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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.**

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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 (Brandtzaeg *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 scurfin, 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 (Brandtzaeg *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 palantine 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 germinal 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

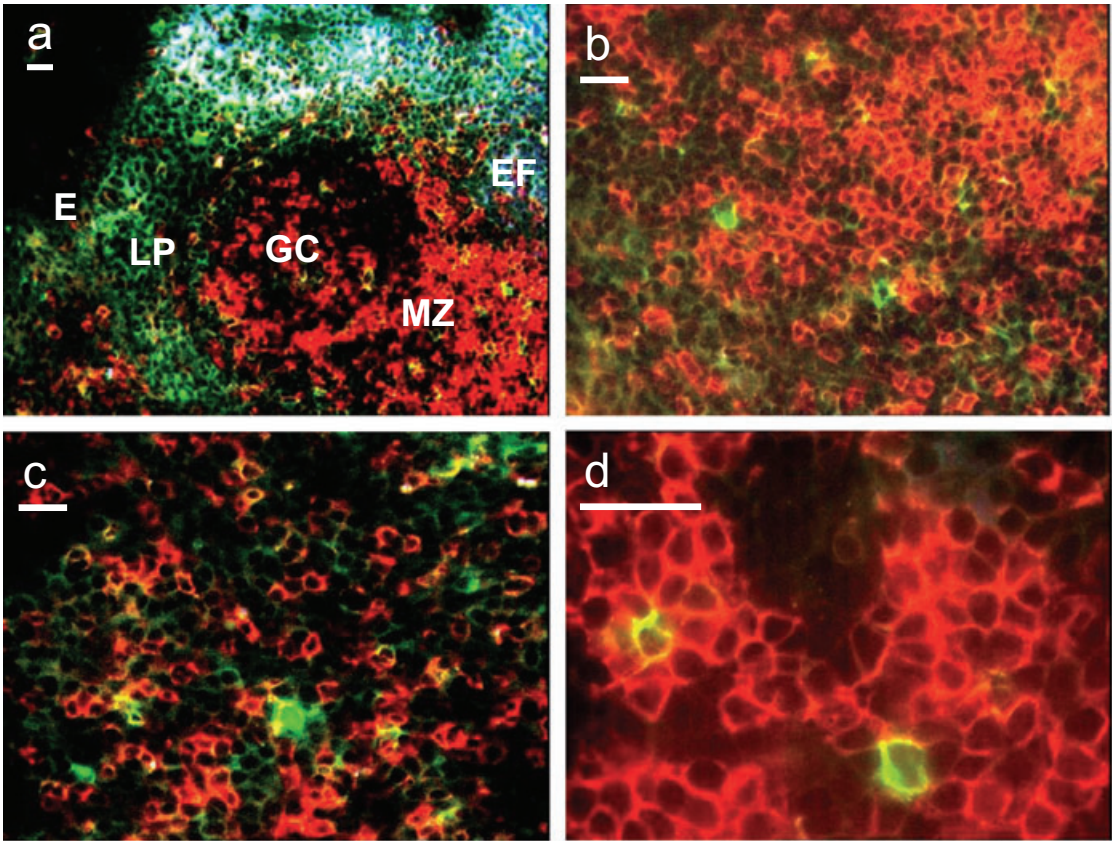

Fig. 1. Distribution of mucosal $CD4^+CD25^+$ regulatory T cells and $CD4^+CD45RO^+$ memory T cells in human palatine tonsil by immunocytochemical analysis.
A. Low power view of the PT from a representative adult subject to show the distribution of $CD4^+$ (red) $CD25^+$ (green) cells within the epithelium (E), lamina propria (LP), germinal centre (GC), mantle zone (MZ) and extrafollicular region (EF).
B–D. Medium and higher power views of GC and EF to demonstrate (B) $CD4^+$ (red), $CD25^+$ (green) and $CD4^+CD25^+$ cells (yellow), (C) $CD45RO^+$ (red), $CD25^+$ (green) and $CD45RO^+CD25^+$ cells (yellow), and (D) $CD45RO^+$ (red), $CD25^+$ (green) and $CD45RO^+CD25^+$ cells (yellow). Scale bar represents 50 μm.

