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Absence of Mucosal Immunity in the Human Upper Respiratory Tract to the Commensal Bacteria *Neisseria lactamica* but Not Pathogenic *Neisseria meningitidis* during the Peak Age of Nasopharyngeal Carriage

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The normal flora that colonizes the mucosal epithelia has evolved diverse strategies to evade, modulate, or suppress the immune system and avoid clearance. *Neisseria lactamica* and *Neisseria meningitidis* are closely related obligate inhabitants of the human upper respiratory tract. *N. lactamica* is a commensal but *N. meningitidis* is an opportunistic pathogen that occasionally causes invasive disease such as meningitis and septicaemia. We demonstrate that unlike *N. meningitidis*, *N. lactamica* does not prime the development of mucosal T or B cell memory during the peak period of colonization. This cannot be explained by the induction of peripheral tolerance or regulatory CD4+CD25+ T cell activity. Instead, *N. lactamica* mediates a B cell-dependent mitogenic proliferative response that is absent to *N. meningitidis*. This mitogenic response is associated with the production of T cell-independent polyclonal IgM that we propose functions by shielding colonizing *N. lactamica* from the adaptive immune system, maintaining immunological ignorance in the host. We conclude that, in contrast to *N. meningitidis*, *N. lactamica* maintains a commensal relationship with the host in the absence of an adaptive immune response. This may prolong the period of susceptibility to colonization by both pathogenic and nonpathogenic *Neisseria* species. *The Journal of Immunology*, 2009, 182: 2231–2240.

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2 Address correspondence and reprint requests to Dr. Andrew Vaughan, Department of Cellular and Molecular Medicine, School of Medical Science, University Walk, Bristol, United Kingdom. E-mail address: andrew.vaughan@emory.edu

3 Abbreviations used in this paper: Treg, regulatory T cell; URT, upper respiratory tract; Nm, *Neisseria meningitidis*; OMV, outer membrane vesicle; PT, palatine tonsil; NI, *Neisseria lactamica*; LOS, lipooligosaccharide; flu, trivalent inactivated split-virion influenza vaccine; SAC, *Staphylococcus aureus* cowan I strain; ODN, oligodeoxyribonucleotide; TMNC, tonsillar mononuclear cell; KLH, keyhole limpet hemocyanin; ASC, Ab secreting cell; AP, alkaline phosphatase.

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Carriage of Nm is infrequent in infancy and does not increase until after the age of 5 years, peaking in early adulthood. Nm is carried by ~10% of the population at any one time (12). Asymptomatic carriage primes an adaptive immune response within 2 wk of colonization (13) and is associated with increased titers of salivary IgA (14) and serum IgG (15). We have demonstrated the presence of memory T cell responses to Nm outer membrane vesicles (OMV) in human palatine tonsils (PT) which increase with age (16). However, although colonization by Nm induces a rapid immune response that protects against invasive disease, carriage studies have shown that single strains may colonize for several months before being cleared (17, 18). It is unclear how Nm can colonize for prolonged periods of time while priming adaptive immunity without inducing an aberrant inflammatory response and associated immunopathology. We have recently demonstrated the presence of Nm-specific Treg activity in the PT that is able to suppress effector T cell responses to Nm in the mucosa (19). The induction and differentiation of Treg may be one mechanism by which Nm avoids inducing immunopathology and clearance by the immune system.

Neisseria lactamica (NI) is a closely related commensal species to Nm and also colonizes the nasopharyngeal epithelium but has only caused disease in exceptional circumstances (20, 21). Colonization by NI peaks at ~18 mo of age, before colonization by Nm (17, 18, 22), with >40% of infants becoming carriers in the first year of life (17). Like Nm, single isolates of NI typically colonize the nasopharynx for several months (17, 18) but the development of cellular immunity to this organism has not been studied.

In this study, we investigate the development of cellular immunity to NI and compare the response with Nm to further understand how the commensal relationship with the host is maintained during asymptomatic carriage. We show that unlike Nm, NI does not prime the development of mucosal memory during the peak period of asymptomatic carriage. This cannot be explained by the induction of peripheral tolerance or Treg activity. Conversely, NI mediates a B cell-dependent proliferative response that is absent to Nm. This response is associated with the production of T cell-independent polyclonal Ig that we propose may be critical for shielding NI from the adaptive immune system and maintaining the commensal relationship with the host.

