Regulation of Th-1 T cell-dominated immunity to *Neisseria meningitidis* within the human mucosa

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Summary

*Neisseria meningitidis* is commonly carried asymptomatically in the upper respiratory tract and only occasionally invades the bloodstream and meninges to cause disease. Naturally acquired immunity appears protective but the nature of the cellular immune response within the mucosa is uncertain. We show that following *in vitro* stimulation with *N. meningitidis* serogroup B (MenB) antigens, approximately 66% of the dividing mucosal CD4\(^+\)CD45RO\(^+\) memory population express the Th1-associated IL18-R while the remainder express CRTH2, a Th2-associated marker. The pro-inflammatory bias of this anti-MenB response is not evident in blood, demonstrating compartmentalization at the induction site; and occurs in the presence or absence of lipopolysacharide indicating that these responses are already fully committed. Depletion of CD25\(^+\) cells reveals suppression of the effector CD4\(^+\) T cell response restricted to the mucosa and most marked in children (i.e. those at greatest risk of disease). Mucosal T-regulatory cell (Treg) activity is partially overcome by blocking the human glucocorticoid-induced TNF receptor (GITR) and is not seen following stimulation with antigens from another mucosal pathogen, influenza virus. Pro-inflammatory, antimeningococcal T cell responses may limit invasive disease at the mucosa but Treg induction while reducing immunopathological damage, may also restrict the effectiveness of the protective response, particularly in children.

Introduction

*Neisseria meningitidis* is carried in the nasopharynx of between 5% and 40% of the population, yet outside epidemic periods meningococcal disease occurs comparatively rarely (one to five cases per 100 000 per year) (Rosenstein *et al*., 2001). In industrialized countries these carriage rates are lower in children under 5 years (<1–5%) and peak in teenagers and young adults (Goldschneider *et al*., 1969; Gold *et al*., 1978; Cartwright *et al*., 1987). The disparity between the high frequency of carriage and the low incidence of disease, and the observed reduction of disease with age are probably due in part to the development of naturally acquired immunity. In most individuals this is thought to be induced through carriage of *N. meningitidis* itself or related bacteria, such as *Neisseria lactamica*, for which carriage typically peaks at 1–2 years. Protective immunity has been linked to levels of serum bactericidal complement-fixing antibodies (SBA) (Goldschneider *et al*., 1969). However, pre-clinical studies in animals and human vaccine trials indicate that SBA underestimates the level of protection afforded by the immune response to the meningococcus and suggests that other immune mechanisms are important (Perkins *et al*., 1998; Vermont and van den Dobbelsteen, 2002).

The first point of contact for many microorganisms with the human host is the mucosa. The mucosal immune system must be able to distinguish between harmless antigens derived from food or commensal organisms, and antigens from potential pathogens (Cheroutre and Madakamutil, 2004; Holmgren and Czerkinsky, 2005). The default response to mucosal antigen encounter appears to be the induction of tolerance, yet in certain circumstances strong immune responses are generated. Immune induction following most bacterial encounters occur at mucosa-associated lymphoid tissue (e.g. Peyer’s patches and the tonsils), from where effector CD4\(^+\) T cells enter the circulation. Subsequent trafficking and compartmentalization of effector CD4\(^+\) T cells is strongly linked to their site of induction, is dictated by homing receptors and their cognate ligands (Campbell *et al*., 2001), and differs for gut, upper respiratory tract and bronchial mucosa (Brandzaeg *et al*., 1997; Kiyono and Fukuyama, 2004).
The classical view of an active mucosal immune response to bacteria is the induction of IgA-promoting Th2-dominated immunity (Murray et al., 1987; Holmgren and Czerkinsky, 2005). However, Th1-dominated pro-inflammatory immune responses to several microbial pathogens that chronically or recurrently infect mucosal surfaces have been reported (Agren et al., 1998; Higgins et al., 1999; D’Elios et al., 2005; Windle et al., 2005).

