The Balancing Act between Colonisers and Inflammation: T regulatory and T_H17 Cells in Mucosal Immunity during Otitis Media

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Abstract: Inflammation of the middle ear, otitis media, is a significant cause of pain and reduced auditory acuity in children. Recurrent episodes may delay development of speech, learning and social behaviour. *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* are most often implicated. These bacteria colonise the nasopharynx asymptomatically but host tolerance of high nasopharyngeal load contributes to onset of inflammation. Immunosuppression is evident in susceptible children which may contribute to tolerance and therefore to progression to chronic disease. While the causative factors involved in the immunosuppressive response are not known, evidence from other mucosal sites suggests that T regulatory (T_{reg}) lymphocytes, a subset of T helper (T_H) lymphocytes, contribute to regulation of immunosuppression to commensal bacteria and promote advancement of infection. The major function of T_{reg} lymphocytes is induction of immune tolerance via immunosuppression in the periphery to foreign and self antigen. They have been identified in adenoids and tonsils and are known to have a positive association with pneumococcus nasopharyngeal colonisation. Interestingly, the pro-inflammatory T_H17 lymphocyte response to *S. pneumoniae* is reduced in pneumococcal-positive children. Furthermore, inadequate T lymphocyte proliferation to non-typeable *H. influenzae* is evident in otitis media-prone children. A weak T lymphocyte repertoire in young children may explain high nasopharyngeal bacterial carriage observed in this population. However, T_H17 and T_H1 lymphocyte responses may be subdued due to T_{reg} lymphocyte suppression. The immune factors that regulate nasopharyngeal colonisation are not well understood and further research is required to elucidate the immunological mechanism that underlies development of otitis media.

Keywords: commensal; immunity; mucosal; nasopharynx; otitis media; T regulatory lymphocytes.

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INTRODUCTION

"*Inasmuch as the nasopharyngeal tonsil is the critical site for the early events that will lead to the development of both otitis media and sinusitis, it appears that manipulation of this area with strategies other than antibiotics could be successful in the prevention of colonization and the subsequent development of inflammation of the upper respiratory tract*" [1]. This statement provides the critical reasoning for the importance of investigating characteristics of the adenoid that may contribute to the pathogenesis and persistence of otitis media (OM), inflammation and dysfunction of the middle ear. For centuries, dating back as far as Hippocrates in 400 BC, OM has been documented, yet approximately 2500 years on OM remains a prevalent disease within a modern society, but it has evolved into a well-defined condition [2]. The aetiology of OM is complex and may be attributed to multiple factors including, age, viral and bacterial milieu, congenital or acquired immunodeficiency, allergy, Eustachian tube dysfunction or facial structure abnormalities, genetic, racial, socio-economic and environmental exposures [3].

OM is a major burden on health services worldwide. Native American, Alaskan, Canadian and Australian Aboriginals are ethnic populations at high risk for developing OM [4-6]. More than 80% of Australian children will have suffered with an OM infection by age three. Of these, almost 40% of children will develop recurring infections, with little relief experienced from antibiotic therapies [7]. Children suffering with these infections experience pain and decreased hearing that may be acute or chronic, with long-term consequences including poor hearing and speech, and under developed learning and social behaviours [5]. The current estimated burden of OM on the Australian Health Services exceeds $100 million annually and is due largely to a lack of available preventative therapies [7].

It has become evident through rigorous clinical research that immunosuppression is evident in OM-prone children, which may contribute to the pathogenesis of OM and the progression to chronic disease [8, 9]. What determines these children to have an immunosuppressive response is still unclear. However, there is evidence at other mucosal sites under similar microbial loads that T regulatory (T_{reg}) lymphocytes, a subset of T helper (T_H) lymphocytes, contribute to the regulation of immunosuppression to commensal bacteria and contribute to the progression of infection and chronic disease [10]. The adenoids and tonsils are the only secondary lymphoid organs that are localised to the nasopharynx and middle ear. There is evidence that adenoidectomy in children suffering from chronic OM improves the clinical outcome by reducing the incidence of disease [1]. It is often suggested that these improvements are due to the removal of the inflamed, and sometimes necrotic, tissue and the associated microbiological reservoir [11]. It remains unknown however, whether or not this phenomenon

occurs due to factors associated with local cellular immunity due to removal of the secondary lymphoid organs of the nasopharynx.

This paper presents the microbiology and pathophysiology of OM, with an overview of the current understanding of cellular immune factors associated with OM. The T_{reg} and T_H17 cellular populations are a particular focus, with discussion of their importance in balancing the inflammatory response to common commensals and opportunistic pathogens at mucosal sites.

THE RESPIRATORY SYSTEM

The three major compartments of the respiratory system include the lower respiratory tract, nasopharynx and middle ear (Fig. 1). These three sites of the respiratory system are vulnerable to many infections, as the nasopharynx is host to a milieu of viral and bacterial organisms [12]. The epithelium, mucous secretions, ciliary clearance and various immunological cells are important physical, cellular and chemical defences the body employs to clear the respiratory tract of foreign particles and harmful microbes. When these defences become compromised, infection can occur, causing discomfort, pain and impaired function [13]. Although it is evident that the physiology of the Eustachian tube influences the development of middle ear infections, what is not well understood is what immune factors mediate the progression from nasopharyngeal colonisation to middle ear infections [14].

Immunocytology and Physiology of the Nasopharyngeal Lymphoid Tissue

The secondary lymphoid organs in the upper respiratory tract (URT) are localised in the Weldeyer's ring. This is an arrangement of four secondary lymphoid organs in a circular rotation of the throat that consists of the palatine, tubal, lingual and nasopharyngeal (adenoid) tonsils [15]. The palatine and lingual tonsil and the adenoid are the dominant lymphoid organs of the Weldeyer's ring with the palatine tonsil and adenoid most studied due to their availability from tonsillectomy and adenoidectomies [16]. Although the tonsils and adenoids have a complex physiology, two regions of interest are the extrafollicular areas and mantle zones as this is where cellular and humoral acquired immunity is most active within these tissues. In the extrafollicular areas, dendritic cells (DC), interdigitating DC, macrophages, mast cells and lymphocytes are found [16-19]. Within the lymph nodes of the adenoids and tonsils are the follicular zones and the germinal centres (Fig. 2). Follicular DC with long processes extending out to lymphocytes and plasma cells, and mast cells are found in the lymph nodes. However, the follicular zones consist mainly of T lymphocytes while the germinal centres are abundant in naive B lymphocytes [17, 20]. As the adenoids and tonsils are the primary source of immune cells in the

upper airways, it is speculated that local immune regulation of the middle ear and nasopharynx may come from these secondary lymphoid organs, although this concept is very under-researched and lacks understanding [1].

Immune Factors of the Nasopharynx and Middle Ear

Immune system factors present in the nasopharynx include interferon (IFN) types I and III, β-defensins, lactoferrin, lysozyme, cathelicidins and mucins, all of which have antimicrobial properties, although the IFNs and cathelicidins are yet to be identified in the human middle ear or nasopharyngeal lymphoid tissue [13, 21-31]. Down-regulation of mRNA or protein expression of the microbial molecule-specific pattern recognition receptors (PRR) retinoic acid-inducible gene 1, NACHT, LRR and PYD domains-containing protein 3 (NALP3), and Toll-like receptor (TLR) 3, 4, 7 and 9 is evident in the accumulation of fluid (effusion) in the middle ear and in the middle ear mucosa of OM-prone children compared to non OM-prone children [32, 33]. Lymphoepithelial tissue of the tonsil and adenoid express TLR4, 7, 9, and, especially strongly, TLR3, which is a significant PRR for antiviral responses [34, 35]. Additionally, a large number of IFN inducible genes and signal and regulatory factors are up-regulated in human middle ear epithelial cell (EC) cultures in a dose- and time-dependent manner in response to Influenzae A virus infection [36]. These include myeloid differentiation primary response gene 88 (MyD88), a signal transducing adaptor protein used by most TLRs to activate the 'rapid-acting' primary transcription factor NF-κB in response to harmful cellular stimuli, and interferon regulatory factors 1 and 7, signalling factors for the production of pro-inflammatory cytokines and IFNs [36]. Other examples include genes that encode for the proteins vipirin, myxovirus resistance 1 and 2, and 2',5'-oligoadenylate synthetase 1 and 2, all of which are involved with degradation of viral components and inhibition of viral replication [36]. Based on the evidence that human middle ear mucosa and nasopharyngeal lymphoid tissue have EC that express PRR, signal and regulatory factors and IFN inducible genes designed for viral detection and inhibition, and that both sites are known for the isolation of common respiratory viruses such as respiratory syncitial virus (RSV) and rhinovirus (RV) that often predispose bacterial OM, it is possible that the human middle ear and the nasopharyngeal lymphoid tissue are potential sites for type I and III IFN production. This is due to the fact that the above innate immune response molecules are necessary for downstream IFN production following viral pathogen-associated molecular pattern (PAMP) recognition. Supporting this notion are studies in mice which have shown the ability of the nasal-associated lymphoid tissue (NALT) to express IFN-α and IFN-β mRNA and multiple IFN-stimulated genes following pneumococcal colonisation [37]. The detection of IFNs in the middle ear mucosa of rodents has also been reported [38].

