duan, Neeraj K. Surana, Roger Lu, and Colette Cywes-Bentley et al. 2015. "Intestinal Microbiota of Mice Influences Resistance to Staphylococcus aureus Pneumonia". Infection and Immunity 83 (10), 4003-4014.

55. Geremia, A., and Arancibia-Cárcamo, C.V. 2019. Innate Lymphoid Cells in Intestinal Inflammation. Frontiers in Immunology 8 (OCT).

56. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, Jensen BJ, Killgore G, Tenover FC, Kuehnert MJ. 2008. Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004. J Infect Dis 197:1226–1234. doi:10.1086/533494.

57. Gorwitz, R. J., Kruszon-Moran, D., McAllister, S. K., McQuillan, G., McDougal, L. K., Fosheim, G. E., Jensen, B. J., Killgore, G., Tenover, F. C., Kuehnert, M. J. 2008. Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001-2004, J Infect Dis 197,1226–1234.

58. Goto, Y., Panea, C., Nakato, G., Cebula, A., Lee, C., Diez, M.G., Laufer, T.M., Ignatowicz, L., Ivanov, II, 2014. Segmented filamentous bacteria antigens presented by intestinal dendritic cells drive mucosal Th-17 cell differentiation. Immunity 40, 594–607.

59. Granstrom, M., I. Julander, and R. Mollby. 1983. Serological diagnosis of deep Staphylococcus aureus infections by enzyme-linked immunosorbent assay (ELISA) for staphylococcal hemolysins and teichoic acid. Scand. J. Infect. Dis. Suppl.41:132–139.

60. Happel KI, Dubin PJ, Zheng M, et al. 2005. Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae. The Journal of Experimental Medicine.; 202:761-9

61. Hawiger, J., Timmons, S., Strong, D. D., Cottrell, B. A., Riley, M., & Doolittle, R. F. 1982. Identification of a region of human fibrinogen interacting with staphylococcal clumping factor. Biochemistry, 21(6), 1407-1413.

62. Hazenberg, M. D., and Spits, H. 2014. Human innate lymphoid cells. Blood, 124(5), 700-709.

63. Henrichsen, J. 1995. Six newly recognized types of Streptococcus pneumoniae. Journal of Clinical Microbiology, 33(10), 2759-62.

64. Hepworth, M, Fung, T, Masur, S, Kelsen, J, McConnell, F, Dubrot, J, Withers, D, Hugues, S, Farrar, M, Reith, W, Eberl, G, Baldassano, R, Laufer, T, Elson, C, and Sonnenberg, G n.d. 2015. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific CD4(+) T cells, Science, 348(6238), 1031-1035.

65. Hepworth, M, Monticelli, L, Fung, T, Ziegler, C, Grunberg, S, Sinha, R, Mantegazza, A, Ma, H, Crawford, A, Angelosanto, J, Wherry, E, Koni, P, Bushman, F, Elson, C, Eberl, G, Artis, D, and Sonnenberg, G. 2013. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria, Nature, 498 (7452), 113-117.

66. Herman A, Kappler J, Marrack P et al. 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. Annu Rev Immunol 9: 745–72.

67. Holt, J. G, Bergey, D. H. 1994. Bergey's manual of determinative bacteriology [Internet]. Williams & Wilkins.

68. Hoshino, H., Laan, M., Sjöstrand, M., Lötvall, J., Skoogh, B., & Lindén, A. (2000). Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. Journal of Allergy and Clinical Immunology, 105(1), 143-149.

69. Huang, C., Wu, P., Chen, C., Wang, C., Lee, T., Tsai, C., & Chiu, C. (2018). IL-17A expression in the adenoid tissue from children with sleep disordered breathing and its association with pneumococcal carriage. Scientific Reports, 8(1).

70. Hueber, W, Sands, B, Lewitzky, S, Vandemeulebroecke, M, Reinisch, W, Higgins, P, Wehkamp, J, Feagan, B, Yao, M, Karczewski, M, Karczewski, J, Pezous, N, Bek, S, Bruin, G, Mellgard, B, Berger, C, Londei, M, Bertolino, A, Tougas, G, and Travis, S. 2012, 'Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial', Gut, 61, 12, pp. 1693-1700.

71. Infante-Duarte, C., Horton, H. F., Byrne, M. C., & Kamradt, T. 2000. Microbial Lipopeptides Induce the Production of IL-17 in Th Cells. The Journal of Immunology, 165(11), 6107-6115.

72. Jacoby P, Watson K, Bowman J, et al. 2007. Modelling the co-occurrence of Streptococcus pneumoniae with other bacterial and viral pathogens in the upper respiratory tract. Vaccine.; 25:2458-2464.

73. Jaffar, Z., Ferrini, M. E., Herritt, L. A., & Roberts, K. 2009. Cutting Edge: Lung Mucosal Th-17-Mediated Responses Induce Polymeric Ig Receptor Expression by the Airway Epithelium and Elevate Secretory IgA Levels. The Journal of Immunology, 182(8), 4507-4511.