Several types of peripherally generated CD4+ T-regulatory cell (Treg) populations may influence the immune response to microbial colonization, including IL-10-secreting Tr1, TGFβ-secreting Th3 and contact-dependent CD4+CD25+ (Sakaguchi et al., 1995; Weiner, 2001). Although CD4+CD25+ can be thymically derived self-reactive cells, they are also capable of inhibiting responses to foreign antigens not normally in the neonatal thymus (Taams and Akbar, 2005). CD4+CD25+ Treg express the Foxp3 gene which encodes the transcription factor scurfen, are CD25 high, CD45RB low (Taams et al., 2001; Taams and Akbar, 2005) and have been found in association with a range of chronic microbial infections, including the gut mucosal pathogen Helicobacter pylori (Mills, 2004; Belkaid and Rouse, 2005; Lundgren et al., 2005). These peripherally induced CD4+CD25+ Treg are present in a range of mucosal-associated lymphoid tissues including the human tonsil and suppress both CD4+ T cell and monocytes/macrophage responses (Taams et al., 2001; Taams and Akbar, 2005). They are generated by repetitive antigen stimulation of CD4+CD25+ effector T cells, by CD4+ T cell stimulation with non-professional antigen presenting cell (APC), by stimulation in the presence of regulatory cytokines TGF-β or IL-10, or by MHC class II-associated antigen presentation by CD4+ T cells to CD4+ T cells (Ng et al., 2001; Taams and Akbar, 2005).

The characteristics of both effector and regulatory mucosal immunity to the meningococcus are not well understood. Observations of antimeningococcal immunity made in the periphery (Holland et al., 1992; Arva and Andersson, 1999; Pollard et al., 1999; Robinson et al., 2002; 2005) may not reflect initial or recall responses at a mucosal surface, where as discussed differential homing and differences in cellular composition may result in significant compartmentalization (Brandtzæg et al., 1997). Previously we have demonstrated age-associated increases in salivary IgA and mucosal T cell proliferative responses to meningococcal antigens (Davenport et al., 2003; Horton et al., 2005). Here we exploit our human tonsil model to investigate the hypothesis that during the acquisition of natural immunity to N. meningitidis, the balance between CD4 T cell immune memory and regulation is dynamic, changing with age and antigen exposure to achieve immune protection while limiting tissue damage. We show that adult responses to meningococcal antigens are heavily Th1 dominated and limited by CD25+ regulatory T cell activity. In contrast to young children who have little cellular immunity to N. meningitidis, we show that older children have surprisingly marked mucosal Treg activity masking a meningococcal antigen-specific effector response. Both the Th1 bias and the CD25-associated regulatory cell activity against these antigens are absent in blood, indicating that this antimeningococcal immune profile is compartmentalized at the site of immune induction.

**Results**

**Distribution of mucosal CD4+CD25+ regulatory T cells and CD4+CD45RO+ memory T cells in human palatine tonsil**

To examine the distribution of CD4+CD25+ T cells and memory CD4+CD45RO+ cells in the palatine tonsil (PT), immunohistochemical analysis was performed (Fig. 1). CD4 T cells were highly concentrated in the extrafollicular region (EF) and the mantle zone (MZ) but were also sparsely distributed in the germinall centre (GC) (Fig. 1A). CD4+ T cells migrate to the GC in response to B cell receptor (BCR) engagement and chemo-attractant gradients (Krzysiek et al., 1999). Activated cells, expressing CD25 (green), were heavily concentrated near the mucosal surface (E and LP) but double-labelled CD4+CD25+ cells (yellow) were mainly distributed in the EF, MZ and the GC (Fig. 1A and B). In these regions CD4+CD25+ cells are reported to suppress GC-Th cell responses and GC-Th cell-driven B cell responses (Lim et al., 2004). A small number of CD25+ cells in these areas also displayed a memory CD45RO+ phenotype (red) (Fig. 1C and D). This CD4+CD25+CD45RO+ phenotype is a reported characteristic of tonsillar CD4+CD25+ regulatory cells in the GC (Taams et al., 2001), activated effector memory CD4+ T cells in the EF (Eriksson et al., 2003) and activated CD4+ Th2 effector memory precursors in the GC (Nagata et al., 1999; Johansson-Lindbom et al., 2003). The remaining CD45RO+ cells are typically comprised of memory T cells, B cells and small numbers of macrophages. There was no clear change in histological appearance with age.