TLRs and nucleotide-binding oligomerization domain-containing proteins (NODs), also involved in pathogen recognition, are expressed in the middle ear mucosa, although at reduced levels in OM-prone children [33]. The distribution of these PRR between the nasopharynx and middle ear is unknown, although research in rodents has demonstrated that PRR expression increases at the proximal end of the Eustachian tube [39]. This could indicate that the innate immune mechanisms of the nasopharynx have evolved to prevent colonisation at this site in order to maintain the sterile environment of the middle ear and thereby prevent infections. The immune processes in the URT contributing to such host homeostasis and colonisation are, however, poorly understood [39, 40]. TLR, γδ T lymphocytes, intraepithelial lymphocytes, natural killer (NK) cells, DC, T lymphocytes, B lymphocytes, neutrophils and macrophages are also distributed throughout the nasal mucosa [34, 35, 41, 42]. Some of these cell types have been identified in the middle ear mucosa and in middle ear effusion, although they are not as well characterised in this region of the nasopharynx [32, 33, 43, 44]. In animal models of OM it is known that lymphocytes enter the middle ear via blood circulation [45]. However, what remains to be established is how local or distal lymph trafficking influence the acquired response in this site. Furthermore, the immune trafficking of lymphocytes to the middle ear in the human is not understood.

OTITIS MEDIA

OM is defined as inflammation of the middle ear associated with an effusion within the middle ear [46]. OM presents in a range of pathologies from acute OM (AOM) through to chronic suppurative OM (CSOM). Symptoms differ in the degree of severity from mild inflammation to tympanic membrane perforation with effusion [46]. AOM presents with at least one of the acute signs of inflammation of the middle ear such as otalgia, irritability, bulging or redness of the tympanic membrane, otorrhoea or fever. In the early stages of infection middle ear effusion may not be present. However, quite often infection progresses in which middle ear effusion (MEE) accompanies the signs of inflammation and is visible as cloudiness within the middle ear [47]. AOM may progress to OM with effusion (OME) that is defined due to the presence of MEE, although unlike AOM, there are no obvious signs of acute inflammation. Some temporary hearing loss may be evident in both conditions [47, 48]. AOM and OME may evolve to a chronic condition if it is recurrent for more than 3 times in 6 months, known as recurrent AOM. If the infection persists for 3-12 weeks it becomes subacute and if longer than 12 weeks it is known as chronic OM (COM) or COM with effusion (COME) [47]. Acute exacerbations may be evident during the course of COM and COME, and this often presents with a purulent discharge or suppuration, hence the term CSOM. Acute suppurative OM may also occur depending on the severity of the infection [47].

Otitis Media Pathophysiology

OM is a complex polymicrobial disease in which infections occur with virus, bacteria or sometimes both concurrently. The respiratory viruses that cause OM or predispose to the bacterial infections that cause OM include RSV, influenza A and B virus, parainfluenza virus type 1, 2 and 3, adenoviruses, enteroviruses and RV [49]. Clinical investigations have confirmed that the aetiology and pathogenesis of OM is associated with upper respiratory viral infections [50]. These infections exacerbate the clinical and bacteriological outcome of OM by compromising mucosal physical barriers, enhancing bacterial adherence to respiratory EC, and altering immune cell function and gene expression [49, 51, 52]. *Streptococcus pyogenes*, group A *Streptococcus*, *Pseudomonas aeruginosa*, *Staphylococcus aureas* and *Alloiococcus otitidis* are bacteria that may cause OM, however not predominantly [53-55]. The three most common species of bacteria cultured from OM infections are non-typeable *Haemophilus influenzae*, *Moraxella catarrhalis* and most commonly cultured, *Streptococcus pneumoniae*, [52, 54]. A factor in common to all these bacteria is that they are commensals of the nasopharynx which become aggressive opportunistic pathogens when physiological conditions are compromised and/or there is a shift in immune homeostasis. This may be influenced by carriage load, changes in the nasopharyngeal mucosa integrity, microbial community in the nasopharynx and the associated immune response. All of these factors are intricate and involve multifactorial microbial and immunological processes that are not entirely understood [56-59].

S. pneumoniae, *M. catarrhalis* and non-typeable *H. influenzae* colonise the nasopharynx asymptomatically but host tolerance of their high nasopharyngeal loads contributes to the development of OM. While *M. catarrhalis* naturally colonises healthy children, carriage rates may be as high as 100% by three months of age [60]. Likewise, pneumococcal colonisation establishes early in life and also occurs in high loads, accounting for up to 70% of nasopharyngeal bacterial colonisation in some high risk populations [52, 61]. Non-typeable *H. influenzae* colonisation of the nasopharynx constitutes a large proportion of the *Haemophilus* species that account for around 10% of nasopharyngeal normal flora [62]. Like *M. catarrhalis* and *S. pneumoniae*, non-typeable *H. influenzae* has a dominant nasopharyngeal carriage rate of approximately 60% in infants in some ethnic populations but colonises the nasopharynx after *M. catarrhalis* and *S. pneumoniae*. As the immune system develops, particularly cellular immunity, pneumococcal carriage will decrease to between 2-10% by 10 years of age, and by adulthood *M. catarrhalis* colonisation will occur in only 1-5% of individuals. However, during OM and other respiratory tract infections, nasopharyngeal colonisation of these commensals increases [60, 63]. Higher carriage rates of *M. catarrhalis* may also be evident in persons with pre-existing respiratory

conditions including allergic sinusitis and chronic obstructive pulmonary disease [58, 64]. Collectively, this research indicates a shared characteristic of these bacteria to colonise nasopharyngeal mucosa in high loads during infancy and early childhood, with colonisation levels diminishing into adulthood. Although this may coincide with maturation of immunity, the immunological factors that regulate bacterial colonisation are not well understood and therefore merit a greater focus in current research of OM [52, 65].

THE IMMUNE RESPONSE TO NASOPHARYNGEAL COLONISATION

The polymicrobial features and carriage load variations which have been described are characteristics of nasopharyngeal colonisation that influence immune responses at this site. Nasopharyngeal colonisation studies of *S. pneumoniae* and of *H. influenzae*, in either murine colonisation models or human respiratory epithelial *in vitro* models, demonstrate enhanced local acute inflammatory responses during dual colonisation compared to single colonisation. Neutrophil influx is increased in the nasal mucosa of mice during dual colonisation compared to single colonisation. Furthermore, *S. pneumoniae* nasal colonisation was reduced in the presence of *H. influenzae*, and this was found to be mediated through components of *H. influenzae* activating complement-dependant neutrophil phagocytic killing of *S. pneumoniae* [66]. The inflammatory cytokines involved in neutrophil recruitment, macrophage inhibitory protein (MIP) 2 in mice and interleukin (IL)-8 in humans, are also elevated during dual colonisation [66, 67]. In this rodent model it was found that the MIP 2 induction was dependent on *S. pneumoniae* production of pneumolysin and the activation of the p38 mitogen-activated protein kinase [67]. In single bacteria nasal colonisation rodent *in vivo* and *ex vivo* models, complement-mediated neutrophil phagocytosis has also been shown to have a role in the clearance of *H. influenzae* nasal colonisation [68].

During the inflammatory response, the platelet-activating factor receptor (PAFR) expression is up-regulated on epithelial and endothelial surfaces [69]. *S. pneumoniae* binds to the PAFR through its ligand phosphorylcholine, which upon ligation, *S. pneumoniae* undergoes translocation into the cell via endocytosis [70]. The early inflammatory process associated with this infection state is due largely to neutrophil infiltration into the mucosa from transendothelial migration [71]. A study using a transmigration *in-vitro* model has shown that neutrophils migrate across an endothelial monolayer in response to live wild-type *S. pneumoniae* in a dose-dependent manner, however killed wild-type *S. pneumoniae* and mutant pneumolysin-deficient *S. pneumoniae* only induce neutrophil migration at a minimal level, indicating that the pneumococcal toxin, pneumolysin, and live *S. pneumoniae* are important factors in eliciting a potent early inflammatory response in the mucosa [71].