74. Josefsson E, McCrea KW, Ní Eidhin D, O'Connell D, Cox J et al. 1998. Three new members of the serine-aspartate repeat protein multigene family of Staphylococcus aureus. Microbiology 144(12): 3387-3395.

75. Julander, I. G., M. Granstrom, S. A. Hedstrom, and R. Mollby. 1983. The role of antibodies against alpha-toxin and teichoic acid in the diagnosis of staphylococcal infections. Infection 11:77–83.

76. Kastelein, R. A., Hunter, C. A., Cua, D. J. 2007. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol 25:221–242.

77. Kim, H. K., Dedent, A., Cheng, A. G., Mcadow, M., Bagnoli, F., Missiakas, D. M., & Schneewind, O. 2010. IsdA and IsdB antibodies protect mice against Staphylococcus aureus abscess formation and lethal challenge. Vaccine, 28(38), 6382-6392.

78. Kiss, E. A., Vonarbourg, C., Kopfmann, S., Hobeika, E., Finke, D., Esser, C., & Diefenbach, A. 2011. Natural Aryl Hydrocarbon Receptor Ligands Control Organogenesis of Intestinal Lymphoid Follicles. Science, 334(6062), 1561-1565.

79. Klose, C., Flach, M., Möhle, L., Rogell, L., Hoyler, T., Ebert, K., . . . Diefenbach, A. 2014. Differentiation of Type 1 ILCs from a Common Progenitor to All Helper-like Innate Lymphoid Cell Lineages. Cell, 157(2), 340-356.

80. Kluytmans J, van Belkum A, Verbrugh H. 1997. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev 10:505–520.

81. Kluytmans, J., van Belkum, A., Verbrugh, H. 1997. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clin Microbiol Rev, 10, 505–520.

82. Kneuper, H., Cao, Z. P., Twomey, K. B., Zoltner, M., Jäger, F., Cargill, J. S., . . . Palmer, T. 2014. Heterogeneity in Ess transcriptional organization and variable contribution of the Ess/Type VII protein secretion system to virulence across closely related Staphylococcus aureus strains. Molecular Microbiology, 93(5), 928-943.

83. Koh, B., Hufford, M. M., Pham, D., Olson, M. R., Wu, T., Jabeen, R., . . . Kaplan, M. H. 2016. The ETS Family Transcription Factors Etv5 and PU.1 Function in Parallel to Promote Th9 Cell Development. The Journal of Immunology, 197(6), 2465-2472.

84. Kolls, J. K., & Linden, A. 2004. Interleukin-17 family members and inflammation. Immunity 21 (4):467-76.

85. Korea CG, et al. 2014. Staphylococcal Esx proteins modulate apoptosis and release of intracellular Staphylococcus aureus during infection in epithelial cells. Infect Immun 82(10):4144–4153.

86. Korea, C., Balsamo, G., Pezzicoli, A., Merakou, C., Tavarini, S., Bagnoli, F., Serruto, D. and Unnikrishnan, M. 2014. Staphylococcal Esx Proteins Modulate Apoptosis and Release of Intracellular Staphylococcus aureus during Infection in Epithelial Cells. Infection and Immunity, 82(10), pp.4144-4153.

87. Korn. T., Bettelli, E., Oukka, M., Kuchroo, V. K. 2009. IL-17 and Th-17 Cells. Annu Rev Immunol,27, 485-517.

88. Laan, M., Prause, O., Miyamoto, M., Sjöstrand, M., Hytönen, A., Kaneko, T., . . . Lindén, A. 2003. A role of GM-CSF in the accumulation of

neutrophils in the airways caused by IL-17 and TNF-α. European Respiratory Journal, 21(3), 387-393.

89. Lamikanra, A., B. D. Paul, O. B. Akinwole, and M. O. Paul. 1985. Nasal carriage of *S. aureus* in a population of healthy Nigerian students. J. Med. Microbiol. 19:211–216.

90. Lee, J. S., Cella, M., Mcdonald, K. G., Garlanda, C., Kennedy, G. D., Nukaya, M., . . . Colonna, M. 2011. AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nature Immunology, 13(2), 144-151.

91. Li, J, Doty, AL, Tang, Y, Berrie, D, Iqbal, A, Tan, SA, Clare-Salzler, MJ, Wallet, SM & Glover, SC, 2017. 'Enrichment of IL-17A(+) IFN-(+) and IL-22(+)IFN-(+) T cell subsets is associated with reduction of NKp44(+)ILC3s in the terminal ileum of Crohn's disease patients', Clinical and Experimental Immunology, vol. 190, no. 1, pp. 143–153.

92. Lowy, F. D. 1998. Staphylococcus aureus infections. N Engl J Med 339:520–532.

93. Lu YJ, Gross J, Bogaert D, et al. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. PLoS Pathogens.

94. Luci, C, Reynders, A, Cognet, C, Chiche, L, Chasson, L, Dalod, M, Vivier, E, Tomasello, E, Ivanov, I, Littman, D, Hardwigsen, J, Anguiano, E, Banchereau, J, & Chaussabel, D, 2009, 'Influence of the transcription factor RORγt on the development of NKp46+ cell populations in gut and skin', Nature Immunology, 10, 1, p. 75-82.

95. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. 2010. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC Infectious Diseases.; 10:304-.

96. Madhi SA, Adrian P, Kuwanda L, Cutland C, Albrich WC, Klugman KP. 2007. Long-Term Effect of Pneumococcal Conjugate Vaccine on

Nasopharyngeal Colonization by Streptococcus pneumoniae—and Associated Interactions with Staphylococcus aureus and Haemophilus influenzae Colonization—in HIV-Infected and HIV-Uninfected Children. Journal of Infectious Diseases; 196:1662-1666.

97. Mancini, F., Monaci, E., Lofano, G., Torre, A., Bacconi, M., Tavarini, S., . . . Bertholet, S. 2016. One Dose of Staphylococcus aureus 4C-Staph Vaccine Formulated with a Novel TLR7-Dependent Adjuvant Rapidly Protects Mice through Antibodies, Effector CD4 T Cells, and IL-17A. Plos One, 11(1).

98. Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. & Weaver, C. T. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature, 441, 231–234.

99. Margolis, E. 200). Hydrogen Peroxide-Mediated Interference Competition by Streptococcus pneumoniae Has No Significant Effect on Staphylococcus aureus Nasal Colonization of Neonatal Rats. Journal of Bacteriology, 191, 571-575.

100. Mascanfroni, I. D., Takenaka, M. C., Yeste, A., Patel, B., Wu, Y., Kenison, J. E., . . . Quintana, F. J. 2015. Metabolic control of type 1 regulatory T cell differentiation by AHR and HIF1-α. Nature Medicine, 21(6), 638-646.

101. Matsuzaki, G., and Umemura, M. 2007. Interleukin-17 as an effector molecule of innate and acquired immunity against infections, Microbiol Immunol, 51(12), 1139-1147.

102. McAdow M, Kim H., K, Dedent A., C, Hendrickx A., P, Schneewind O, Missiakas D., M .2011. Preventing Staphylococcus aureus sepsis through the inhibition of its agglutination in blood. PLoS Pathog 7.

103. Mebius, R. E., Rennert, P., & Weissman, I. L. 1997. Developing Lymph Nodes Collect CD4 CD3 − LTβ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells. Immunity, 7(4), 493-504.

104. Menzies, B. E., Kernodle, D. S.1994. Site-directed mutagenesis of the alpha-toxin gene of Staphylococcus aureus: role of histidines in toxin activity in vitro and in a murine model. Infect Immun 62: 1843–1847.

105. Mishra, R., Mariotti, P., Fiaschi, L., Nosari, S., Maccari, S., & Liberatori, S. et al. 2012. Staphylococcus aureus FhuD2 Is Involved in the Early Phase of Staphylococcal Dissemination and Generates Protective Immunity in Mice. The Journal of Infectious Diseases, 206(7), 1041-1049.

106. Mocca, C. P., Brady, R. A., & Burns, D. L. 2014. Role of Antibodies in Protection Elicited by Active Vaccination with Genetically Inactivated Alpha Hemolysin in a Mouse Model of Staphylococcus aureus Skin and Soft Tissue Infections. Clinical and Vaccine Immunology, 21(5), 622-627.

107. Mubarak A., 2014, Characterisation of Treg and Th-17 cells in nasopharynx-associated lymphoid tissue and their association with pneumococcal carriage in children and adults. PhD thesis. University of Liverpool, UK.

108. Mubarak, A., Ahmed, M., Upile, N., Vaughan, C., Xie, C., Sharma, R., . . . Zhang, Q. 2016. A dynamic relationship between mucosal T helper type 17 and regulatory T-cell populations in nasopharynx evolves with age and associates with the clearance of pneumococcal carriage in humans. Clinical Microbiology and Infection, 22(8). doi: 10.1016/j.cmi.2016.05.017

109. Mulcahy, M. E., Leech, J. M., Renauld, J., Mills, K. H., & Mcloughlin, R. M. 2016. Interleukin-22 regulates antimicrobial peptide expression and keratinocyte differentiation to control Staphylococcus aureus colonization of the nasal mucosa. Mucosal Immunology, 9(6), 1429-1441.

110. Narita, K., Hu, D. L., Mori, F., Wakabayashi, K., Iwakura, Y., Nakane, A. 2010. Role of interleukin-17A in cell-mediated protection against Staphylococcus aureus infection in mice immunized with the fibrinogen-binding domain of clumping factor A. Infect Immun, 78, 4234–4242.

111. Ni Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Höök, M., & Foster, T. J. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-

binding adhesin of Staphylococcus aureus. Molecular Microbiology, 30(2), 245-257.

112. Nikoopour, Enayat, Stacey M. Bellemore, & Bhagirath Singh. (2015). IL-22, Cell Regeneration and Autoimmunity. Cytokine 74 (1), 35-42.