**Compartmentalization of mucosal and peripheral CD4 T cell recall responses to N. meningitidis**

Having identified memory CD45RO+CD4+ T cells in the EF and MZ of the upper respiratory tract mucosa, we sought to characterize the memory T cell reactivity to N. meningitidis serogroup B (MenB) outer membrane vesicles (OMV). Previously we have demonstrated that mucosal proliferative responses to MenB OMV are acquired with age (Davenport et al., 2003). We have subsequently also detected proliferative T cell responses to...
MenB antigens in the peripheral blood of healthy adults with no prior history of meningococcal disease (results not shown). Analysis of carboxy-fluorescein diacetate succinimidyl ester (CFSE)-labelled PT mononuclear cells (MNC) (Fig. 2A) and peripheral blood mononuclear cells (PBMC) (results not shown) from adults demonstrated that the proliferating cells observed were predominantly CD4$^+$ T cells. This was independent of the presence (H44/76) or absence (LpxA–) of the inflammatory mediator lipopolysaccharide (LPS) (Fig. 2A).

We next compared the phenotype of the dividing memory cell population in cultures of CD45RO$^+$ PT MNC and CD45RO$^+$ PBMC generated by CD45RA depletion. Proliferating CD4$^+$CD45RO$^+$ PT T cells stimulated with MenB OMV were biased towards the expression of the Th1-associated marker IL-18R ($P < 0.05$) (Fig. 2B). Only 31.2–37.2% expressed the Th2-associated marker, CRTH2. In contrast, equivalent numbers of IL-18R-expressing and CRTH2-expressing CD4$^+$CD45RO$^+$ PBMC T cells responded to MenB stimulation (Fig. 2B), thus demonstrating phenotypic differences in the distribution of MenB antigen-responsive CD4$^+$ memory T cells between the mucosal and systemic compartments. Subsequent experiments performed using PT and peripheral blood from the same individuals have confirmed this compartmentalization (V. Davenport, R.S. Heyderman and N.A. Williams, unpublished results).

We speculated that the presence of meningococcal LPS in the cultures may influence the bias of the CD4 T helper cell responses, by triggering Th1-polarizing cytokine release from cells in vitro (Dixon et al., 2001; Al-Bader et al., 2004; Uronen-Hansson et al., 2004). Initial $^3$H-thymidine incorporation experiments demonstrated that CD45RO$^+$ proliferation in response to stimulation with membranes generated from a LPS-deficient, LpxA– mutant was comparable with responses induced by OMV from the parent strain H44/76 (containing around 10% LPS), and that purified LPS alone has no proliferative effect on human MNC in culture (Davenport et al., 2003; A. Vaughn, N.A. Williams and R.S. Heyderman, unpublished). CFSE analy-
sis demonstrated that the distribution of IL-18R and CRTH2 expression was identical in cultures stimulated by the LPS-deficient LpxA– membranes to those stimulated with the wild-type H44/76 OMV (Fig. 2B).

The cytokine profile of the mucosal immune response to MenB OMV

To support the phenotypic evidence from surface marker staining, we quantified the levels of cytokines released into the mucosal and peripheral CD45RO+ culture supernatants (Fig. 3A and B). Despite detectable cell proliferation to MenB antigens in these cultures (see above), IL-2 and IL-4 levels were consistently below the 10 pg ml⁻¹ threshold of detection (data not shown). Following initial time-course experiments IFN-γ, IL-5 and IL-10 were measured on day 7 of culture and TNFα on day 3 (Fig. 3A). Despite considerable interindividual variation, a different cytokine profile emerged for each compartment, with high levels of secreted IFN-γ and little IL-5, IL-10 or TNFα secretion detectable in mucosal cultures compared with elevated levels of IFN-γ, IL-5, IL-10 and TNFα in PBMC cultures (Fig. 3B). Cytokine responses to the LpxA– mutant membranes were comparable with responses induced by OMV from the H44/76 parent strain. These results support the CD4+CD45RO+ T cell surface marker staining, are consistent with a Th1-dominated immune response in the mucosa and a more balanced Th1/Th2 response in the periphery.