The PRR TLR2, TLR 4 and NOD1 are important in the clearance of encapsulated strains of *H. influenzae*, however neutrophils were found to enhance the killing of *H. influenzae* when accompanied by TLR4 signalling pathways [68]. Mice lacking TLR4 had enhanced nasal colonisation loads of *H. influenzae*, however not as effectively as mice lacking TLR2 or NOD1. The TLR4 knockout mice did however exhibit significantly higher levels of *H. influenzae* prolonged survival (colonisation levels detected at 14 days post inoculation) compared to the TLR2 and NOD1 knockout mice. Interestingly, TLR2 knockout mice had diminished neutrophil activation compared to TLR4 and NOD1 knockout mice, suggesting that the TLR2 signalling pathway may be important in controlling encapsulated *H. influenzae* colonisation through neutrophil activation. Taken together, these findings indicate that TLR2, TLR4 and NOD1 signalling pathways are important in the hosts innate immunity to nasal colonisation by encapsulated strains of *H. influenzae* [68]. In contrast, the lack of expression of TLR2, TLR4 or NOD1, and a deficiency in neutrophils, did not alter the clearance of non-typeable *H. influenzae* (non-encapsulated strains) indicating that there are redundancies in place that can eliminate this coloniser, likely complement-induced antibody opsonisation-mediated phagocytosis as the lack of the polysaccharide capsule render the bacteria more susceptible to such immune mechanisms [68]. The signalling molecule MyD88 that is common to the TLR family signalling cascade has been found to be crucial in host immunity to *S. pneumoniae* nasal colonisation and systemic infection [72]. In nasal colonisation and infection rodent models, mice lacking MyD88 had higher *S. pneumoniae* nasal colonisations loads, more severe lower respiratory infections and systemic infections indicated by a quick onset and high bacterial loads, significantly decreased survival rates, and decreased innate immune responses indicated by reduced neutrophil and polymorphonuclear leukocyte lung infiltration, decreased tumour necrosis factor (TNF)-α, IL-6 and chemokine ligand 1 (KC) and reduced signs of an inflammatory reaction in the lungs. These results clearly indicate that MyD88 in the TLR signalling pathway is crucial in the local and systemic cytokine and leukocyte-associated immune response to *S. pneumoniae* colonisation and infection [72].

Cytokine Responses to Nasopharyngeal Colonisation

The middle ear mucosa is capable of mounting an inflammatory response to OM pathogens including *S. pneumoniae* and *A. otitidis*. During such an inflammatory response cytokines including IL-8, IL-1β, IL-6 and TNF-α are generated which represent both innate and adaptive immunity lineages [73]. Children with recurrent OM have demonstrated impaired IL-2 and IL-4 production by adenoidal lymphocytes after restimulation with *S. aureus*. Furthermore IFN-γ is released by adenoidal lymphocytes in response to the same restimulation. However, this T_H1 response is subdued in such lymphocytes compared to peripheral blood lymphocytes,

indicating that mucosal immunity to nasopharyngeal colonisation is suppressed when compared to the systemic response [74]. Mice starting out at 1, 2 and 6 weeks old, representing neonatal, infant and adult mice, respectively, showed significant differences in their macrophage, chemokine and cytokine responses when challenged with *S. pneumoniae* in a 7 or 14 day colonisation model. Overall, the neonatal and infant mice had reduced granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, monocyte chemotactic protein-1 and the neutrophil attractant chemokine (C-X-C motif) ligand-1. IL-1α, IL-6, TNF-α and IFN-γ cytokines were also significantly reduced compared to the response of adult mice. The only exception to this trend was the response of IL-10, which was 15-fold higher in neonates than in adult mice [65]. These experimental findings indicate that in infancy, neutrophil recruitment and activation, T lymphocyte development and survival, and induction of T_H1 and T_H17 responses may be impaired as the cytokines and chemokines that are restricted in the infant mouse are important to these innate and adaptive immune processes. This is particularly important in relation to pneumococcal nasal colonisation since neutrophils and T_H17 lymphocytes are regarded as important mediators of *S. pneumoniae* clearance [75].

Adaptive Immunity to Nasopharyngeal Colonisation

The adaptive component of the immune system involves a complex network of many cell types and molecules that act in concert to mount an aggressive, quick and effective response to counter a challenge with a pathogenic microbe. In addition to EC, the key immunological cells include activated myeloid cells, such as macrophages, monocytes and DC, as well as B lymphocytes and a variety of subsets of T lymphocytes of polarised function. During T_H lymphocyte activation the cells will adopt a particular cytokine profile and mature into effector lymphocytes of the phenotype T_H1, T_H2, T_H3, T_H9, T_H17, T_H22 or T follicular helper lymphocytes (T_{FH}). Through cell to cell contact or messenger molecules such as chemokines and cytokines, these cell types communicate with each other to generate an optimal response to clear an infection [76]. In some cases immune tolerance may be induced for non-threatening commensals at mucosal sites or to suppress excessive inflammatory responses that may be damaging to host tissue [10, 77, 78].

In the URT it remains unclear exactly what adaptive immune pathways and mechanisms regulate the response to bacterial colonisation. The bacterial load that is present at any given time may influence immune function in the nasopharynx as there is a direct correlation between nasopharyngeal bacterial load and increased proliferation of T and B lymphocytes [79]. Since larger lymphocyte populations do not necessarily equate to greater lymphocyte activation, this often contributes to the problem of hypertrophic adenoids in diseased

patients [80]. Investigations into the effects of pneumococcal colonisation has revealed that control of pneumococcal carriage may be independent of antibody neutralisation [75]. Recent reports have shown that in mice T_H17 $CD4^+$ T lymphocytes produce the pro-inflammatory cytokine IL-17A to induce a monocyte/macrophage and neutrophil cellular-mediated reduction in pneumococcal colonisation [75, 81]. An increase of IL-17A has also been identified in human tonsillar tissue stimulated with pneumolysin-producing pneumococcal whole cell antigen. *In vitro* studies using human neutrophils demonstrate an 1L-17A dose-dependent-neutrophil-mediated killing of *S. pneumoniae* [75]. Pneumococcal carriage in children has also been shown to induce $CD4^+$ T lymphocyte-mediated protective immunity in peripheral blood and adenoidal mononuclear cells. Interestingly, a decrease in the $CD4^+$ T lymphocyte response was evident in pneumococcal culture-positive children compared to children with an absence of pneumococcal nasopharyngeal culture [82]. Collectively, these results support the notion that pneumococcal colonisation is mediated via $CD4^+$ T lymphocytes but that *S. pneumoniae* itself may influence the dynamics of this response. Although non-typeable *H. influenzae* has been demonstrated to activate T and B lymphocytes from tonsillar tissue and to induce a T_H1 lymphocyte response, inadequate T lymphocyte proliferation to the P6 antigen was evident in OM-prone children compared to non OM-prone children [83, 84]. A weak T lymphocyte repertoire in young children may explain the high pneumococcal and non-typeable *H. influenzae* carriage observed in this population, or the T_H17 lymphocyte response may be subdued due to T_{reg} lymphocyte suppression. Further research in this area is needed to elucidate fully the mechanisms involved.

Whereas *S. pneumoniae* and non-typeable *H. influenzae* appear to induce T lymphocyte responses, *M. catarrhalis* may be able to induce lymphocyte responses that are thymus-independent. *M. catarrhalis* immunoglobulin (Ig) D-binding (MID) protein has been demonstrated to induce B lymphocyte proliferation and activation with T_H2 cytokine co-stimulation, in the absence of T lymphocytes. Supplementing the cultures with recombinant CD40 ligand enhanced both the B lymphocyte proliferative response and antibody production, indicating that T lymphocytes may enhance a *M. catarrhalis* MID protein-induced B lymphocyte response. Of note, further activation of T lymphocytes by *M. catarrhalis* MID protein was poor, supporting the view of a B lymphocyte cellular response to *M. catarrhalis* [85].

Antibody Responses to Nasopharyngeal Colonisation

Adenoidal tissue from children with OM has been demonstrated to generate *S. pneumonia* and *H. influenzae* type b-specific IgG and IgA antibody, with production of IgG dominating over that of IgA. These

antibody responses to the two nasopharyngeal colonisers are also more prominent in the adenoidal secretions compared to the peripheral blood, indicating that the pathogen-specific humoral response is compartmentalised in the mucosa [86]. Interestingly, reduced immunity is often evident following respiratory bacterial colonisation, especially in individuals prone to respiratory infection. Poor inflammatory responses have been associated with *M. catarrhalis* colonisation of the luminal regions of the lower respiratory tract [87]. A lack of secretory antibody to *M. catarrhalis* and *S. pneumoniae* outer membrane proteins has been reported in children aged from birth to 2 years who show active nasopharyngeal colonisation of both bacteria. In adults, however, secretion of salivary IgA to multiple outer membrane proteins of *M. catarrhalis* has been demonstrated, indicating that repeated or prolonged exposure enhances the mucosal antibody response [88, 89]. Furthermore, there is a significant reduction in antibody responses to the non-typeable *H. influenzae* P6 antigen in OM-prone children compared to non OM-prone children [90]. Although an underdeveloped immune system in early childhood may contribute to impaired immunity, it does not explain sufficiently why OM-prone children do not mount an adequate Ig response against the nasopharyngeal flora, as demonstrated in non OM-prone children of the same age. Collectively, this may indicate immunosuppression during nasopharyngeal colonisation with the aforementioned bacteria and hence host tolerance of high carriage loads contributing to the development of OM or other URT infections. Unfortunately, very little is understood of the crosstalk between innate and adaptive immunity in the tolerance of nasopharyngeal flora or how the co-colonisation of many microbes in the nasopharynx may affect immune dynamics [35]. By investigating the role of T_{reg} lymphocytes in URT colonisation and how these cells are induced at this site, a clearer understanding may emerge of host tolerance to nasopharyngeal colonisation and progression to chronic disease.