113. O'Connell, D.P., Nanavaty, T., McDevitt, D., Gurusiddappa, S., Ho¨o¨k, M., and Foster, T. J. 1998. The fibrinogen-binding MSCRAMM (clumping factor) of Staphylococcus aureus has an integrin-like Ca2þ-dependent inhibitory site. J Biol Chem 273: 6821–6829.

114. Oshea, J. J., & Paul, W. E. 2010. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4 T Cells. Science, 327(5969), 1098-1102.

115. Popov Lauren M., Marceau Caleb D., Starkl Philipp M., Lumb Jennifer H., Shah Jimit, Guerrera Diego, Cooper Rachel L., Merakou Christina, Bouley Donna M., Meng Wenxiang, Kiyonari Hiroshi, Takeichi Masatoshi, Galli Stephen J., Bagnoli Fabio, Citi Sandra, Carette Jan E. & Amieva Manuel R. 2015. The adherens junctions control susceptibility to Staphylococcus aureus α-toxin, Proceedings of the National Academy of Sciences of the United States of America. 112 (46),14337-14342.

116. Qiu, J. and Zhou, L. 2013. Aryl hydrocarbon receptor promotes RORγt+ group 3 ILCs and controls intestinal immunity and inflammation. *Semin. Immunopathol.* 35, 657–670.

117. Qiu, J., Guo, X., Chen, Z., He, L., Sonnenberg, G., Artis, D., . . . Zhou, L. 2013. Group 3 Innate Lymphoid Cells Inhibit T-Cell-Mediated Intestinal Inflammation through Aryl Hydrocarbon Receptor Signaling and Regulation of Microflora. Immunity, 39(2), 386-399.

118. Qiu, J., Heller, J., Guo, X., Chen, Z., Fish, K., Fu, Y., & Zhou, L. 2012. The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells. Immunity, 36(1), 92-104.

119. Ragle, B. E., and Wardenburg, J. B. 2009. Anti-Alpha-Hemolysin Monoclonal Antibodies Mediate Protection against Staphylococcus aureus Pneumonia. Infection and Immunity, 77(7), 2712-2718.

120. Ramachandran, G. 2014. Gram-positive and gram-negative bacterial toxins in sepsis: A brief review. Virulence, 5, 213–218.

121. Russ, B. E., Prier, J. E., Rao, S., & Turner, S. J. 2013. T cell immunity as a tool for studying epigenetic regulation of cellular differentiation. Frontiers in Genetics, 4.

122. RW, Wu R 2004. IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappa B signaling pathways. J Immunol 173:3482–3491.

123. Sabat A, Melles DC, Martirosian G, Grundmann H, van Belkum A et al. 2006. Distribution of the serine-aspartate repeat protein-encoding sdr genes among nasal-carriage and invasive Staphylococcus aureus strains. J Clin Microbiol 44: 1135-1138.

124. Satoh-Takayama, N, Vosshenrich, C, Lesjean-Pottier, S, Sawa, S, Lochner, M, Rattis, F, Mention, J, Thiam, K, Cerf-Bensussan, N, Mandelboim, O, Eberl, G, and Di Santo, J n.d., 2008. 'Microbial Flora Drives Interleukin 22 Production in Intestinal NKp46(+) Cells that Provide Innate Mucosal Immune Defense', Immunity, 29, 6, pp. 958-970.

125. Satpathy, A.T., Briseno, C.G., Lee, J.S., Ng, D., Manieri, N.A., Kc, W., Wu, X., Thomas, S.R., Lee, W.L., Turkoz, M., McDonald, K.G., Meredith, M.M., Song, C., Guidos, C.J., Newberry, R.D., Ouyang, W., Murphy, T.L., Stappenbeck, T.S., Gommerman, J.L., Nussenzweig, M.C., Colonna, M., Kopan, R., Murphy, K.M., 2013. Notch2dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. Nat. Immunol. 14, 937–948.

126. Schaffer, A. C., Solinga, R. M., Cocchiaro, J., Portoles, M., Kiser, K. B., Risley, A., . . . Lee, J. C. 2006. Immunization with Staphylococcus aureus Clumping Factor B, a Major Determinant in Nasal Carriage, Reduces Nasal

Colonization in a Murine Model. Infection and Immunity, 74(4), 2145-2153.

127. Schluepen, C., Malito, E., Marongiu, A., Schirle, M., Mcwhinnie, E., Surdo, P. L., . . . Liberatori, S. 2013. Mining the bacterial unknown proteome: Identification and characterization of a novel family of highly conserved protective antigens inStaphylococcus aureus. Biochemical Journal, 455(3), 273-284.

128. Schneewind, O., & Missiakas, D. M. 2012. Protein secretion and surface display in Gram-positive bacteria. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 367(1592), 1123–1139.

129. Sebulsky, M. T., & Heinrichs, D. E. 2001. Identification and Characterization of fhuD1 and fhuD2, Two Genes Involved in Iron-Hydroxamate Uptake in Staphylococcus aureus. Journal of Bacteriology, 183(17), 4994-5000.