Identification of mucosal CD25+ regulation of T cell proliferation to MenB OMV

The finding that mucosal memory to MenB antigens was Th1-dominated led us to speculate that there may be concomitant induction of regulation in order to limit the inflammatory consequences of this response. The removal of CD25+ cells by MACS sorting prior to the establishment of PBMC cultures from adult donors did not alter the proliferative responses to MenB antigens (Fig. 4A). Indeed, modest reductions in peak proliferation were observed, consistent with the removal of some CD25+-activated effector cells. In a complimentary approach, the addition of a blocking antibody to the human glucocorticoid-induced TNF receptor (GITR) which mediates CD4+CD25+-mediated suppression (Shimizu et al., 2002) was used to confirm the absence of regulatory activity in this compartment (Fig. 4B). Levels of proliferation were equivalent in cultures with and without the anti-GITR antibody.

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In contrast to peripheral cultures, depletion of CD25+ cells from adult PT MNC from adults resulted in increases in the level of proliferation to both PorA-containing and PorA-deficient MenB OMV (Fig. 5). Levels of mucosal suppression were not dependent on the total number of CD25+ cells within the cultures prior to depletion (range 2.3–9.7%), the number of CD25+CD45RBlow regulatory cells (range 1.2–3.1%), or age of the tonsil donor (range 21–49 years) (data not shown). Regulatory activity was also not dependent on the presence or absence of meningococcal LPS (data not shown).

Acquisition of mucosal CD25+ Treg activity to MenB OMV with age

Treg activity was assessed using either anti-human GITR antibody blockade or CD25+ cell depletion. Although there were some differences in the peak and kinetics of the response with the two approaches, they both gave comparable results (Fig. 6A). These data confirmed the presence of a GITR-regulatable cell population [as expected, monoclonal antibody (mAb) suppressed approximately 50% of the total activity demonstrated by CD25+ depletion]. Given that acquisition of natural mucosal T cell immunity to N. meningitidis is initiated in childhood (Davenport et al., 2003), we next examined the relationship between mucosal CD25+ regulatory activity and age. We removed CD25+ cells from mucosal MNC populations prior to culture and examined the effects on proliferative responses to MenB OMV across a wide range of ages (2 years 7 months to 49 years) (Fig. 6B). MenB responses were detectable in undepleted tonsillar MNC cultures from children aged 2–11 years although generally at a low level [range 1727–6647 corrected cpm (ccpm)]. As previously reported (Davenport et al., 2003), a significant age-associated increase in MenB response was demonstrated (P < 0.05), with adult responses ranging from 11 869 to 21 580 ccppm. An age-associated increase in Treg activity was also demonstrated (P < 0.01) (Fig. 6B). Following CD25 depletion, no significant differences in response were detected to MenB antigens in 2- to 7-year-old children. When responses of older children were analysed, the depletion of CD25+ cells led to a dramatic increase in proliferation to MenB OMV, indicating the presence of MenB antigen-responsive cells almost completely inhibited by CD25+ Treg.

The specificity of the mucosal regulation observed was investigated in parallel studies with antigens from an obligate mucosal pathogen, influenza virus. A strong response to influenza virus antigens was seen in adults...
of meningococcal colonization currently available and both mucosal lymphoid organization and regulatory responses differ between rodent and human systems. We have therefore used human PT, a frequent location for meningococcal carriage and a likely site of immune induction (Drucker et al., 1979; Brandtzaeg et al., 1997), to investigate the cellular immune response to meningococcal antigens. We show that maturation of mucosal immunity to the meningococcus is characterized by the emergence of predominantly pro-inflammatory CD4+CD45RO+ memory T cells, controlled by cells with a GITR-regulatable CD4+CD25+ phenotype. Mucosal CD4+CD25+ cells were found distributed in the EF, MZ and GC of the PT but interestingly not close to the epithelial surface. Surprisingly, in older children who are likely to be in the early phases of immune induction, regulatory activity dominated. We speculate that the low level mucosal immune responses to MenB antigens detected in children may have been induced by non-pathogenic commensal Neisseria such as N. lactamica, or by early contact with N. meningitidis itself. We suggest that particularly in the context of marked Treg activity and the predominance of meningococcal disease in childhood, these weak naïve or cross-reactive responses offer at best only partial protection.