Materials and Methods

Subjects

PT were collected from individuals undergoing routine tonsillectomy for a variety of conditions including recurrent tonsillitis, hypertrophy, and sleep apnea. Immunodeficient patients or those that had previously been diagnosed with invasive meningococcal disease were excluded from this investigation. Although our observations are limited by the use of tonsillar tissue obtained from patients with tonsillar disease, the tonsils were not removed during periods of active inflammation. In addition, immunohistochemical analyses have not revealed marked neutrophil infiltration or areas of necrosis in the excised tissue (16, 19), which is immunologically responsive to experimental stimuli. Also, there is no evidence of reduced colonization rates of Neisseria species in individuals requiring tonsillectomies as demonstrated by the high frequency of Nm beneath the mucosal surface of tonsil specimens when examined by immunohistochemistry (23). Written informed consent was obtained from all individuals or from a legal guardian. The collection of samples and the research described complies with relevant guidelines and institutional practices (Central and South Bristol Research Ethics Committee, E4388).

Ags and reagents

OMV of NI (strain Y92-1009, ST-613) were prepared from late log-phase cultures under iron-limited growth using a sodium-deoxycholate detergent extraction method as described previously (24). OMV of Nm (strain H44/76, B: P1.7, 16; L3, 7, 9, ST-32) were prepared by Dr. J. Findlow (Health Protection Agency North West, Manchester, U.K.) using the same method (24). OMV provide a useful tool for the investigation of immune responses to Neisseria species because they contain a representative sample of the bacterial outer membrane proteins embedded in a phospholipid vesicle, containing lipooligosaccharide (LOS). They are naturally produced and are therefore physiologically relevant in vivo (25). In addition, OMV have been used extensively by ourselves (16, 19) and others (26, 27) for the evaluation of naturally acquired and vaccine-induced immunity to Neisseria species. Inactivated split-virion influenza vaccine (flu) (Fluzone 2002–2003 formula) was purchased from Sanofi-Pasteur MSD.

Staphylococcus aureus cowan 1 strain (SAC) was purchased from Merck Chemicals. CpG oligodeoxynucleotide (ODN) type B, 2006 was obtained from Autogen Bioclear, PWM, keyhole limpet hemocyanin (KLH), BSA, Tween 20, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma-Aldrich.

Abs and flow cytometry

The following anti-human Abs conjugated to PE, PE-Cy5.5, or alkaline phosphatase (AP) were used: CD13 PE (Miltenyi Biotec), CD19 PE-Cy5.5, IgM AP. IgA AP (Caltag), CD45RA PE (BD Biosciences), and IgG AP (Sigma-Aldrich). Affinity purified anti-human IgM, IgG, and IgA were purchased from Invitrogen. Analytical flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). CellQuest software (BD Biosciences) was used for data acquisition and the data were analyzed using FlowJo software (Tree Star). Phenotypic acquisition was performed as previously described (16).

Isolation of tonsillar mononuclear cells (TMNC)

TMNC were isolated from PT using a previously described method (16) and resuspended in complete cell culture medium (RPMI 1640; Invitrogen) supplemented with 4 mM l-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 20 mM HEPES (all from Sigma-Aldrich), and either 10% (v/v) FCS (Harlan Laboratories) or 1% (v/v) heat-inactivated human AB serum (National Blood Service) as indicated.

Cellular separation by magnetic depletion

All separations were conducted according to the manufacturer’s instructions by negative depletion (Miltenyi Biotec). To assess the suppressive role of Treg on effector T cell responses, CD19+ or both CD19+ and CD25+ cells were depleted from the TMNC population. CD25 microbeads were used at a final concentration of 2.5 µl per 1 × 107 cells. This concentration was sufficient to remove only the highest 10% of CD25-expressing CD4+ cells from the TMNC pool, which contains the majority of Foxp3+ Treg (28). Typically, depletion of CD25+ cells removed >90% of Foxp3+ cells (data not shown). For this experiment, the CD19 microbeads were used at a final concentration of 10 µl per 1 × 107 cells to minimize the loss of CD19+ cells by nonspecific binding. The purity of the depleted fractions was assessed by flow cytometry.

T cell proliferation assays

T cell proliferation was measured in TMNC using a previously described method (16). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 in complete cell culture medium supplemented with human serum and either no Ag, Nm OMV (1 µg/ml protein), NI OMV (1 µg/ml protein), or flu (0.09 µg/ml protein), BSA (100 mg/ml), anti-human IgM (20 µg/ml), and anti-human IgA (10 µg/ml), and anti-human IgA (10 µg/ml) at 4°C overnight.

Preparation of ELISPOT plates

Where required, 96-well flat-bottom filter plates (Multiscreen-HHT, 0.45 µm, Millipore) were coated with the following reagents dissolved in PBS: Nm OMV (10 µg/ml protein), NI OMV (10 µg/ml protein), influenza vaccine (0.9 µg/ml hemagglutinin), KLH (10 µg/ml), BSA (100 mg/ml), anti-human IgM (20 µg/ml), anti-human IgG (10 µg/ml), and anti-human IgA (10 µg/ml) at 4°C overnight.