130. Seilie, E. S., & Wardenburg, J. B. 2017. Staphylococcus aureus pore-forming toxins: The interface of pathogen and host complexity. Seminars in Cell & Developmental Biology, 72, 101-116.

131. Seth A, Stern L, Ottenhoff T et al. Binary and ternary complexes between T-cell receptor, class II MHC and superantigen in vitro. Nature 1994; 369: 324–7.

132. Sonnenberg, G, Monticelli, L, Alenghat, T, Fung, T, Grunberg, S, Sinha, R, Bushman, F, Artis, D, Hutnick, N, Weiner, D, Kunisawa, J, Shibata, N, Kiyono, H, Zahm, A, Friedman, J, Tardif, M, Tessier, P, Sathaliyawala, T, Kubota, M, Farber, D, Collman, R, Chang, K, Shaked, A, and Fouser, L., 2012, 'Innate lymphoid cells promote anatomical containment of lymphoid-resident commensal bacteria', Science, 336, 6086, p. 1321-1325.

133. Spits, H, Artis, D, Colonna, M, Diefenbach, A, Di Santo, J, Eberl, G, Koyasu, S, Locksley, R, McKenzie, A, Mebius, R, Powrie, F, & Vivier, E 2013, 'Innate lymphoid cells-a proposal for uniform nomenclature', Nature Reviews Immunology, vol. 13, no. 2, p. 145-149.

134. Spits, H., and Cupedo, T. (2012). Innate lymphoid cells: Emerging insights in development, lineage relationships, and function. Annual Review of Immunology, 30, 647-675.

135. Stoll, S., Delon, J., Brotz, T.M., Germain, R.N., 2002. Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. Science 296, 1873–1876.

136. Stranger-Jones, Y. K., T. Bae, and O. Schneewind. 2006. Vaccine assembly

137. Takagi, R., T. Higashi, K. Hashimoto, K. Nakano, Y. Mizuno, Y. Okazaki, and S. Matsushita. 2008. B cell chemoattractant CXCL13 is preferentially expressed by human Th-17 cell clones. J. Immunol. 181:186-189.

138. Tuazon CU, Perez A, Kishaba T, Sheagren JN. Staphylococcus aureus among insulin-injecting diabetic patients: an increased carrier rate. JAMA 1975; 231:1272-1272.

139. Tuffs, S., Haeryfar, S., & Mccormick, J. 2018. Manipulation of Innate and Adaptive Immunity by Staphylococcal Superantigens. Pathogens, 7(2), 53. doi:10.3390/pathogens7020053.

140. Van Maele, L, Carnoy, C, Cayet, D, Ivanov, S, Porte, R, Deruy, E, Trottein, F, Faveeuw, C, Sirard, J, Eberl, G, Benecke, A, Chabalgoity, J, and Renauld, J . 2014. Activation of type 3 innate lymphoid cells and interleukin 22 secretion in the lungs during streptococcus pneumoniae infection. Journal of Infectious Diseases, 210(3), 493-503.

141. Veldhoen, M. Hocking, R. J. Atkins, C. J. Locksley, R. M. & Stockinger, B. 2006. TGF beta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24:179–189

142. Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J., & Stockinger, B. 2008. The aryl hydrocarbon receptor links TH-17-cell-mediated autoimmunity to environmental toxins. Nature, 453(7191), 106-109.

143. Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M., Stockinger, B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity, 24, 179–189.

144. Vely, F, Barlogis, V, Vallentin, B, Neven, B, Piperoglou, C, Perchet, T, Petit, M, Yessaad, N, Touzot, F, Bruneau, J, Mahlaoui, N, Zucchini, N, Farnarier, C, Michel, G, Moshous, D, Blanche, S, Dujardin, A, Spits, H, Distler, J, Ramming, A, Picard, C, Golub, R, Fischer, A, &and Vivier, E n.d., 2016. 'Evidence of innate lymphoid cell redundancy in humans', Nature Immunology, 17, 11, pp. 1291-1299.

145. Verkaik, N. J., Boelens, H. A., Vogel, C. P., Tavakol, M., Bode, L. G., Verbrugh, H. A., . . . Wamel, W. J. 2010. Heterogeneity of the humoral immune response following Staphylococcus aureus bacteremia. European Journal of Clinical Microbiology & Infectious Diseases, 29(5), 509-518.

146. Vivier, E, Artis, D, Colonna, M, Diefenbach, A, Di Santo, J, Eberl, G, Koyasu, S, Locksley, R, McKenzie, A, Mebius, R, Powrie, F, and Spits, H 2018, 'Innate Lymphoid Cells: 10 Years On', Cell, 174, 5, p. 1054-1066.

147. Vivier, Eric, Hergen Spits, and Tom Cupedo. 2009. "Interleukin-22-Producing Innate Immune Cells: New Players In Mucosal Immunity And Tissue Repair?". Nature Reviews Immunology 9 (4): 229-234.