Mucosal Immunity versus Systemic Immunity

Although both cellular and humoral immunity are important to the homeostasis of nasopharyngeal bacterial colonisation, there is evidence to suggest that immune responses at mucosal sites are compartmentalised from systemic responses such as those in the peripheral blood [91]. While nasopharyngeal colonisation of *S. pneumoniae* is controlled by a T_H17 $CD4^+$ T lymphocyte response, systemic infections such as bacteraemia are combated via an antibody-mediated opsonisation inducing phagocytosis that is independent of a $CD4^+$ T lymphocyte response [91]. Interestingly, however, pre-nasopharyngeal colonisation of *S. pneumoniae* confers an enhanced antibody-mediated protection to systemic challenge that acts via natural immunity [91]. Recent studies on nasopharyngeal pneumococcal colonisation detected induction of several pneumococcal antigen-specific serum IgG responses in children 12-24 months of age. Unfortunately, this humoral immunity

failed to protect against pneumococcal nasopharyngeal recolonisation albeit due to the polymorphic and capsular shielding nature of *S. pneumoniae* or the shortfall of systemic immunity to confer mucosal protection. Hence, it seems that T lymphocytes of the mucosa are more promising in control of pneumococcal colonisation in the nasopharynx [92].

The OM pathogens *S. pneumoniae*, *M. catarrhalis* and non-typeable *H. influenzae* have been shown to induce cellular responses including the activation of B and T lymphocytes from peripheral blood. Induction of cytokines with a T_H1 lymphocyte signature was also evident, although T lymphocytes were found not to be the cellular source [93]. It is known that these OM pathogens can activate NK cells and that the cytokine profile observed may originate from NK cells in an early innate response that aids the activation of T lymphocytes for a downstream T_H1 lymphocyte cascade [93-95]. Of the little that is known of cellular immunity to OM pathogens, most relates to systemic responses which at best play a very limited role in regulation of bacterial colonisation at the nasopharyngeal mucosal site. It is important to note that microbial challenge at one mucosal site can often confer a certain protection from microbial challenge or hypersensitivity at another mucosal site within the body. This is evident in patients suffering from chronic *Helicobacter pylori* infection of the gastrointestinal mucosa, who demonstrate protection from hypersensitivity disorders such as asthma in the lower respiratory mucosa [96]. In a rodent model of experimentally-induced allergic airway disease, this phenomenon has been recently attributed to *H. pylori*-induced T_{reg} lymphocytes migrating to the lungs and conferring protection to hypersensitivity via the immunosuppressive effects of intrinsic IL-10 production, as evidenced by reduced T_H2 and T_H17 cellular responses (Fig. 3) [97, 98].

γδ T lymphocytes are a small subset of T lymphocytes that represent approximately 2% of the total T lymphocyte population. These cells differ from conventional T lymphocytes in the structure of their T cell receptor (TCR), switching the conventional α and β chains with one γ and one δ chain. γδ T lymphocytes are not restricted to major histocompatibility complex (MHC) class I or II recognition as they can identify whole proteins without the requirement for these to be processed and presented via antigen-presenting cells (APC). γδ T lymphocytes are found abundantly in the intestinal, nasal and bronchial mucosa where they work in close association with local intraepithelial lymphocytes in the epithelial mucosal layer and are therefore important contributors to mucosal immunity [42, 99]. A role has been reported for γδ T lymphocytes in regulation of pulmonary inflammation in a *S. pneumoniae* infection model [100]. At 7-10 days post *S. pneumoniae* intranasal challenge, *c*learance of bacteria was evident but lungs remained inflamed. By this time, γδ T lymphocyte populations had infiltrated the lungs significantly, with more than a 30-fold increase compared to naïve mice,

observed as a localised mucosal response [100]. Numbers of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and NK lymphocytes were up to double those found in controls, suggesting that due to their dominant presence γδ T lymphocytes have an important role in reducing *S. pneumoniae*-associated pulmonary inflammation [100]. γδ T lymphocyte-deficient mice were equally efficient at clearing *S. pneumoniae* from the lungs compared to wild type mice. It was determined that γδ T lymphocytes reduce lung inflammation and granuloma formation by inhibiting the alveolar macrophage and pulmonary DC response through direct cytotoxicity [100]. Although this indicates that γδ T lymphocytes are not associated with immunity to *S. pneumoniae*, it does point to their importance for controlling the inflammatory pathology associated with *S. pneumoniae* infections and that this response is limited to the pulmonary mucosa. This highlights how γδ T lymphocytes are instrumental to a controlled local response that avoids compromising immune function at a systemic level.

Antigen Influences T Helper Lymphocyte Maturation

T_H lymphocytes are evidently important in nasopharyngeal colonisation. The binding of microbial antigen to a TLR initiates a complex cellular signal and a pro-inflammatory response is generated that will influence a T_H lymphocyte response [101]. The effector state that is adopted is influenced largely by the type of infection, PRR and APC [102, 103]. Nasopharyngeal tissue from children has been shown to express T_H1, T_H2, T_H17 and T_{reg} lymphocyte responses to allergen and antigen stimulation [75, 101, 104]. Additionally, the level of exposure of antigen is a strong influencing factor in what effector phenotype a T_H lymphocyte will adopt. Studies with bee venom have demonstrated that T_H lymphocytes with a T_H1 or T_H2 phenotype expressing IFN-γ or IL-4, respectively, switched to expression of the immunosuppressive cytokine IL-10 upon high dose, continual exposure to the bee venom allergen phospholipase A [105]. Evidently, the microbial or allergen environment that activates a DC and in turn primes a T_H lymphocyte will have a great impact on the outcome of the T_H lymphocyte phenotype and therefore of the effector response. It is known that microbial colonisation of the nasopharynx is high, but it remains to be determined if these elevated carriage loads influence the T_H lymphocyte phenotype and the nature of the effector response [104].

Linking Innate Immunity to T Helper Lymphocyte Responses in the Mucosa

In understanding immune regulation of nasopharyngeal colonisation, it is important to appreciate how the T_H lymphocyte response is influenced at a mucosal site abundant in microbial flora. An important component of the mucosa that detects microbes and signals to T_H lymphocytes are TLR of the innate immune system. The role of TLR in T_H lymphocyte activation has been elucidated in detail [76]. Experiments using MyD88-deficient

knockout mice immunised with ovalbumin and complete Freund's adjuvant revealed that stimulated T lymphocytes failed to proliferate or to produce detectable levels of IFN-γ, a dominant cytokine of the T_H1 response. Furthermore, DC from these animals, when treated with mycobacteria, failed to exhibit up-regulated expression of any of the co-stimulators CD80, CD86, MHC class II and IL-12, all of which are important to formation of a T_H1 response. Taken together, these results demonstrate that the TLR signalling pathways in DC are influential in activating T_H lymphocytes and developing a T_H1 effector response [106]. It has also been shown that IL-6 from TLR-activated DC renders antigen-specific T lymphocytes to overcome the suppressive activity of T_{reg} lymphocytes, thereby skewing the immune response away from a T_H3 phenotype [107].

The concept of microbe and PRR-driven activation of DC and T_H lymphocyte function in the mucosa are supported by studies with probiotics and *H. pylori* used to activate DC via the DC-specific intracellular adhesion molecule 3 (DC-SIGN). Such stimulation was shown to facilitate T_{reg} lymphocyte priming, a weakened T_H1 response marked by a decrease in IL-6 and an increased suppressive response via IL-10 (Fig. 4) [108, 109]. In human gastric biopsy samples this phenomenon of *H. pylori*-induced T_{reg} lymphocyte tolerogenic responsiveness by way of DC is corroborated by the demonstration that *H. pylori* binding to DC-SIGN causes a dampened T_H1 cytokine profile, characterised by reduced IFN-γ and IL-6 and an increased IL-10 immunosuppressive response. It is reasonable to speculate that the production of IL-10 may originate from a T_{reg} lymphocyte profile, although confirmation of this requires further research [110]. The mechanism of *H. pylori*-induced survival via DC to direct the T_H lymphocyte response to an immunosuppressive T_{reg} phenotype is clarified further by recent rodent model studies and supportive findings from human gastric biopsy samples [98]. Bone marrow-derived DC (BM-DC) and mesenteric lymph node (MLN)-DC co-cultured with *H. pylori* and *Escherichia coli* lipopolysaccharide (LPS) resulted in DC expressing high MHC class II and lower levels of CD80, CD86 and CD40 (MHC $II^{HI}CD80^{LO}CD86^{LO}CD40^{LO}$) compared to DC exposed to *E. coli* LPS alone, indicating *H. pylori* impaired DC maturation. Furthermore, IL-12 and IL-6 were decreased and IL-10 inversely elevated in the *H. pylori*-infected, *E. coli* LPS-stimulated cells compared to *E. coli* LPS stimulation only [98]. Interestingly, unlike reports that demonstrate DC-SIGN to be the ligand for *H. pylori* to DC, this study suggests that *H. pylori*-impaired DC maturation is independent of DC-SIGN. This conclusion should be treated with some circumspection since it is drawn from experiments conducted in mice transgenically expressing human DC-SIGN, in which *H. pylori* induced immature DC similar to those evident in wild type mice, with no alternative receptors explored. Firstly, it is feasible that human DC-SIGN does not signal or function in mice as it does in humans and, secondly, if DC in mice express SIGNR3, the functional homologue of human DC-