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 lipopolysacharide (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-

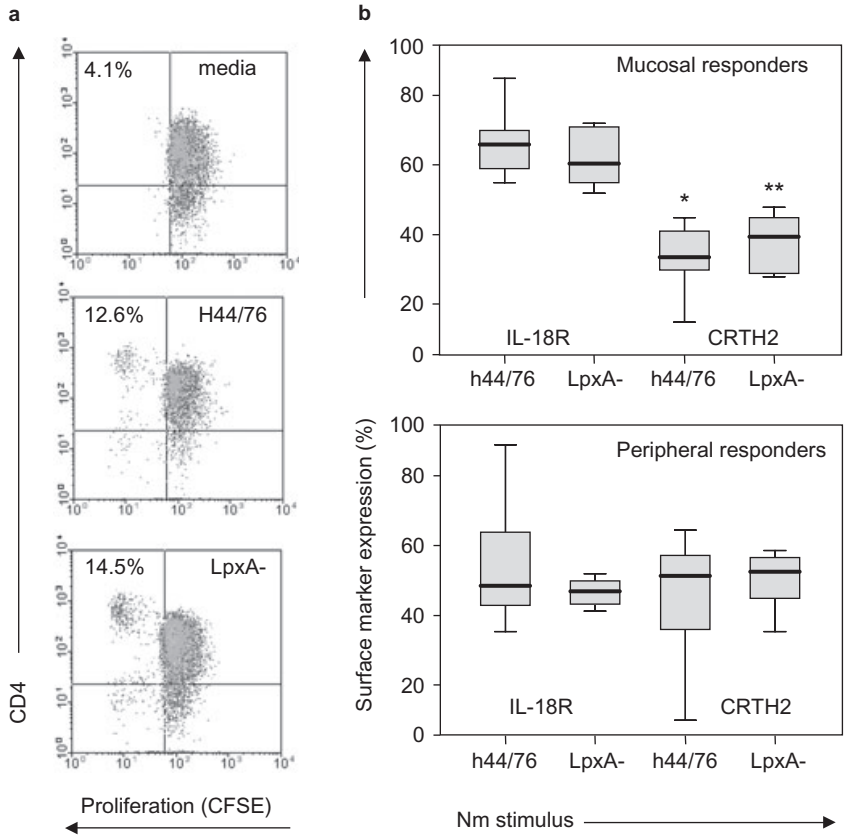

Fig. 2. Characterization of mucosal CD4⁺ T cell memory responses to MenB antigens by CFSE labelling and cell surface marker staining. CFSE-labelled CD45RO⁺ cells from blood and PT were stained with anti-CD4 mAb after 6 days of stimulation with MenB OMV (H44/76) or LPS-deficient MenB membranes (LpxA⁻).
A. Representative data showing PT CD4⁺ T cell proliferation in response to MenB stimulation.
B. Collated data showing the proportion of dividing memory CD4 T cells expressing Th1-associated IL-18R and Th2-associated CRTH2 surface markers in mucosa and the peripheral blood. In CD45RA-depleted PT cultures, proliferating CD4⁺ T cells stimulated with either antigen were biased towards the expression of IL-18R (*$P < 0.05$). Medians of normalized values, interquartile range and full range for six mucosal (age range 20–49 years) and eight peripheral (age range 21–44 years) samples. Nm, *Neisseria meningitidis*.

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.