148. Von Eiff C, Becker K, Machka K, Stammer H, Peters G. 2001. Nasal carriage as a source of Staphylococcus aureus bacteremia. N Engl J Med. 344(1):11–16.

149. Walker, J. N., Crosby, H. A., Spaulding, A. R., Salgado-Pabón, W., Malone, C. L., Rosenthal, C. B., . . . Horswill, A. R. 2013. The Staphylococcus aureus ArlRS Two-Component System Is a Novel Regulator of Agglutination and Pathogenesis. PLoS Pathogens, 9(12).

150. Wang, X., Theodore, M. J., Mair, R., Trujillo-Lopez, E., Plessis, M. D., Wolter, N., . . . Mayer, L. W. 2011. Clinical Validation of Multiplex Real-Time PCR Assays for Detection of Bacterial Meningitis Pathogens. Journal of Clinical Microbiology, 50(3), 702-708.

151. Wardenburg, J. B. and Schneewind, O. 2008. Vaccine protection against Staphylococcus aureus pneumonia. The Journal of Experimental Medicine 205(2): 287-294.

152. Weaver, C. T., Hatton, R. D., Mangan, P. R. and Harrington. L. E. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25:821-52.

153. Weinke, T., Schiller, R., Fehrenbach, F. J., Pohle, H. D. 1992. Association between Staphylococcus aureus nasopharyngeal colonization and septicemia in patients infected with the human immunodeficiency virus. Eur J Clin Microbiol Infect Dis, 11, 985-989.

154. Wilke, G. A., and Wardenburg, J. B. 2010. Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus-hemolysin-mediated cellular injury. Proceedings of the National Academy of Sciences, 107(30), 13473-13478.

155. Wilson, N. J., Boniface, K., Chan, J. R., Mckenzie, B. S., Blumenschein, W. M., Mattson, J. D., . . . Malefyt, R. D. 2007. Development, cytokine profile and function of human interleukin 17–producing helper T cells. Nature Immunology, 8(9), 950-957.

156. Withers, D. R., Hepworth, M. R., Wang, X., Mackley, E. C., Halford, E. E., Dutton, E. E., . . . Sonnenberg, G. F. 2016. Transient inhibition of ROR-γt therapeutically limits intestinal inflammation by reducing TH-17 cells and preserving group 3 innate lymphoid cells. Nature Medicine, 22(3), 319-323.

157. Xiao, S, Yosef, N, Zhu, C, Wu, C, Kuchroo, V, Regev, A, Yang, J, Baloglu, E, Schmidt, D, Ramesh, R, Lobera, M, Sundrud, M, Ghosh, S, Wang, Y, Zhou, L, Xiang, Z, Wang, J, Xu, Y, Lin, X, Zhong, Z, Tsai, P, Kretschmer, K, Rahl, P, Young, R, Hafler, D, and Marson, A. 2014. 'Small-molecule RORγt antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms', Immunity, 40, 4, p. 477-489.

158. Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., Dong, C. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 282, 9358–9363.

159. Yang, X. O., Pappu, B. P., Nurieva, R., Akimzhanov, A., Kang, H. S., Chung, Y., . . . Dong, C. 2008. T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors RORα and RORγ. Immunity, 28(1), 29-39.

160. Ye, P., Rodriguez, F. H., Kanaly, S., Stocking, K. L., Schurr, J., Schwarzenberger, P., . . . Kolls, J. K. 2001. Requirement of Interleukin 17 Receptor Signaling for Lung Cxc Chemokine and Granulocyte Colony-Stimulating Factor Expression, Neutrophil Recruitment, and Host Defense. The Journal of Experimental Medicine, 194(4), 519-528.

161. Yoshida, H., Honda, K., Shinkura, R., Adachi, S., Nishikawa, S., Maki, K., . . . Nishikawa, S. 1999. IL-7 receptor α CD3– cells in the embryonic intestine induces the organizing center of Peyers patches. International Immunology, 11(5), 643-655.

162. Zhang, F., Ledue, O., Jun, M., Goulart, C., Malley, R., & Lu, Y. 2018. Protection against Staphylococcus aureus Colonization and Infection by B- and T-Cell-Mediated Mechanisms. MBio, 9(5).

163. Zhang, F., Ledue, O., Jun, M., Goulart, C., Malley, R., & Lu, Y. 2018. Protection against Staphylococcus aureus Colonization and Infection by B- and T-Cell-Mediated Mechanisms. MBio, 9(5).

164. Zhang, P., Lee, J. S., Gartlan, K. H., Schuster, I. S., Comerford, I., Varelias, A., . . . Hill, G. R. 2017. Eomesodermin promotes the development of type 1 regulatory T (TR1) cells. Science Immunology, 2(10).

165. Zhang, Q., Bernatoniene, J., Bagrade, L., Pollard, A. J., Mitchell, T. J., Paton, J. C., & Finn, A. 2006. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. European Journal of Immunology, 36(1), 46-57.