SIGN, it may be speculated that *H. pylori* preferentially binds SIGNR3 in mice as it does DC-SIGN in humans [109, 111]. Therefore, the above study conducted in SIGNR3$^{-/-}$ mice, and compared to SIGNR3$^{-/-}$ mice with transgenic expression of human DC-SIGN, will explore more accurately the possibility of *H. pylori*-impaired DC maturation independent of C-type lectin binding receptors. This research also demonstrated that *H. pylori*-experienced, semi-mature BM-DC and MLN-DC are adept at converting naive CD4$^+$ T_H lymphocytes to Fork-head box P3 (FoxP3)$^+$CD25$^+$ T_{reg} lymphocytes. Further, these tolerogenic DC are less able than non-*H. pylori*-experienced DC at activating an effector T lymphocyte response, indicated by reduced IFN-γ and T lymphocyte proliferative measures [98]. The ability of immature DC to generate FoxP3$^+$CD25$^+$ T_{reg} lymphocytes was found to be dependent on contact and transforming growth factor (TGF)-β, with DC-derived and T lymphocyte-derived IL-18 necessary to skew this response away from a T_H17, T_H1 profile to that of a T_{reg} lymphocyte. This was demonstrable in wild type mice developing *H. pylori* tolerance while *Il18r*$^{-/-}$ mice had lower *H. pylori* colonisation levels together with higher gastric leukocytes, INF-γ and IL-17 production [98]. Together, these studies demonstrate how microbes and PRR are essential to directing adaptive cellular responses, often resulting in microbial tolerance at mucosal sites and prevention of inflammatory pathology.

The oropharynx and nasopharynx are other mucosal sites where T_H lymphocytes are induced via TLR-activated APC to induce host tolerance to commensal microbiota. Isolated oral Langerhans cells (LC) from human oral mucosa specimens are adept at inducing T_{reg} lymphocytes in the oral mucosa with immunosuppressive functionality. The process requires oral LC to mature via TLR4 activation, whereby up-regulation of co-stimulatory factors including CD80 and IL-10 occurs [112]. Co-culture of these active oral LC induces a T_{reg} lymphocyte phenotype producing IL-10 and TGF-β. Commensal oral bacteria are also reported to activate DC that in turn induce an immunosuppressive T_{reg} lymphocyte phenotype [112, 113]. The mechanism of T_{reg} lymphocyte activation in the human tonsil by DC has been recently elucidated (Fig. 4). Immunohistochemical analysis of tonsil sections clearly shows the co-localisation of FoxP3$^+$ lymphocytes with CD123$^+$ plasmacytoid DC (pDC). Stimulation of pDC with TLR7 and TLR9 ligands up-regulates pDC expression of MHC II, CD80 and CD83 co-receptors [114]. Co-culture of these mature pDC with naive CD4$^+$ T lymphocytes, with or without TLR secondary stimulation resulted in CD4$^+$ CD25$^+$ FoxP3$^+$ CD127$^-$ T_{reg} lymphocytes that secrete predominantly secrete IL-10 and suppress proliferation of autologous T cells [114]. It is evident from these studies that TLR on APC such as DC are crucial to the crosstalk between innate and adaptive immunity that may influence the cellular response to microbes in the mucosa. These findings, taken together, provide strong evidence that T_{reg} lymphocytes may be activated at mucosal sites via DC sampling of

the surrounding microenvironment. A comprehension of the induction of T_{reg} lymphocytes in the nasopharynx is crucially important for understanding the activation pathways and function(s) of T_{reg} lymphocytes in host tolerance to the milieu of microbial flora that colonises this site. Further investigation of T_{reg} lymphocyte induction mechanisms in different pathological states and in response to common commensals of the URT could provide novel approaches to coaching the immune system, through therapeutic interventions, towards faster recovery from infection and prevention of chronic illness.

T REGULATORY LYMPHOCYTES IN THE MUCOSA

T_{reg} lymphocytes are a subtype of $CD4^+$ T lymphocytes, the main function of which is the induction of peripheral immune tolerance to both foreign antigen and self antigen. A dysfunction or imbalance in T_{reg} lymphocyte numbers has been shown to contribute to the development of conditions such as allergy, cancer, autoimmune disorders and allograft rejection [115-118]. Chronic infections and inflammation-derived tissue damage may also arise from abnormal T_{reg} lymphocyte function [119, 120]. The cellular characteristics of T_{reg} lymphocytes are similar to those of a T_H lymphocyte, but they may be distinguished by their high level expression of FoxP3, a transcription factor belonging to the Fork-head – winged helix family. FoxP3 is necessary to maintaining the suppressive function of T_{reg} lymphocytes, its deletion resulting in the loss of suppressive capacity [121]. T_{reg} lymphocytes also express mid to high levels of the surface receptor CD25 (the IL-2 receptor α-chain), CD152, TNF receptor 2, membrane-bound TGF-β and particularly in humans, low level expression of CD127 (the IL-7 receptor α-chain). This low level CD127 expression is used, together with the other receptors mentioned, to distinguish T_{reg} lymphocytes from effector T lymphocytes. This is because FoxP3 expression cannot be used as a unique identifier of T_{reg} lymphocytes since effector T lymphocytes share FoxP3 expression [122, 123]. T_{reg} lymphocytes have been identified in the nasopharynx and are known to have a positive association with nasopharyngeal colonisation by *S. pneumoniae*. However, currently it is not known if host tolerance to *M. catarrhalis* and non-typeable *H. influenzae* colonisation is associated with a T_{reg} lymphocyte immunosuppressive response [104, 114]. Furthermore the influence of polymicrobial colonisation of the nasopharynx on T_{reg} lymphocyte phenotype and effector response is yet to be elucidated.

T_{reg} Lymphocyte Subtypes

Two functional types of T_{reg} lymphocytes exist; naturally occurring, thymus-derived T_{reg} lymphocytes (nT_{reg}) and naive, $CD4^+$ $CD25^+$ inducible T_{reg} lymphocytes (iT_{reg}) [103]. iT_{reg} lymphocytes are generated from mature $CD4^+$ T lymphocytes, both conventional $CD4^+$ T and nT_{reg} lymphocytes, in the periphery at certain times

of antigenic and cytokine stimulation. Both the cytokines TGF-β and IL-2 and retinoic acid (RA) are necessary for the transition of $CD4^+$ T lymphocytes to iT_{reg} lymphocytes. Type 1 iT_{reg} lymphocytes (Tr1) are known to secrete predominantly IL-10 during active immunosuppression, while Type 3 iT_{reg} lymphocytes (T_H3) exert immunosuppression via a biased secretion of TGF-β [124, 125]. All iT_{reg} lymphocytes may secrete these cytokines but the profile depends on the prevailing physiological setting [103, 124, 125]. The thymus-derived nT_{reg} lymphocytes possess a large TCR repertoire to self and non-self antigens in which their main role is to induce suppression of T lymphocytes and APC during autoimmune responses in a cell to cell contact-dependent fashion via CD152 and membrane-bound TGF-β [118]. They therefore have a strong influence in maintaining homeostasis. iT_{reg} lymphocytes on the other hand, have a more non-self-specific TCR repertoire which, when activated, exerts a suppressive effect on T lymphocytes and APC via soluble factors including IL-10 and TGF-β, rather than through a direct cell to cell contact mechanism [126]. As iT_{reg} lymphocytes are actively induced in the periphery via non-self antigen presentation and cytokine signals to suppress pro-inflammatory cellular responses, it is thought that they are involved in the tolerance to microbes at mucosal sites. Evidence of this has been discussed with regard to the gastrointestinal, oropharynx and nasopharynx mucosa where ligation of microbial receptors on DC induces a skewed T_{reg} immunosuppressive effector response, although precise mechanisms of this process in relation to nasopharyngeal colonisation require clarification (Fig. 4) [104, 109, 110, 112-114].