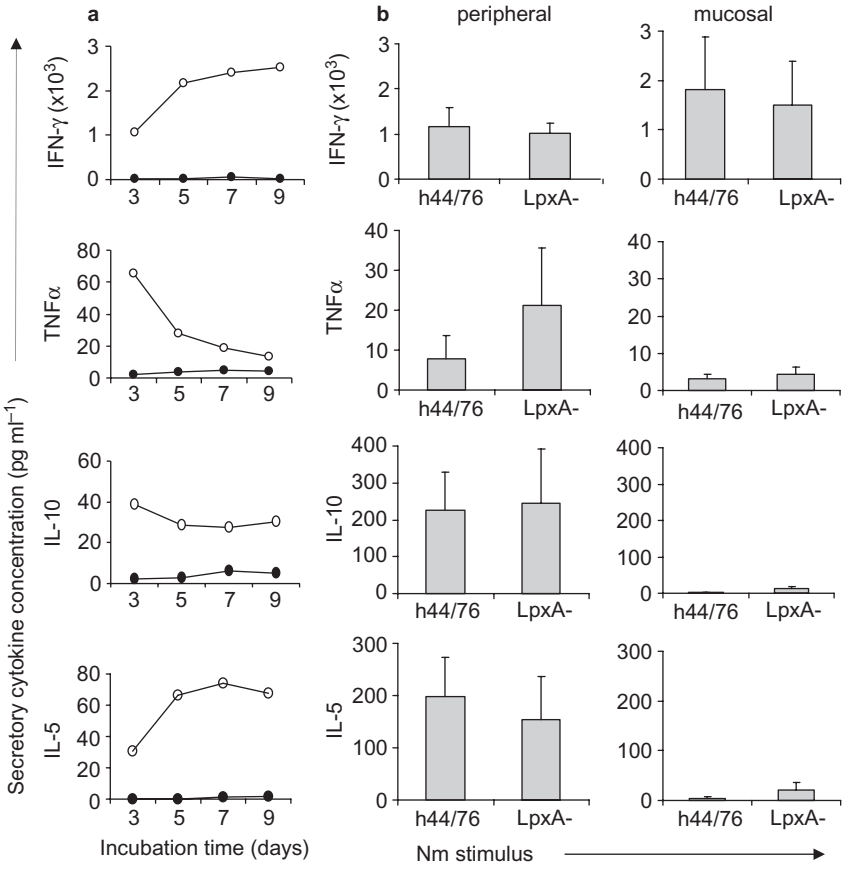

Fig. 3. Secreted cytokine profiles in MenB OMV-stimulated peripheral and mucosal cultures.
A. Representative data showing the time-course IFN-γ, TNFα, IL-10 and IL-5 by PBMC stimulated with MenB OMV (open circles) or media control (closed circles).
B. Collated cytokine data for six mucosal and five peripheral samples (means ± SEM). IL-2 and IL-4 levels were consistently below the 10 pg ml^{-1} threshold of detection (data not shown).

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$^+$CD45RB^{low} 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 ccpm. 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

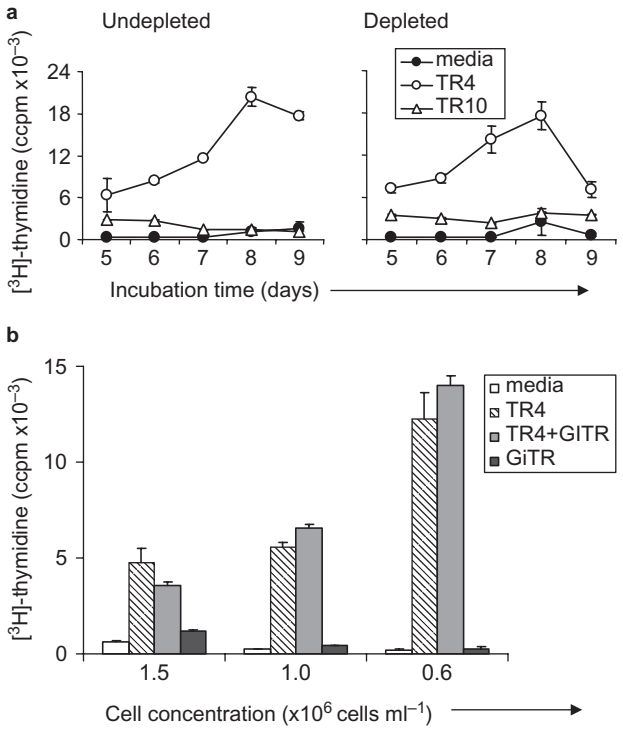

Fig. 4. Absence of CD25$^+$ regulatory activity against T cell proliferative responses to MenB OMV in blood.
A. Proliferation of PBMC following stimulation with MenB OMV (TR4 and TR10) before and after depletion of CD25$^+$ cells. Representative experiment showing means of triplicate wells ±SEM.
B. Inhibition 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.

(Fig. 7A) and in children (Fig. 7B). Unlike MenB responses in older children and adults, CD25 depletion did not lead to an increase in proliferation to influenza control antigens. In some cases depletion led to a slight reduction in the level of the anti-influenza response suggesting that some CD25$^+$ effector T cells were removed.