Protection against invasive disease by mucosal pathogens depends on the ability of the human host to generate long-lived, antigen-specific memory T and B cells that can be rapidly mobilized to mediate microbial clearance. In adults, we have found a predominance of mucosal Th1 responses to MenB antigens, similar to those reported to Gram-negative bacteria in the gut of both animals and humans (Higgins et al., 1999; D'Elios et al., 2005; Windle et al., 2005). The Th1 bias in the mucosa identified is ideally suited for eliminating invading bacteria through cytology, phagocytosis and microbial killing by activation of macrophages and NK cells, enhanced FcγR1 expression on phagocytes and induction of complement-fixing and opsonizing antibodies. Upregulation of chemokines and endothelial adhesins will also facilitate the entry of monocytes, macrophages and T cells into the site of infection. However, this bias also carries the risk of inducing tissue destruction and inflammation that may compromise the integrity of the mucosal barrier and upregulate host receptors that may be exploited by the meningococcus for attachment and invasion (Virji, 1996). It has been suggested that within the mucosa, populations of Treg may be generated or activated to inhibit both naïve and memory CD4+ T cell function and help to control such damage (Kong et al., 1996; Hauben and Roncarolo, 2005; Tama and Akbar, 2005). In the case of the meningococcus, this Treg activity may preclude effective elimination of colonizing bacteria from their mucosal niche. Classically microbial induced Th1 polarization is associated with

Fig. 4. Absence of CD25+ regulatory activity against T cell proliferative responses to MenB OMV in blood.
A. Proliferation of PBMC following stimulation with MenB OMV (TR4 and TR10) before and after depletion of CD25+ cells. Representative experiment showing means of triplicate wells ±SEM.
B. Inhibition of CD4+CD25+ mediated suppression using anti-GITR mAb (means ± SEM of triplicate wells with backgrounds subtracted). Representative experiment of three adults (age range 21–36 years). Proliferation was determined by assessment of [3H]-thymidine incorporation on day 7 of culture.

Discussion

Neisseria meningitidis frequently colonizes the upper respiratory tract as a commensal and only occasionally invades the mucosal barrier. Rapid bloodstream multiplication may result in severe sepsis, invasion of the central nervous system and meningitis. Most of our knowledge of such upper respiratory tract pathogens and their interaction with the human immune system has been derived from studies of peripheral blood (Pollard et al., 1999; Robinson et al., 2002; 2005) and has been inferred from animal models or studies of the gut (D’Elios et al., 2005; Windle et al., 2005). There is no appropriate animal model
IL-12 release from APC following the interaction between microbial factors including LPS with cellular toll-like receptor (TLR) (Pridmore et al., 2001; Uronen-Hansson et al., 2004) and may be influenced by pro-inflammatory cytokine release from the colonized epithelium. The presence of LPS and other molecular patterns on the meningococcus are therefore likely to underlie the apparent Th1 bias in the mucosal responses observed in our studies (Al-Bader et al., 2004; Steeghs et al., 2006). The lack of dependence on the presence of LPS in our ex vivo cultures suggests that the MenB antigen-responsive CD4^+CD45RO^+ T cells detected are already fully committed memory cells.

The IL-10 and TNFα detected in the peripheral MNC cultures stimulated with MenB antigens could have emanated from monocytes (Mirlashari et al., 2001). However, the lack of an LPS effect (the most potent pro-inflammatory TLR ligand on OMV) and the presence of IL-5 suggest that these cells are not wholly responsible for the difference in cytokine profile between the two environments. Why CRTH2-dominated IL-5-secreting Th2 cells reactive to MenB antigens are more highly represented in the systemic than in the mucosal compartment is unclear but may reflect a diminished influence of Th1-inducing and a greater influence of Th2-inducing microbial patterns (Al-Bader et al., 2004) on antigen presentation in the systemic compartment. This phenotypic difference between compartments may also result from differences in the homing characteristics of Th1 and Th2 cells. Th1 cells may preferentially track back to the tonsils leading to a relative pro-inflammatory bias. Th2 cells may home to the diffuse lymphoid tissues underlying the upper airway mucosa rather than entering the tonsil, promoting the generation of anti-\textit{N. meningitidis} salivary IgA antibodies (Horton et al., 2005). The balanced systemic Th1/Th2 phenotype seen in adults is consistent with the high levels of antimeningococcal IgG found in serum and the development of bactericidal activity.