The Role of IL-10 and TGF-β in T_{reg} Lymphocyte Immunosuppression

The cytokines that T_{reg} lymphocytes generate to down-regulate pro-inflammatory responses by T_H and cytotoxic T lymphocytes, NK cells and APC exert immunosuppressive properties through a variety of mechanisms. IL-10 inhibits antigen presentation by blocking the co-receptors CD28 and CD80, thereby interrupting T lymphocyte stimulation, proliferation and cytokine production [127]. TGF-β and IL-10 are powerful immunosuppressors that can disrupt antigen presentation via changes in MHC Class I and II, CD40, CD80/CD86 and IL-12 co-stimulatory molecule expression on APC. These cytokines may inhibit the inflammatory process by disrupting effector macrophages and monocyte responses as well as affecting T_H1 and T_H2 responses by inhibiting potent pro-inflammatory cytokines and chemokines. IL-10 and TGF-β may also impair T lymphocyte activation via altering the CD28 signalling cascade [128, 129]. Overall, the pleiotropic immunosuppressive effects these cytokines exert under certain conditions of physiological stress inhibits antigen presentation and consequently T lymphocyte proliferation, activation and cytokine secretion. The actions of IL-

10 and TGF-β driven by T_{reg} lymphocyte stimulation has been shown to be influenced by the physiological state (type of infection, allergy or autoimmune response), type of antigen and antigen exposure [105, 108, 128].

Maintenance of T_{reg} Lymphocyte Phenotype and Plasticity Characteristics

High expression of FoxP3, TGF-β and IL-2 each plays a role in maintaining iT_{reg} phenotype, inhibiting its conversion to a T_H17 lymphocyte phenotype under IL-6 stimulation [125]. Interestingly, the combination of these cytokines also has an impact on IL-6 signalling on nT_{reg} lymphocytes and can interrupt their switch to T_H17 lymphocytes [125, 130]. In light of this, T_{reg} lymphocytes may switch their function from an immunosuppressive role to an aggressive pro-inflammatory one depending on the cytokine environment and transcription factor activation. Under the influence of low levels of TGF-β and high levels of IL-6, IL-21 and IL-23, driven by the transcription factor retinoic acid-related orphan receptor γt, nT_{reg} but not iT_{reg} lymphocytes may switch to an IL-17-producing T_H17 pro-inflammatory response (Fig. 4). The ability of these cells to switch their role from passive, anti-inflammatory mediators to aggressive, pro-inflammatory inducers, under the influence of a fine balance of regulating factors, makes them key players in maintaining optimal health at mucosal sites where constant microbial stimulation is endured [130].

T_{reg} Lymphocytes and Microbial Interactions

Several studies performed in gastrointestinal tissue have outlined the relationship among colonising bacteria and T_{reg} lymphocytes. Positive correlations are evident with *H. pylori* colonisation, infection and related inflammation [10, 78]. In the state of inflammation it may be reasoned that T_{reg} lymphocytes are present to control the inflammatory response, yet asymptomatic *H. pylori* colonisation associates positively with the presence of T_{reg} lymphocytes [10, 78]. It is thought that this positive correlation is driven by the ability of *H. pylori* to induce naive $CD4^+$ T lymphocytes via DC, TGF-β and IL-10 to mature into $CD4^+$ $CD25^+$ $FoxP3^+$, IL-10-producing iTregs and away from a T_H17 phenotype. This results in prevention of a targeted T_H17 response to *H. pylori* colonisation via a skewed iT_{reg} phenotype (Fig. 4) [110]. Intestinal colonisation in mice with altered Schaedler flora has also contributed to knowledge of the role of T_{reg} lymphocytes in host-microbe homeostasis. These mice show increased iT_{reg} lymphocytes with established colonisation and consequently T_H17 and T_H1 responses are down-regulated, thereby preventing inflammation and promoting bacterial colonisation in the intestine [77]. Interestingly, the influence of probiotics on improved gastrointestinal health, in particular the anti-inflammatory benefits, may be though induction of IL-10-producing T_{reg} lymphocytes via stimulation of DC

and naive $CD4^+$ T lymphocytes by probiotics such as *Lactobacillus casai*, *L. reuteri* and *Bifidobacterium infantis* [108, 131].

T_{reg} lymphocytes have been isolated from the oral cavity of patients with periodontitis and gingivitis lesions, although their association with bacterial colonisation in this site is still poorly understood. Recent evidence indicates T_{reg} lymphocytes with tolerogenic functions are induced in the oral mucosa via oral LC or DC activated with TLR4 or *Streptococcus mitis*, *Propionibacterium acnes* and *Bacteroides fragilis*, respectively [112, 113, 132]. Although this suggests a link between oral commensals and T_{reg} lymphocytes, further research needs to be undertaken to confirm the T_{reg} lymphocyte phenotype since FoxP3 data were lacking in these studies.

Mycobacterium tuberculosis has been demonstrated to induce T_{reg} lymphocytes with suppressive capacity *in vitro* through monocyte activation and prostaglandin E2 production [133]. Of note, T_{reg} lymphocytes were elevated in peripheral blood mononuclear cells (PBMC) of tuberculosis patients compared to PBMC from healthy tuberculin reactors, indicating a direct relationship between the suppressive T lymphocyte subset and susceptibility to primary tuberculosis [133]. The persistence of malaria and human papillomavirus (HPV) infections has also been linked to levels of with functional T_{reg} lymphocytes. In patients with a clinical *Plasmodium falciparum* infection, blood parasitaemia increased simultaneously with TGF-β peaks in serum, $CD4^+$ $CD25^{hi+}$ T lymphocyte increases and raised expression of FoxP3 in PBMC. IL-6 and IFN-γ were measured at lower concentrations and a slower release indicating that a T_H1 response was under the suppressive effects of a T_{reg} lymphocyte response and consequently favouring a persistent *P. falciparum* infection. Persistent HPV16 infection was also shown to have a positive association with an increased percentage of circulating T_{reg} lymphocytes [134, 135]. However, not all persistent infections are a result of T_{reg} lymphocyte-mediated tolerance. *P. aeruginosa* induces T_{reg} lymphocytes in the spleen and lungs of infected mice, but no relationship is evident between T_{reg} lymphocytes and *P. aeruginosa* infection [136]. This may be due to the high levels of IL-6 in this type of aggressive infection, thereby overcoming a T_{reg} lymphocyte response. This highlights the different roles of T_{reg} lymphocytes in colonisation and infection [136].

Several insightful studies have been published recently which focus on T_{reg} lymphocyte responses to commensals of the nasopharynx. In human tonsils, *Neisseria meningitidis*-specific T_{reg} lymphocytes have been identified that display regulatory activity towards suppression of the T_H1 dominant response to *N. meningitidis* [137]. Even more surprising was that this activity was observed only in the tonsil mononuclear cells and not in PBMC, indicative of a compartmentalised mucosal response [137]. Similar findings have also been reported

with adenoid-derived T_{reg} lymphocytes responsive to *S. pneumoniae* colonisation of the nasopharynx, but in this example a positive correlation was also evident between the suppressive activity and frequency of T_{reg} lymphocytes and the increased carriage of *S. pneumoniae* [104]. Both of these studies on nasopharyngeal commensals highlight the ability of upper respiratory mucosal-derived T_{reg} lymphocytes to induce host tolerance and hence to promote survival of commensals in the nasopharynx. Furthermore, T_{reg} lymphocyte-mediated immune suppression to nasopharyngeal commensals is compartmentalised to the mucosa, leaving one to speculate that T lymphocyte responses to bacterial colonisers (and in events of physiological stress, potential pathogens) of the upper airways may be regulated at the site of induction as opposed to systemically controlled responses [104, 137].

CONCLUSION

While the first steps to dissecting tolerance to commensals in the upper respiratory tract have been taken, there are still many cellular mechanisms involved in this process that are yet to be characterised (Fig. 4). Investigation of the adenoid and peripheral blood T_{reg} lymphocyte responses to nasopharyngeal colonisers, in conjunction with clinical nasopharyngeal culture outcomes, would be beneficial to understanding colonisation tolerance in the respiratory mucosa. This would increase our knowledge of host tolerance to nasopharyngeal colonisation in OM-prone children. From a clinical research perspective, this may reveal novel strategies for immune therapy to regulate nasopharyngeal colonisation, with the ultimate goal of preventing progression to chronic disease.

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CONFLICT OF INTEREST

None declared.