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 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 cytolysis, 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; Taams 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

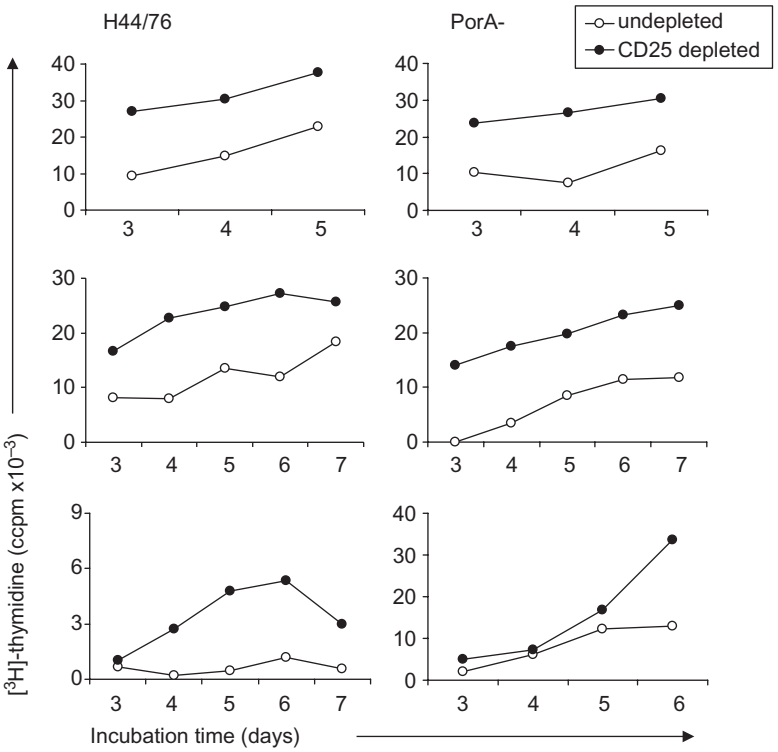

Fig. 5. Detection of mucosal CD25$^+$ regulatory activity against T cell proliferative responses to MenB OMV. Mucosal cell proliferation following stimulation with MenB OMV (H44/76 and PorA$^-$) and before and after depletion of CD25$^+$ cells, assessed by [3H]-thymidine incorporation between days 3 and 7 of culture (means ± SEM of triplicate wells with backgrounds subtracted), three representative adults (age range 21–35 years).

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-*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$^+$-mediated regulation of immunity to a bacterial colonizer of the upper respiratory tract. The Treg 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$^+$ Treg (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 Treg, this would not explain the observed enhancement of