166. Zhang, Q., Choo, S., & Finn, A. 2002. Immune Responses to Novel Pneumococcal Proteins Pneumolysin, PspA, PsaA, and CbpA in Adenoidal B Cells from Children. Infection and Immunity, 70(10), 5363-5369.

167. Zook, E, and Kee, B., 2016, 'Development of innate lymphoid cells', Nature Immunology, 17, 7, p. 775-782.

Appendix

1.Appendix I

List of the Clinical samples used in the study indicating age, gender and the reason for tonsillectomy.

Number	ID (new)	Age (years)	Sex (Male=1, Female= 2)	Reasons for adeno/tonsillectomy (OSA=obstructive sleep apnea, RT=recurrent tonsillitis, SDB= sleep disorder breathing, Un= unknown)
1.	NC-08	14	2	RT, Nasal obstruction
2.	NC-09	11	1	OSA
3.	NC-10	10	2	Sore Throats
4.	NC-11	3	1	Un
5.	NC-12	1.2	1	OSA
6.	NC-13	4	1	OSA, RT
7.	NC-14	14	1	RT
8.	NC-15	2	1	OSA, RT
9.	NC-16	3	2	OSA
10.	NC-17	4	1	OSA
11.	NC-18	4	2	OSA
12.	NC-22	8	1	RT
13.	NC-23	3	1	RT, Snoring
14.	NC-25	Un	1	Un
15.	NC-26	4	2	RT, Snoring
16.	NC-27	1	1	OSA
17.	NC-28	5	2	RT
18.	NC-29	8	1	SDB

19.	NC-30	4	2	SDB
20.	NC-31	3	2	SDB
21.	NC-32	7	2	RT
22.	NC-33	11	1	RT, SDB
23.	NC-34	4	2	RT
24.	NC-35	3	1	RT
25.	NC-36	4	1	RT
26.	NC-37	5	2	SDB
27.	NC-38	3	2	SDB
28.	NC-39	5	1	SDB
29.	NC-40	6	1	SDB
30.	NC-41	Un	1	SDB
31.	NC-42	5	2	SDB
32.	NC-44	5	1	SDB
33.	NC-45	4	1	OSA
34.	NC-46	3	2	OSA
35.	NC-47	6	1	Tonsillitis, SiSnoring
36.	NC-48	6	1	OSA
37.	NC-49	10	2	RT
38.	NC-50	10	1	OSA
39.	NC-51	5	1	OSA
40.	NC-52	6	2	OSA
41.	NC-53	4	2	SDB + RT
42.	NC-54	4	1	Moderate sleep apnea
43.	NC-55	15	2	Quinsy, RT
44.	NC-56	6	2	SDB

45.	NC-57	3	1	SDB
46.	NC-58	2	1	SDB
47.	NC-59	5	1	OSA
48.	NC-60	7	1	RT
49.	NC-61	4	1	SDB
50.	NC-62	2	1	OSA
51.	NC-64	4	2	RT, OSA
52.	NC-65	3	1	RT, OSA
53.	NC-66	5	1	OSA
54.	NC-67	4	1	OSA
55.	NC-68	6	2	RT, Snoring
56.	NC-69	7	2	RT
57.	NC-70	2	2	RT
58.	NC-71	3	1	RT
59.	NC-72	1.9	1	RT
60.	NC-73	5	1	RT
61.	NC-74	6	2	OSA
62.	NC-75	8	2	OSA
63.	NC-76	1	1	OSA
64.	NC-77	3	1	RT/Snoring
65.	NC-78	3	1	RT/Snoring
66.	NC-79	6	1	RT
67.	NC-80	16	1	OSA
68.	NC-81	3.5	2	SDB
69.	NC-82	5	2	RT
70.	NC-83	5	2	SDB

71.	NC-84	2	1	OSA, RT
72.	NC-85	7	1	OSA, RT
73.	NC-86	3	1	OSA
74.	NC-87	9	1	OSA
75.	NC-88	3	2	OSA, RT
76.	NC-89	5	1	RT, SDB
77.	NC-90	2	1	OSA
78.	NC-91	2	2	OSA
79.	NC-92	4	1	SDB
80.	NC-93	2	1	SDB
81.	NC-94	2	2	RT, Snoring
82.	NC-95	3	1	RT, SDB
83.	NC-96	4	2	SDB
84.	NC-97	8	2	RT/ Adenoid hypertrophy
85.	NC-98	3	1	SDB/ Downs syndrom
86.	NC-99	9	1	RT
87.	NC-100	3.5	1	SDB
88.	NC-101	3.5	1	SDB
89.	NC-102	8	2	RT/ Sleep apnea
90.	NC-103	3	1	SDB
91.	NC-104	10	2	RT/ SNORING
92.	NC-105	2	2	Snoring and ear infection
93.	NC-106	2.5	2	RURTI/Sinusitis
94.	NC-107	8	2	RT/ Snoring
95.	NC-108	4	1	OSA
96.	NC-109	3	1	OSA/ Chronic Lung disease