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FIGURE LEGENDS

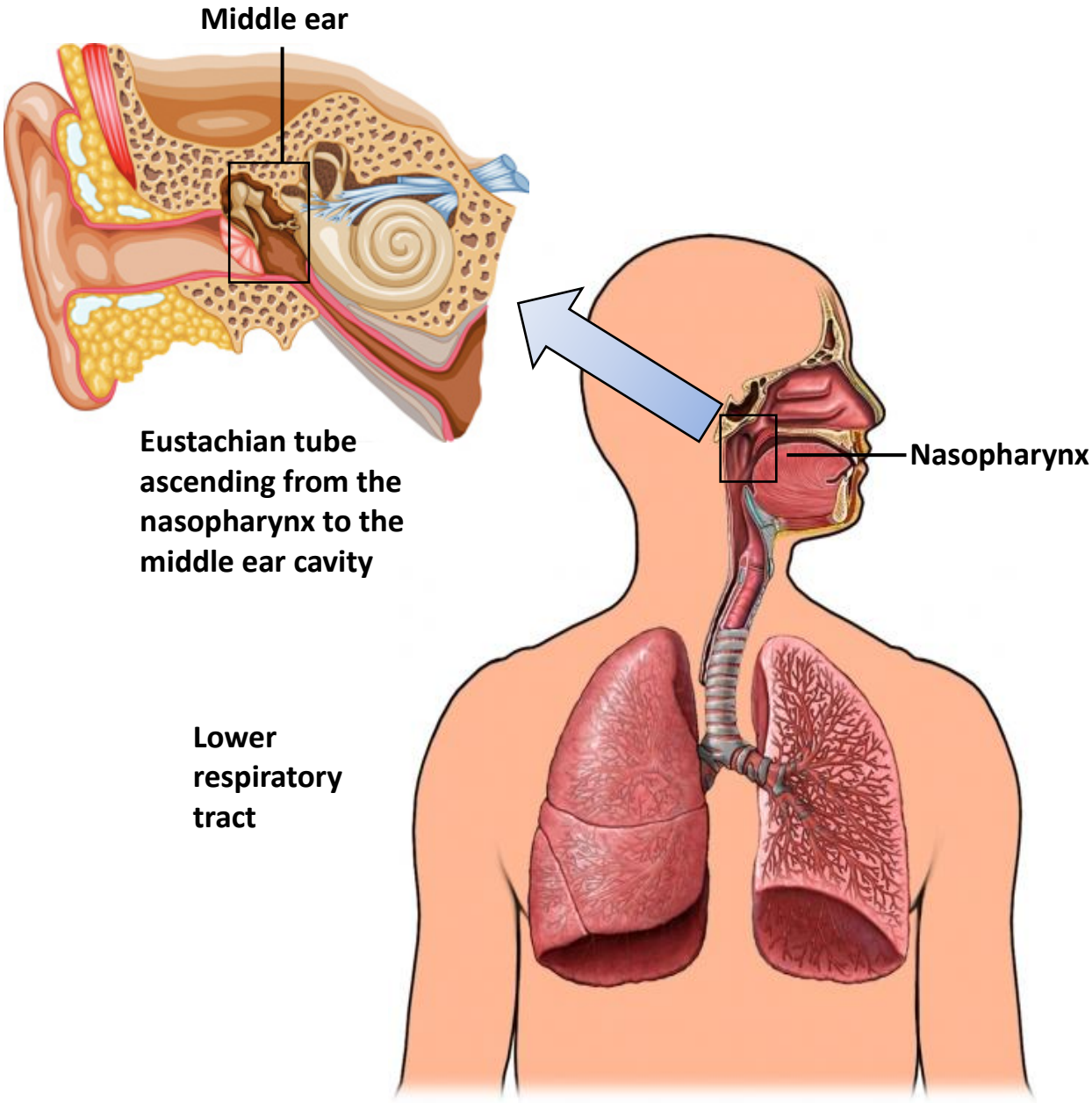
Fig. (1). The respiratory system showing the anatomy of the upper respiratory tract.

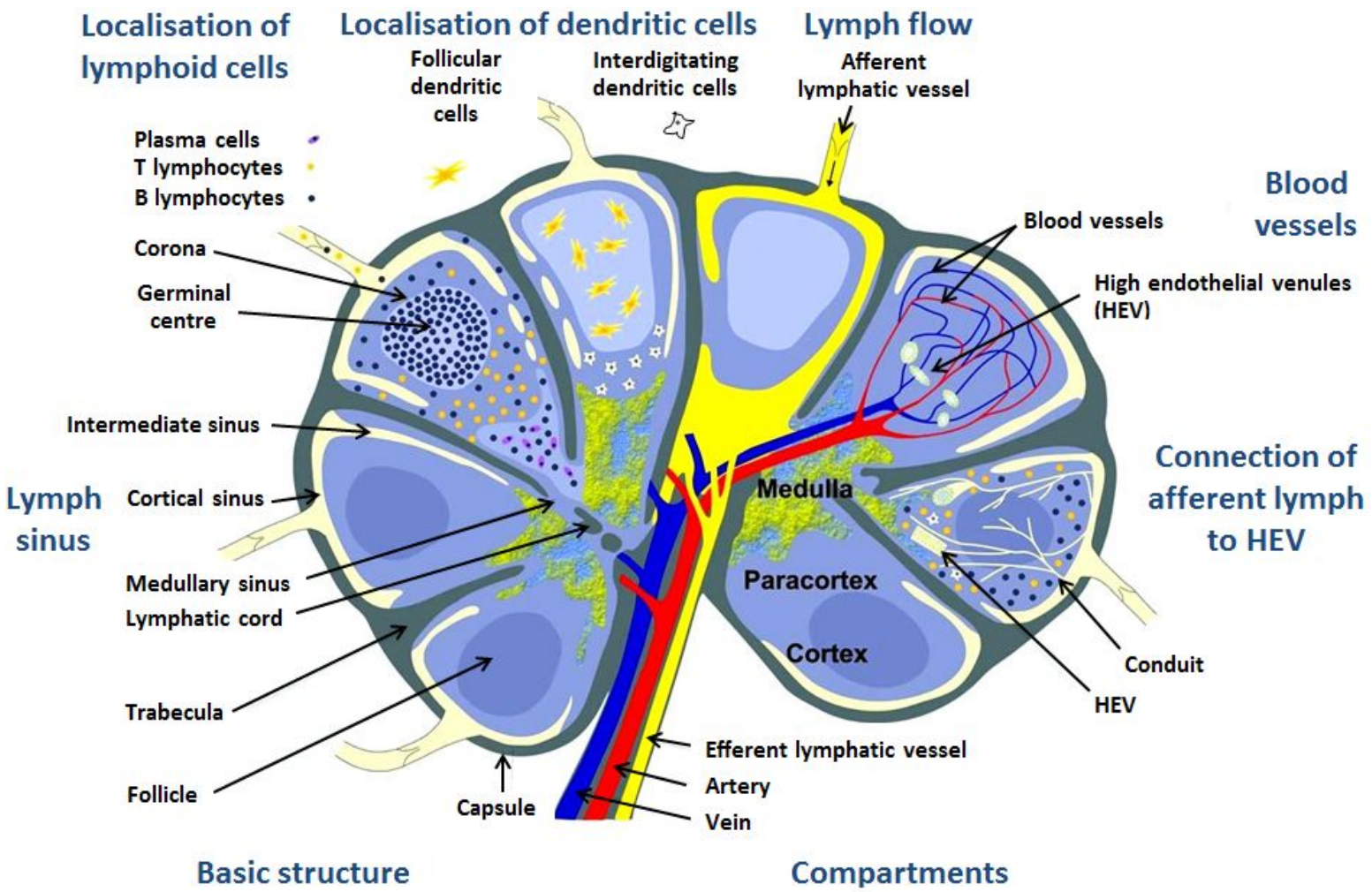
Fig. (2). Schematic view of the substructure of adenoid lymphoid tissue.