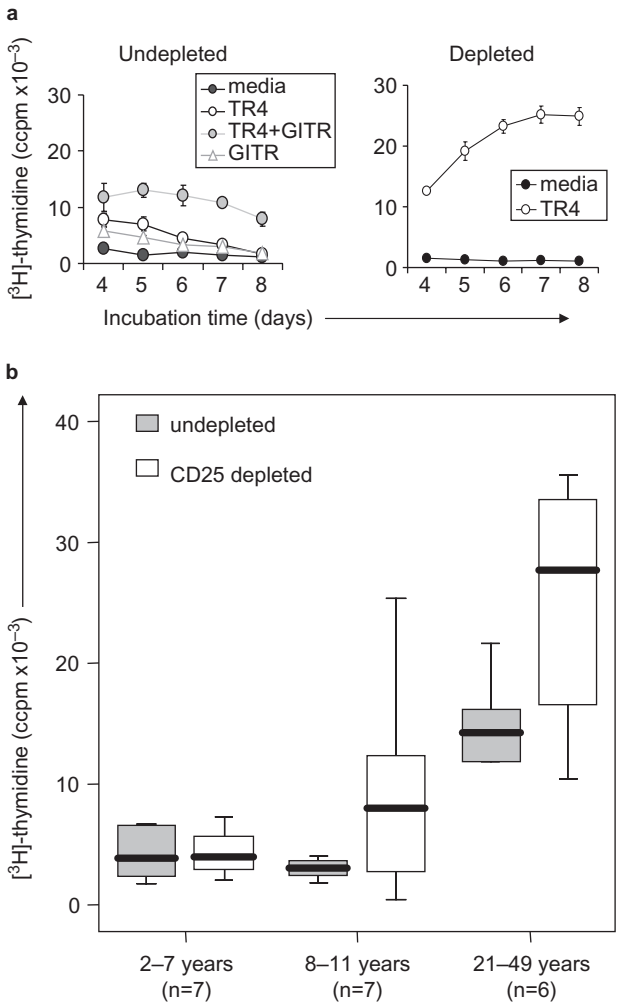

Fig. 6. Age association of mucosal CD25$^+$ regulatory activity against T cell proliferative responses to MenB OMV.
A. Assessment of Treg activity in an adult subject comparing anti-human GITR antibody blockade with CD25$^+$ cell depletion (mean ± SEM of triplicate wells). Data are representative of four experiments.
B. Peak proliferation of PT MNC stimulated with MenB, before and after CD25$^+$ cell depletion. Median proliferation with backgrounds subtracted, interquartile range and full range for 20 subjects (age range 2 years 7 months to 49 years). A significant age-associated increase in the level of CD25$^+$ Treg-mediated suppression was demonstrated ($P < 0.01$). Means ± SEM with backgrounds subtracted.

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 polysaccharea*, *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

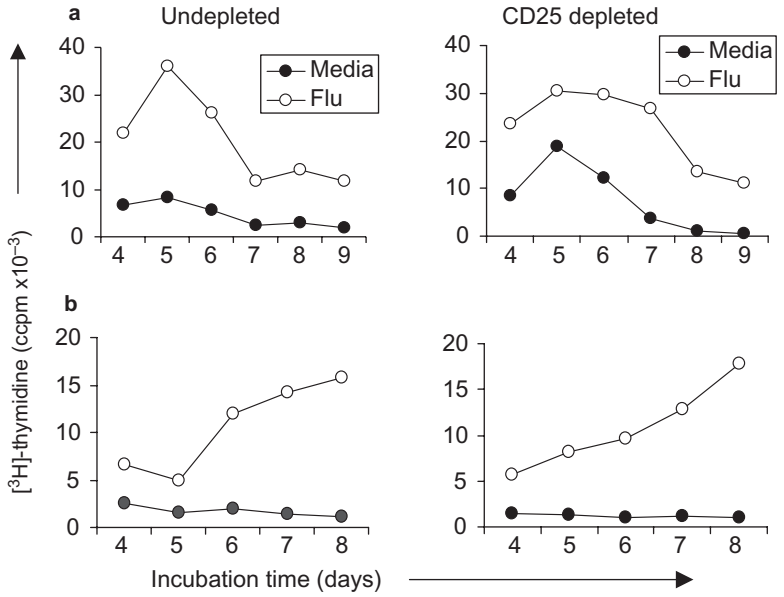

Fig. 7. Absence of mucosal CD25+ regulatory activity against T cell proliferative responses to influenza antigens.
A. Representative data from an adult (age 23 years).
B. Representative data from a child (age 12 years).
Mucosal mononuclear cell proliferation following *in vitro* stimulation with influenza vaccine antigens using cells replete and depleted of CD25+ cells. Proliferation was assessed by [3H]-thymidine incorporation between days 4 and 8 of culture (means ± SEM of triplicate wells).

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 (Davenport *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} (Fluarix® 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

Palantine 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 (IgG2a) 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*.,

2003). MNC were isolated from blood and tonsil cell suspensions by 30 min centrifugation at 400 *g* on a histopaque gradient. Where required, $CD25^+$ and $CD45RA^+$ cells were depleted from isolated MNC using MACS microbeads coated with anti-human CD25 or CD45RA, respectively, and magnetic cell sorting (Miltenyi Biotec) on LS+ columns. Starting populations of $1–2 \times 10^8$ cells were used where possible and depleted according to the manufacturer's instructions. The efficiency of depletion was determined using phycoerythrin-labelled anti-CD25 or anti-CD45RA antibodies (BD Pharmingen) and flow cytometric analysis. Cells were depleted with at least 95% purity, as determined by flow cytometry.

Cell culture and proliferation assays

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% CO_2 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 ^{3}H-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 (^{3}H-thymidine) was quantified using a 1450 microbeta liquid scintillation counter (Wallac), giving results in ccpm.

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 5×10^7 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 $CD45RO^+$ 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-

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.

Al-Bader, T., Jolley, K.A., Humphries, H.E., Holloway, J., Heckels, J.E., Semper, A.E., *et al.* (2004) Activation of human dendritic cells by the PorA protein of *Neisseria meningitidis*. *Cell Microbiol* **6:** 651–662.

Andersson, J., Boasso, A., Nilsson, J., Zhang, R., Shire, N.J., Lindback, S., *et al.* (2005) The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* **174:** 3143–3147.