The acquisition of mucosal immunity to MenB antigens is accompanied by the induction of regulatory activity capable of limiting cellular reactivity to the bacteria. This suppression appears to be a dominant feature of the response in 8- to 11-year-olds, providing novel evidence for CD4^+CD25^+ T reg-mediated regulation of immunity to a bacterial colonizer of the upper respiratory tract. The T reg cells involved could be thymically derived but the absence of regulation against the low level cross-reactive or naïve responses observed in children, and the apparent specificity for MenB rather than influenza virus antigens suggest that these are peripherally induced CD4^+CD25^+ T reg (Taams et al., 2001; Belkaid and Rouse, 2005). The expression of CD25 on human T cells is quite variable and only those expressing very high levels of CD25 are thought to be suppressive (Baecher-Allan et al., 2001; Belkaid and Rouse, 2005). Although our depletion experiments may have removed recently activated cells expressing intermediate levels of CD25 in addition to T reg, this would not explain the observed enhancement of

![Fig. 5. Detection of mucosal CD25^+ regulatory activity against T cell proliferative responses to MenB OMV. Mucosal cell proliferation following stimulation with MenB OMV (H44/76 and PorA^r) and before and after depletion of CD25^+ cells, assessed by [3H]-thymidine incorporation between days 3 and 7 of culture (means ± SEM of triplicate wells with backgrounds subtracted), three representative adults (age range 21–35 years).](image-url)
effector responses to MenB antigens. Identification and depletion of mucosal cells expressing the Foxp3 gene (Lundgren et al., 2005) may have provided better discrimination but these contain both CD25+ and CD25- populations (Ono et al., 2006). Given the concordance with the GITR experiments it is unlikely that an alternative approach would provide a different picture.

Populations of peripherally induced CD4+CD25+ Treg have been found to reduce Th1-dominated responses in the gut mucosa associated with chronic H. pylori infections (Lundgren et al., 2003; 2005). However, our findings are unique in that they establish compartmentalization within the Treg response. Such compartmentalization has been recently described for CD4+CD25+ regulatory cells controlling allograft transplantation tolerance (Ochando et al., 2005) and in the lymphoid organs of HIV-infected individuals (Andersson et al., 2005). The demonstration of robust mucosal immune responses but no regulatory activity to influenza virus even in children implies that the Treg identified in our studies are not entirely non-specific and that their induction may not be universal to immunity against organisms of the nasopharynx. Similar observations have been made in models of graft-versus-host disease (GVHD) where although initial studies suggested that suppression via CD4+CD25+ Foxp3+ Treg was independent of antigen specificity, there is now evidence that antigen specificity of Treg is crucial for effective organ-specific tolerance (Bluestone and Tang, 2004). The differences in Treg activity observed with influenza virus and MenB antigens may reflect differences in the behaviour of N. meningitidis which is largely a commensal and influenza which is an obligate pathogen.

In conclusion, this human model system provides an unique window into the regulation of the immune response to both mucosal pathogens and potentially to novel exogenous vaccines. Although our observations are limited by the use of material taken from subjects with tonsillar enlargement and recurrent tonsillar infection, none of the subjects had clinical tonsillitis, immunohistochemical studies have not revealed marked neutrophil infiltration or necrosis, and the tissue appears immunologically responsive to experimental stimuli. Our data support the hypothesis that naturally acquired immunity to N. meningitidis, a potential upper respiratory tract mucosal pathogen, is a balance between memory and immune regulation, moving in favour of effector function with increasing age. This process enables the development of a pro-inflammatory response over time while protecting the integrity of the mucosal barrier against local tissue damage and reducing bloodstream invasion. We speculate that this shift in balance from regulatory to effector T cell immunity with age could be due to the switch in the relative frequency of colonizing non-disease-causing Neisseria species in the upper respiratory tract from N. lactamica in early childhood to Neisseria polysaccharae, N. cinerea and N. meningitidis in teenagers (O'Dwyer et al., 2004). However, mucosal Treg activity is a double-edged sword, where suppression of the pro-inflammatory response to the meningococcus by Treg may facilitate prolonged colonization and inadvertently lead to invasion and disease in susceptible individuals (Stephens, 1999). These findings have important implications for the design of future vaccines against mucosal pathogens such as N. meningitidis, particularly where
interruption of nasopharyngeal colonization and the promotion of herd immunity is a goal.