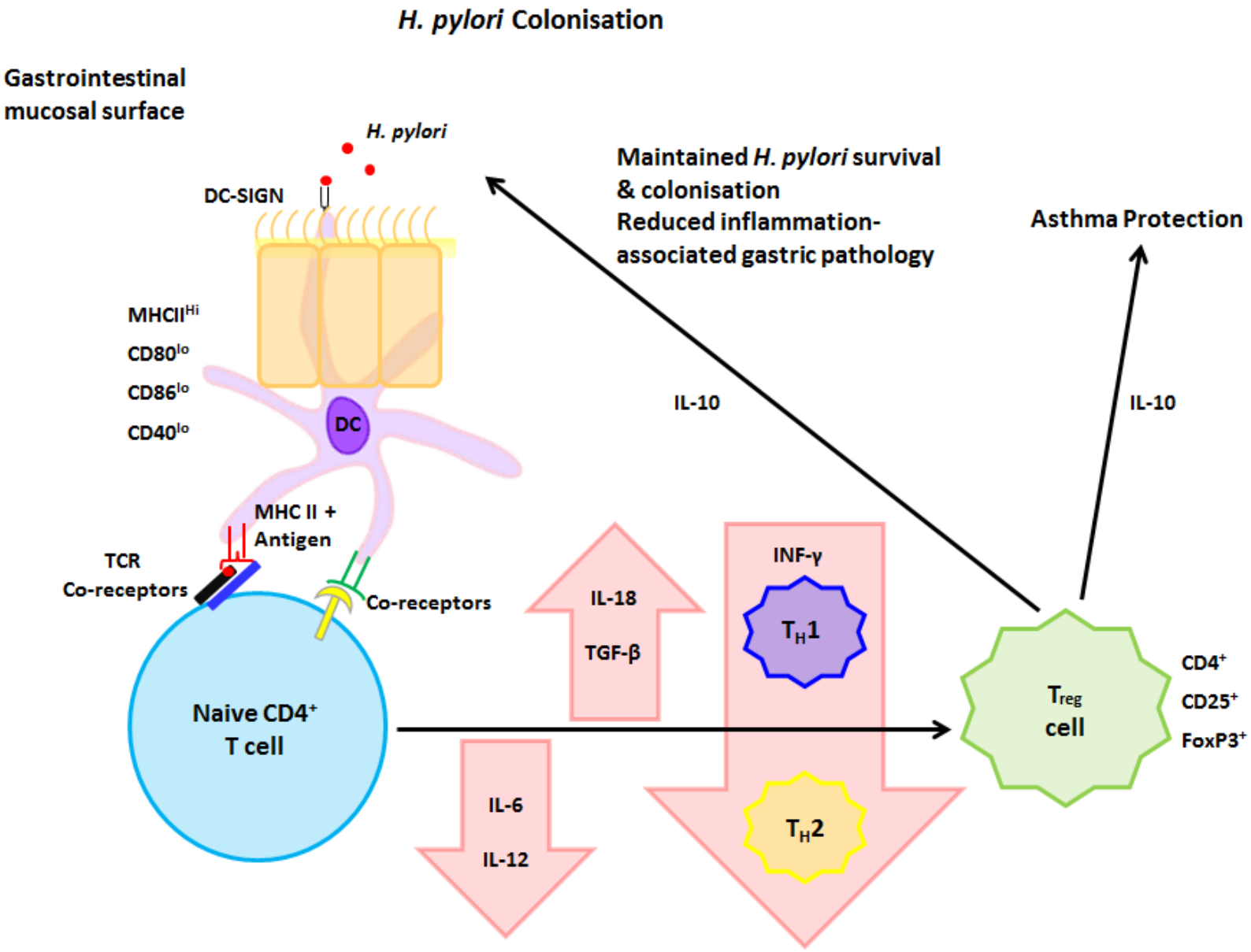
The cortex contains mostly B lymphocytes organised as primary or secondary follicles. Migration of these cells towards the follicles is mediated by follicular dendritic cells also located in the cortex. T lymphocytes migrate to the neighbouring region, the paracortex, where they interact with interdigitating dendritic cells. The central region, the medulla, consists mainly of B lymphocytes and plasma cells. Lymphocytes enter the lymph node via the afferent lymphatic vessel or through transmigration of high endothelial venules. Lymph and blood vasculatures are connected via a conduit system and both drain into the efferent lymph vessel via the medullary sinus. Redrawn from [138].

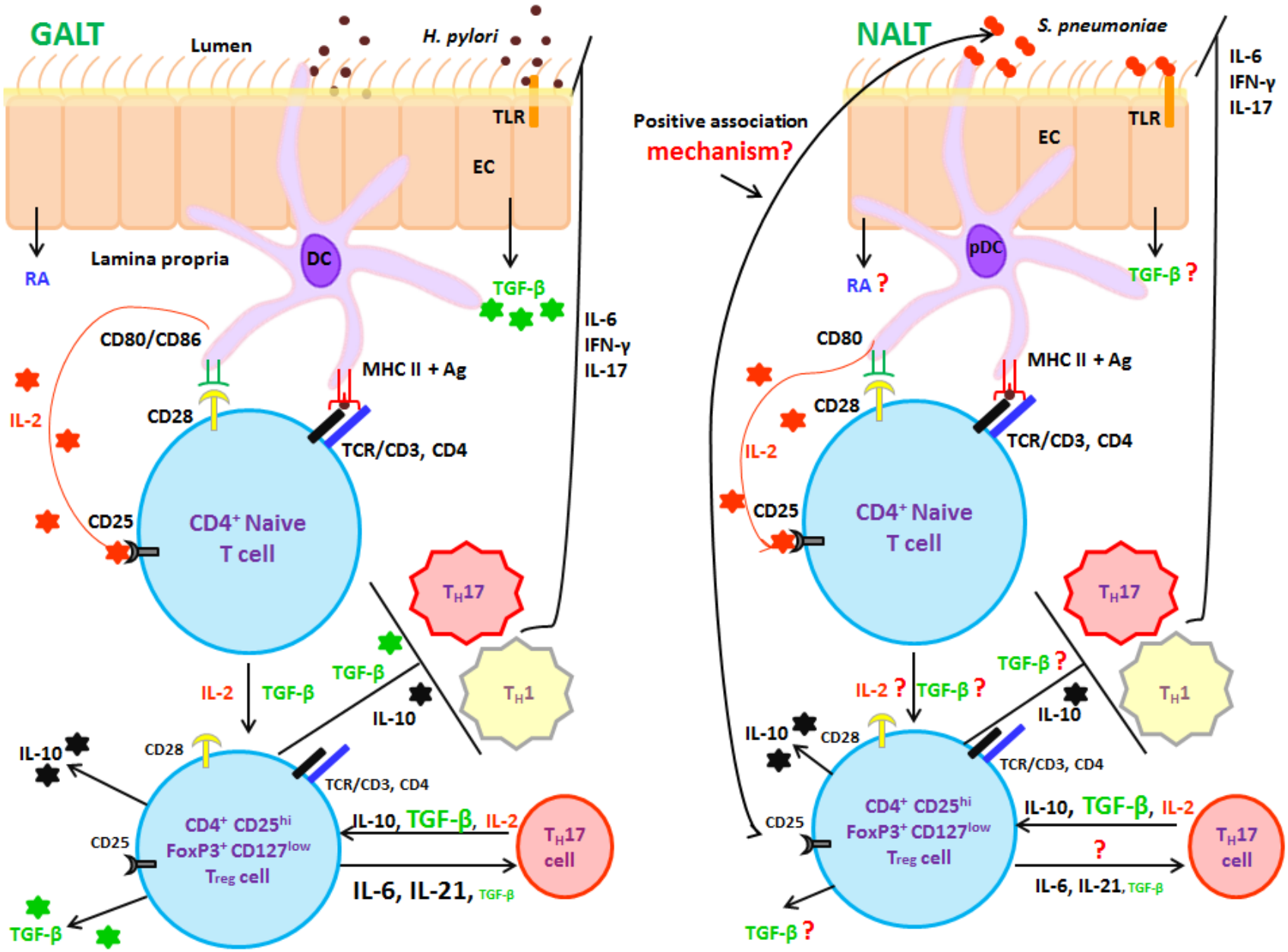
Fig. (3). The influence of *Helicobacter pylori* colonisation on dendritic cell maturation and T_H lymphocyte response in the gastrointestinal mucosa.

Fig. (4). T regulatory lymphocyte-induced host tolerance to commensal bacteria in the mucosa.


Middle ear
Eustachian tube ascending from the nasopharynx to the middle ear cavity
Nasopharynx
Lower respiratory tract


Localisation of lymphoid cells
Localisation of dendritic cells
Lymph flow
Follicular dendritic cells
Interdigitating dendritic cells
Afferent lymphatic vessel
Plasma cells
T lymphocytes
B lymphocytes
Blood vessels
Blood vessels
High endothelial venules (HEV)
Corona
Germinal centre
Intermediate sinus
Lymph sinus
Cortical sinus
Connection of afferent lymph to HEV
Medulla
Medullary sinus
Lymphatic cord
Paracortex
Cortex
Conduit
HEV
Trabecula
Follicle
Capsule
Efferent lymphatic vessel
Artery
Vein
Basic structure
Compartments


H. pylori Colonisation
Gastrointestinal mucosal surface
H. pylori
DC-SIGN
MHCII^{Hi}
CD80^{lo}
CD86^{lo}
CD40^{lo}
DC
MHC II + Antigen
TCR
Co-receptors
Co-receptors
Naive CD4^{+} T cell
IL-18
TGF-β
IL-6
IL-12
INF-γ
T_H1
T_H2
Maintained *H. pylori* survival & colonisation
Reduced inflammation-associated gastric pathology
IL-10
IL-10
Asthma Protection
T_{reg} cell
CD4^{+}
CD25^{+}
FoxP3^{+}


GALT
Lumen
H. pylori
TLR
EC
DC
Lamina propria
RA
TGF-β
IL-6
IFN-γ
IL-17
CD80/CD86
CD28
MHC II + Ag
TCR/CD3, CD4
IL-2
CD25
CD4⁺ Naive T cell
TH17
TH1
IL-2
TGF-β
TGF-β
IL-10
IL-10
CD28
TCR/CD3, CD4
CD4⁺ CD25hi FoxP3⁺ CD127low Treg cell
CD25
IL-10, TGF-β, IL-2
TH17 cell
IL-6, IL-21, TGF-β
TGF-β
NALT
S. pneumoniae
IL-6
IFN-γ
IL-17
Positive association
mechanism?
TLR
EC
pDC
RA ?
TGF-β ?
CD80
CD28
MHC II + Ag
TCR/CD3, CD4
IL-2
CD25
CD4⁺ Naive T cell
TH17
TH1
TGF-β ?
IL-2 ?
TGF-β ?
IL-10
IL-10
CD28
TCR/CD3, CD4
CD25
CD4⁺ CD25hi FoxP3⁺ CD127low Treg cell
IL-10, TGF-β, IL-2
?
TH17 cell
IL-6, IL-21, TGF-β
TGF-β ?