Arva, E., and Andersson, B. (1999) Kinetics of cytokine release and expression of lymphocyte cell-surface activation markers after *in vitro* stimulation of human peripheral blood mononuclear cells with *Streptococcus pneumoniae*. *Scand J Immunol* **49:** 237–243.

Baecher-Allan, C., Brown, J.A., Freeman, G.J., and Hafler, D.A. (2001) CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* **167:** 1245–1253.

Belkaid, Y., and Rouse, B.T. (2005) Natural regulatory T cells in infectious disease. *Nat Immunol* **6:** 353–360.

Bluestone, J.A., and Tang, Q. (2004) Therapeutic vaccination using CD4+CD25+ antigen-specific regulatory T cells. *Proc Natl Acad Sci USA* **101** (Suppl. 2)**:** 14622–14626.

Brandtzaeg, P., Jahnsen, F.L., Farstad, I.N., and Haraldsen, G. (1997) Mucosal immunology of the upper airways: an overview. *Ann N Y Acad Sci* **830:** 1–18.

Campbell, J.J., Murphy, K.E., Kunkel, E.J., Brightling, C.E., Soler, D., Shen, Z., *et al.* (2001) CCR7 expression and memory T cell diversity in humans. *J Immunol* **166:** 877–884.

Cartwright, K.A., Stuart, J.M., Jones, D.M., and Noah, N.D. (1987) The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiol Infect* **99:** 591–601.

Cartwright, K., Morris, R., Rumke, H., Fox, A., Borrow, R., Begg, N., *et al.* (1999) Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins. *Vaccine* **17:** 2612–2619.

Chan, W.L., Pejnovic, N., Lee, C.A., and Al-Ali, N.A. (2001) Human IL-18 receptor and ST2L are stable and selective markers for the respective type 1 and type 2 circulating lymphocytes. *J Immunol* **167:** 1238–1244.

Cheroutre, H., and Madakamutil, L. (2004) Acquired and natural memory T cells join forces at the mucosal front line. *Nat Rev Immunol* **4:** 290–300.

D'Elios, M.M., Amedei, A., Benagiano, M., Azzurri, A., and Del Prete, G. (2005) *Helicobacter pylori*, T cells and cytokines: the 'dangerous liaisons'. *FEMS Immunol Med Microbiol* **44:** 113–119.

Davenport, V., Guthrie, T., Findlow, J., Borrow, R., Williams, N.A., and Heyderman, R.S. (2003) Evidence for naturally acquired T cell mediated mucosal immunity to *Neisseria meningitidis*. *J Immunol* **171:** 4263–4267.

Dixon, G.L., Newton, P.J., Chain, B.M., Katz, D., Andersen, S.R., Wong, S., *et al.* (2001) Dendritic cell activation and cytokine production induced by group B *Neisseria meningitidis*: interleukin-12 production depends on lipopolysaccharide expression in intact bacteria. *Infect Immun* **69:** 4351–4357.

Drucker, M.M., Agatsuma, Y., Drucker, I., Neter, E., Bernstein, J., and Ogra, P.L. (1979) Cell-mediated immune response to bacterial products in human tonsils and peripheral blood lymphocytes. *Infect Immun* **23:** 347–352.

Eriksson, J.C., Davidsson, A., Garberg, H., and Brokstad, K.A. (2003) Lymphocyte distribution in the tonsils prior to and after influenza vaccination. *Vaccine* **22:** 57–63.

Gold, R., Goldschneider, I., Lepow, M.L., Draper, T.F., and Randolph, M. (1978) Carriage of *Neisseria meningitidis* and *Neisseria lactamica* in infants and children. *J Infect Dis* **137:** 112–121.

Goldschneider, I., Gotschlich, E.C., and Artenstein, M.S. (1969) Human immunity to the meningococcus. I. The role of humoral antibodies. *J Exp Med* **129:** 1307–1326.

Guthrie, T., Hobbs, C., Davenport, V., Horton, R., Heyderman, R.S., and Williams, N.A. (2004) Mucosal and systemic T-cell mediated immunity following influenza vaccination in healthy adults. *J Infect Dis* **190:** 1927–1935.

Hauben, E., and Roncarolo, M.G. (2005) Human CD4+ regulatory T cells and activation-induced tolerance. *Microbes Infect* **7:** 1023–1032.