Experimental procedures

Patients and clinical materials

Palatine tonsils were obtained from otherwise healthy individuals undergoing tonsillectomy: 12 adults (age range 20–49 years; nine females) with history of recurrent tonsillitis and 14 children (age range 2 years 7 months to 11 years 9 months; four females) with upper airway obstruction. Individuals with active infection or who were immunosuppressed were excluded. PT were collected into decontamination media comprising HBSS and antibiotics (1000 U ml$^{-1}$ penicillin, 1 mg ml$^{-1}$ streptomycin, Gibco). Peripheral blood from 11 healthy adults (aged 21–44 years; five females) was collected into citrate phosphate dextrose solution (Sigma) to prevent coagulation. None of the participants had a prior history of meningococcal disease and none had clinically inflamed PT at the time of operation. The collection of samples and the research described complies with relevant guidelines and institutional practices (Central and South Bristol Research Ethics Committee E4388). Informed consent of all participating subjects was obtained.

Control and meningococcal antigens

Outer membrane vesicles from MenB were kindly prepared by Dr Jamie Findlow and Dr Ray Borrow, Vaccine Evaluation Unit, Health Protection Agency Northwest (Cartwright et al., 1999; Pollard et al., 1999). OMV were derived from the Norwegian clinical invasive strain H44/76 (B:15: P1.7,16) and the isogenic mutants TR4 (B15:P1.7-8,4) and TR10 (B:15:P1.5-2,10) (Cartwright et al., 1999; Pollard et al., 1999). These differ only in the major outer membrane protein Porin A, and have been selected to be representative of likely colonizing strains in the UK. The nature of the responses was further determined using OMV from a spontaneous PorA$^{-}$ mutant (B:15:P-.-) and using inner and outer membrane preparations from an LPS deficient strain, LpxA$^{-}$ (Steeghs et al., 2001), prepared as previously described (Daveport et al., 2003). OMV were employed in our assays at concentrations of 0.1, 0.5 and 1 μg protein ml$^{-1}$. As a positive control for proliferation we employed dialysed inactive trivalent split virion influenza vaccine (Guthrie et al., 2004) at 0.2–1.8 μg ml$^{-1}$ (Flu-axir® 2002/2003). The vaccine contains 15 μg of haemagglutinin (HA) of A/Moscow/10/99 (H3N2)-like strain, A/New Caledonia/20/99 (H1N1)-like strain and B/Hong Kong/330/2001-like strain but no adjuvant.

Immunocytochemistry of palatine tonsils

Palatine tonsils were mounted and snap frozen in isopentane and stored at −70°C. Five-micrometre cryostat sections were blocked in a mixture of 5% human and goat serum and indirectly stained with anti-human CD4 (IgG2a) and CD25 (IgG1) or CD45RO and CD25 (IgG1) and visualized using a panel of fluorochrome- or biotin-conjugated isotype-specific secondary goat anti-mouse antibodies (anti-IgG1-FITC, anti-IgG2a-PE, anti-IgG2a-biotin). A tertiary step with 1% AMCA avidin (aminomethylcoumarin avidin D, Vector Laboratories, USA) was used to visualize the biotinylated secondary antibody. The sections were mounted using Vectashield (Vector Laboratories, CA, USA) and microscopy was performed using a Leica DMR fluorescence microscope (Leica, Wetzlar, Germany) with a combined red, green and blue filter. Images (20×, 40× and 100× magnification) were captured and digitized using a high-sensitivity colour camera (Color Coolview, Photonic Science, UK) and Image ProPlus software (Media Cybernetics, MD, USA).

Isolation of MNC and cell depletions

Samples of PT were used within 12 h of removal and blood within 1 h. Single-cell suspensions of PT were prepared from decontaminated tonsils as previously described (Davenport et al., 2000).
Peripheral blood and PT MNC proliferative responses were determined using a method adapted from Plebanski (Williams et al., 1990; Plebanski et al., 1992). MNC were cultured in complete RPMI 1640 media containing 1% human AB serum (National Blood Service). PT MNC (0.6, 1.0 and 1.5 million cells per ml) and PBMC (1 million cells per ml) were cultured in 2 ml volumes in 24-well flat-bottom plates (Nunc) with OMV, LpxA- membranes, influenza peptides or media alone at 37°C in 5% CO2 for up to 9 days. On days 3–9 of culture, triplicate 100 µl of samples were transferred from undepleted/CD25-depleted/CD45RA-depleted culture assays into 96-well round-bottom plates. The cells were pulsed with 0.4 mCi 3H-thymidine (Amersham Pharmacia Biotech) for 24 h and then frozen at −20°C. On completion of all 6 days of the assay the plates were thawed and harvested together (Peprotek). Radioactive thymidine incorporation (cpm) was quantified using a 1450 microbeta liquid scintillation counter (Wallac), giving results in cpm.