97.	NC-110	7	2	R LRTI/ Tracheomlasis
98.	NC-111	7	1	Asthma
99.	NC-112	13	2	RT/ Snoring
100.	NC-113	13	2	RT/ SDB
101.	NC-114	3	2	OSA
102.	NC-115	2	2	OSA
103.	NC-116	3	1	OSA
104.	NC-117	2	1	Mild OSA/ Tonsillitis
105.	NC-118	2	2	SDB
106.	NC-119	4	2	Nasal block and choking
107.	NC-120	4	2	OSA
108.	NC-121	3	1	OSA/ Tonsillitis
109.	NC-122	8	2	RT
110.	NC-123	8	2	RT/ snoring
111.	NC-124	16	2	RT/ Chronic fatigue syndrome
112.	NC-125	4	2	OSA/ Cutameous hemangioma_multiphilia
113.	NC-126	3	1	OSA
114.	NC-127	2	2	RT/ SDB
115.	NC-128	6	1	OSA
116.	NC-129	13	2	RT/ Snoring
117.	NC-130	4	Un	OSA/ tonsillitis
118.	NC-131	7	2	RT/ SDB
119.	NC-132	4	1	OSA
120.	NC-133	5	1	SDB/ RT
121.	NC-134	5	1	SDB/ RT
122.	NC-135	3	1	OSA/ Tonsillitis

123.	NC-136	2	1	RT/ Snoring
124.	NC-137	3	1	Recurrent ADM
125.	NC-138	3	1	SDB
126.	NC-139	4	1	OSA
127.	NC-140	2	1	OSA/ RT
128.	NC-141	2	1	OSA
129.	NC-142	7	2	OSA/ RT
130.	NC-143	2	1	OSA/
131.	NC-144	7	2	Nasal obstruction/ RT
132.	NC-145	4	2	SBM (Mild_Mod. OSA)
133.	NC-146	3	2	Tonsillitis/ SDB
134.	NC-147	4	2	Tonsillitis
135.	NC-148	14	1	SDB
136.	NC-149	4	1	RT
137.	NC-150	13	2	RT
138.	NC-151	8	1	SDB
139.	NC-152	6	1	SDB
140.	NC-153	2	1	OSA
141.	NC-154	7	1	SDB
142.	NC-157	3	1	OSA
143.	NC-158	2	1	RT/SDB
144.	NC-159	2	1	SDB
145.	NC-160	5	1	RT
146.	A137	17	Un	Un
147.	A138	17	Un	Un
148.	A139	22	Un	Un

149.	A140	16	Un	Un
150.	A141	20	Un	Un
151.	A142	28	Un	Un
152.	A143	20	Un	Un
153.	A144	23	Un	Un
154.	A145	18	Un	Un
155.	A146	20	Un	Un
156.	A147	24	Un	Un
157.	A148	18	Un	Un
158.	A149	Un	2	RT
159.	A150	60	2	Chronic tonsillitis, Halitosis
160.	A151	32	1	RT
161.	A152	23	2	RT
162.	A153	16	2	RT
163.	A154	20	1	RT
164.	A155	32	2	RT
165.	A156	24	2	Un
166.	A157	17	2	RT
167.	A158	18	2	RT
168.	A159	17	2	RT
169.	A160	39	1	RT
170.	A161	17	2	RT
171.	A162	37	1	RT
172.	A163	19	2	RT
173.	A164	26	2	RT
174.	A165	52	1	Recurrent Quinsy (Peritonsillar abscess)

175.	A166	51	2	RT
176.	A167	29	2	RT
177.	A169	55	1	Recurrent Quinsy
178.	A170	17	2	RT
179.	A171	19	1	RT
180.	A172	44	1	Snoring
181.	A173	27	2	RT
182.	A174	21	2	RT
183.	A175	16	2	RT
184.	A176	28	2	Snoring
185.	A177	25	1	Quinsy
186.	A178	18	2	Tonsillitis
187.	A179	17	1	RT
188.	A180	17	2	RT
189.	A181	21	2	RT
190.	A182	24	1	RT
191.	A183	20	2	Tonsillitis
192.	A184	23	2	RT
193.	A185	17	2	RT
194.	A187	34	2	RT
195.	A188	20	2	RT
196.	A189	28	2	RT
197.	A190	25	2	RT
198.	A191	19	1	RT
199.	A192	26	2	RT
200.	A193	50	1	Snoring

201.	A194	54	2	Snoring
202.	A195	22	2	RT
203.	A196	33	2	RT
204.	A197	17	2	RT/ tonsil infection
205.	A198	31	2	Recurrent Infections + Stones
206.	A199	22	2	Tonsillectomy
207.	A200	18	2	Tonsillectomy
208.	A201	51	2	Tonsillectomy + Uppp
209.	A202	26	1	RT
210.	A203	21	2	RT
211.	A204	50	1	Snoring
212.	A205	27	1	RT
213.	A206	36	2	Bilateral tonsillitis
214.	A207	35	2	RT
215.	A208	26	2	Tonsil stones, tonsillitis
216.	A209	21	2	RT
217.	A210	19	2	RT
218.	A211	24	2	RT