Higgins, L.M., Frankel, G., Douce, G., Dougan, G., and MacDonald, T.T. (1999) *Citrobacter rodentium* infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. *Infect Immun* **67:** 3031–3039.

Holland, J., Langford, P.R., Towner, K.J., and Williams, P. (1992) Evidence for *in vivo* expression of transferrin-binding proteins in *Haemophilus influenzae* type b. *Infect Immun* **60:** 2986–2991.

Holmgren, J., and Czerkinsky, C. (2005) Mucosal immunity and vaccines. *Nat Med* **11:** S45–S53.

Horton, R.E., Stuart, J., Christensen, H., Borrow, R., Guthrie, T., Davenport, V., *et al.* (2005) Influence of age and carriage status on salivary IgA antibody to *Neisseria meningitidis*. *Epidemiol Infect* **133:** 883–889.

Johansson-Lindbom, B., Ingvarsson, S., and Borrebaeck, C.A. (2003) Germinal centers regulate human Th2 development. *J Immunol* **171:** 1657–1666.

Kiyono, H., and Fukuyama, S. (2004) NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* **4:** 699–710.

Kong, Y.Y., Eto, M., Omoto, K., Umesue, M., Hashimoto, A., and Nomoto, K. (1996) Regulatory T cells in maintenance and reversal of peripheral tolerance *in vivo*. *J Immunol* **157:** 5284–5289.

Krzysiek, R., Lefevre, E.A., Zou, W., Foussat, A., Bernard, J., Portier, A., *et al.* (1999) Antigen receptor engagement selectively induces macrophage inflammatory protein-1 alpha (MIP-1 alpha) and MIP-1 beta chemokine production in human B cells. *J Immunol* **162:** 4455–4463.

Lim, H.W., Hillsamer, P., and Kim, C.H. (2004) Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest* **114:** 1640–1649.

Lundgren, A., Suri-Payer, E., Enarsson, K., Svennerholm, A.M., and Lundin, B.S. (2003) *Helicobacter pylori*-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to *H. pylori* in infected individuals. *Infect Immun* **71:** 1755–1762.

Lundgren, A., Stromberg, E., Sjoling, A., Lindholm, C., Enarsson, K., Edebo, A., *et al.* (2005) Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in *Helicobacter pylori*-infected patients. *Infect Immun* **73:** 523–531.

Lyons, A.B., and Parish, C.R. (1994) Determination of lymphocyte division by flow cytometry. *J Immunol Methods* **171:** 131–137.

Mills, K.H. (2004) Regulatory T cells: friend or foe in immunity to infection? *Nat Rev Immunol* **4:** 841–855.

Mirlashari, M.R., Hoiby, E.A., Holst, J., and Lyberg, T. (2001) Outer membrane vesicles from *Neisseria meningitidis*: effects on cytokine production in human whole blood. *Cytokine* **13:** 91–97.

Murray, P.D., McKenzie, D.T., Swain, S.L., and Kagnoff, M.F. (1987) Interleukin 5 and interleukin 4 produced by Peyer's patch T cells selectively enhance immunoglobulin A expression. *J Immunol* **139:** 2669–2674.

Nagata, K., Tanaka, K., Ogawa, K., Kemmotsu, K., Imai, T., Yoshie, O., *et al.* (1999) Selective expression of a novel surface molecule by human Th2 cells *in vivo*. *J Immunol* **162:** 1278–1286.

Ng, W.F., Duggan, P.J., Ponchel, F., Matarese, G., Lombardi, G., Edwards, A.D., *et al.* (2001) Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* **98:** 2736–2744.

O'Dwyer, C.A., Reddin, K., Martin, D., Taylor, S.C., Gorringe, A.R., Hudson, M.J., *et al.* (2004) Expression of heterologous antigens in commensal *Neisseria* spp. preservation of conformational epitopes with vaccine potential. *Infect Immun* **72:** 6511–6518.

Ochando, J.C., Yopp, A.C., Yang, Y., Garin, A., Li, Y., Boros, P., *et al.* (2005) Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. *J Immunol* **174:** 6993–7005.

Ono, M., Shimizu, J., Miyachi, Y., and Sakaguchi, S. (2006) Control of autoimmune myocarditis and multiorgan inflammation by glucocorticoid-induced TNF receptor family-related protein(high), Foxp3-expressing CD25+ and CD25- regulatory T cells. *J Immunol* **176:** 4748–4756.