**GITR blocking assays**

In experiments to demonstrate CD25+ T cell regulatory activity was mediated through GITR, anti-human GITR blocking antibody was employed (R&D Systems). The antibody was used at a concentration of 2 µg ml⁻¹, which is reported to block 50% of GITR binding (Shimizu et al., 2002). PBMC and PT MNC were incubated with anti-human GITR mAb alone, with MenB OMV alone and MenB OMV plus anti-GITR mAb together.

**Phenotyping dividing memory T cells**

In order to phenotype the memory responses to MenB antigens, CD45RA-depleted MNC were labelled with the tracking dye CFSE prior to culture, according to the method of Lyons and Parish (1994). In brief, 3 µM CFSE was used to label 5 x 10⁷ cells for 10 min at 37°C. Samples were removed on day 6 and stained with antibodies against CD4 labelled with APC (Dako) and either IL-18R (Chan et al., 2001) (R&D Systems) or CRTH2 (Nagata et al., 1999) (Miltenyi) both labelled with phycoerythrin for analysis by flow cytometry. Surface expression of IL-18R and CRTH2 was selected to discriminate between Th1 and Th2 cells, respectively, within the responding populations.

**Measurement of cytokine profiles in culture supernatants by Cytometric Bead Array**

Samples (150 µl) were transferred from MNC cultures on days 3, 5, 7 and 9 into 96-well plates. Cells were pelleted by centrifugation and 60 µl of aliquots of culture supernatant decanted for storage in duplicate plates at −80°C. A human Th1 and Th2 Cytometric Bead Array (BD Pharmingen) was employed to quantify the cytokines IFN-γ, TNFα, IL-2, IL-4, IL-5 and IL-10 simultaneously from 50 µl of samples. Samples and standards were prepared according to the manufacturer’s instructions and analysed by flow cytometry.

**Flow cytometry**

Analytical flow cytometry was performed using a FACS Calibur (BD Pharmacia). Cell Quest Software was employed for data acquisition. Flow cytometry was employed to determine the efficiency of CFSE labelling and MACS cell depletions. Live lymphocytes were gated on FSC/SSC. Phenotypic analysis of proliferating CD4+ T cells was performed by gating on CFSE and determining the percentage of CD4+ T cells expressing the Th1-associated IL-18R or the Th2-associated CRTH2 surface markers. One hundred thousand events were acquired where possible and samples were analysed using WinMDI version 2.8 software (Joseph Trotter, Scripps Research Institute, USA). For the quantification of cytokines from culture supernatants by Cytometric Bead Array BD software templates and instructions were followed.

**Statistical analysis**

Non-parametric analysis was performed using SPSS version 11.0 for Windows. Compartmentalization of T cell effector memory responses were examined using Wilcoxon-matched pairs to test for differences between strain H44/76 and LpxA- treatments, and to determine the difference in expression of the two markers, IL-18R and CRTH2 (using means of H44/76 and LpxA- treatment for each individual). Comparisons of proliferative responses before and after CD25 depletion for each age group were similarly made using a Wilcoxon-matched pairs test. The difference in response before and after CD25 depletion across all three age groups was examined by Kruskall Wallis for k-independent samples; and differences between each group were tested using a Mann–Whitney test, with Bonferroni (0.025) correction to allow for multiple sampling.

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**References**

Agren, K., Brauner, A., and Andersson, J. (1998) *Haemophilus influenzae* and *Streptococcus pyogenes* group A chal-

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lenge induce a Th1 type of cytokine response in cells obtained from tonsillar hypertrophy and recurrent tonsillitis. ORL J Otorhinolaryngol Relat Spec 60: 35–41.