Perkins, B.A., Jonsdottir, K., Briem, H., Griffiths, E., Plikaytis, B.D., Hoiby, E.A., *et al.* (1998) Immunogenicity of two efficacious outer membrane protein-based serogroup B meningococcal vaccines among young adults in Iceland. *J Infect Dis* **177:** 683–691.

Plebanski, M., Saunders, M., Burtles, S.S., Crowe, S., and Hooper, D.C. (1992) Primary and secondary human *in vitro* T-cell responses to soluble antigens are mediated by subsets bearing different CD45 isoforms. *Immunology* **75:** 86–91.

Pollard, A.J., Galassini, R., Rouppe van der Voort, E.M., Hibberd, M., Booy, R., Langford, P., *et al.* (1999) Cellular immune responses to *Neisseria meningitidis* in children. *Infect Immun* **67:** 2452–2463.

Pridmore, A.C., Wyllie, D.H., Abdillahi, F., Steeghs, L., van der Ley, P., Dower, S.K., and Read, R.C. (2001) A lipopolysaccharide-deficient mutant of *Neisseria meningitidis* elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. *J Infect Dis* **183:** 89–96.

Robinson, K., Neal, K.R., Howard, C., Stockton, J., Atkinson, K., Scarth, E., *et al.* (2002) Characterization of humoral and cellular immune responses elicited by meningococcal carriage. *Infect Immun* **70:** 1301–1309.

Robinson, K., Wooldridge, K.G., Wells, D.B., Hasan, A., Todd, I., Robins, A., *et al.* (2005) T-cell-stimulating protein A elicits immune responses during meningococcal carriage and human disease. *Infect Immun* **73:** 4684–4693.

Rosenstein, N.E., Perkins, B.A., Stephens, D.S., Popovic, T., and Hughes, J.M. (2001) Meningococcal disease. *N Engl J Med* **344:** 1378–1388.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155:** 1151–1164.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y., and Sakaguchi, S. (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol* **3:** 135–142.

Steeghs, L., de Cock, H., Evers, E., Zomer, B., Tommassen, J., and van der Ley, P. (2001) Outer membrane composition of a lipopolysaccharide-deficient *Neisseria meningitidis* mutant. *EMBO J* **20:** 6937–6945.

Steeghs, L., van Vliet, S.J., Uronen-Hansson, H., van Mourik, A., Engering, A., Sanchez-Hernandez, M., *et al.* (2006) *Neisseria meningitidis* expressing lgtB lipopolysaccharide targets DC-SIGN and modulates dendritic cell function. *Cell Microbiol* **8:** 316–325.

Stephens, D.S. (1999) Uncloaking the meningococcus: dynamics of carriage and disease. *Lancet* **353:** 941–942.

Taams, L.S., and Akbar, A.N. (2005) Peripheral generation and function of CD4+CD25+ regulatory T cells. *Curr Top Microbiol Immunol* **293:** 115–131.

Taams, L.S., Smith, J., Rustin, M.H., Salmon, M., Poulter, L.W., and Akbar, A.N. (2001) Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* **31:** 1122–1131.

Uronen-Hansson, H., Allen, J., Osman, M., Squires, G., Klein, N., and Callard, R.E. (2004) Toll-like receptor 2 (TLR2) and TLR4 are present inside human dendritic cells, associated with microtubules and the Golgi apparatus but are not detectable on the cell surface: integrity of microtubules is required for interleukin-12 production in response to internalized bacteria. *Immunology* **111:** 173–178.

Vermont, C., and van den Dobbelsteen, G. (2002) *Neisseria meningitidis* serogroup B: laboratory correlates of protection. *FEMS Immunol Med Microbiol* **34:** 89–96.

Virji, M. (1996) Microbial utilization of human signaling molecules. *Microbiology* **142:** 3319–3336.

Weiner, H.L. (2001) The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nat Immunol* **2:** 671–672.

Williams, N.A., Hill, T.J., and Hooper, D.C. (1990) Murine epidermal antigen-presenting cells in primary and secondary T-cell proliferative responses to a soluble protein antigen *in vitro*. *Immunology* **71:** 411–416.

Windle, H.J., Ang, Y.S., Athie-Morales, V., McManus, R., and Kelleher, D. (2005) Human peripheral and gastric lymphocyte responses to *Helicobacter pylori* NapA and AphC differ in infected and uninfected individuals. *Gut* **54:** 25–32.