Memory B cell ELISPOT assay

The number of memory B cells present in TMNC to specific Ags was quantified using a method adapted from Crotty et al. (29). TMNC were cultured in 2 ml volumes in 24-well flat-bottom plates at 5 × 106 cells/ml in cell culture medium supplemented with FCS and a mixture of polyclonal mitogens: SAC (10 µg/ml PWM (1 µg/ml), and CpG ODN type B (6 µg/ml)). Cells were incubated at 37°C in a humidified
POT plates at 1 supplemented with FCS then seeded into triplicate wells in the ELISpot. Cells were washed and resuspended in complete cell culture medium Nm OMV (1 not blocked. TMNC were depleted of CD3 \(^+\) and CD45RA \(^+\) cells (E). Points represent mean proliferation of triplicate wells. Note the smaller scale in E, B, D, and F. Flow cytometric analysis of TMNC showing CD19 \(^+\) and CD45RA \(^+\) cell populations during each stage of depletion. G, Peak proliferative responses of whole TMNC populations between days 2–3 to stimulation with Nl OMV and Nm OMV in children ≤5 years (n = 13, median 5 years) and individuals ≥6 years (n = 21, median, 18 years). The response to medium alone was subtracted as background. Horizontal bars represent the median peak proliferation for each group. The Wilcoxon Signed-Ranks test was used to compare responses between Nl and Nm OMV and the Mann-Whitney U test was used to compare responses between age groups. H, Peak proliferative responses of whole TMNC and CD19 \(^+\) cell-depleted TMNC between days 2–3 to stimulation with Nl OMV and Nm OMV in individuals (age range 3–31 years, median 8.5 years). The response to medium alone was subtracted from each data point as background. Horizontal bars represent the median peak proliferation for each group. Groups were compared using the Wilcoxon Signed-Ranks test (n = 20).

**Detection of T cell-independent ASC**

For the detection of total ASC, prepared ELISPOT plates were washed and blocked as for the memory B cell ELISPOT assay. For the detection of Ag-specific IgM \(^+\) and IgG \(^+\) ASC, ELISPOT plates were washed but not blocked. TMNC were depleted of CD3 \(^+\) cells and cultured at 37°C in a humidified atmosphere of 5% CO\(_2\). On day 5 or 6, ELISPOT plates that had been precoated with Nm OMV, Nl OMV, flu, and KLH were washed and blocked with wash buffer supplemented with BSA (10 mg/ml) for 2 h at 37°C. TMNC were washed and placed into complete cell culture medium supplemented with FCS in triplicate wells in the ELISPOT plates at 1 \(\times\) 10\(^5\) cells/well in 100 \(\mu\)l. The plates were then incubated overnight at 37°C and washed with PBS followed by 0.05% (v/v) Tween 20. The plates were then incubated overnight with anti-human IgG AP and IgA AP in PBS supplemented with BSA (10 mg/ml). Finally, the plates were washed and developed using 5-bromo-4-chloro-3-indolyl phosphate. The number of Ag-specific IgM and blocked as for the memory B cell ELISPOT assay. For the detection of total ASC, prepared ELISPOT plates were washed but

**Statistical analysis**

All statistical analyses were made using nonparametric tests using a two-tailed hypothesis. Unpaired samples were analyzed using the Mann-Whitney U test and paired samples were analyzed using the Wilcoxon signed-ranks test using GraphPad Prism version 4.02 for Windows (GraphPad Software).

**Results**

**Low-level mucosal T cell memory to Nl OMV during the peak age of asymptomatic Nl carriage**

We have previously demonstrated the presence of Ag-specific mucosal T cell proliferation and memory responses to Nm OMV in the PT that increase with age (16), coinciding with increased frequency of asymptomatic meningococcal carriage (12). We assumed that mucosal colonization by Nl would induce similar adaptive responses to those observed to Nm and therefore compared the development of mucosal T cell immunity to Nl and Nm OMV in a range of subjects. Responses to flu were measured as a positive control because the majority of children have been primed as a result of natural infection by influenza virus by 3 years of age (30). In addition, we have previously demonstrated flu-specific T cell responses in the PT over a wide range of ages (31).

Cellular proliferation to the Nl, Nm, and flu Ags was detected in TMNC cultures but the magnitude of the response to Nl OMV was much greater and peaked earlier (day 2–3) than the response to Nm.
Effect of adding irradiated TMNC to memory T cell proliferative responses. TMNC were depleted of both CD19<sup>+</sup> and CD45RA<sup>+</sup> cells and cultured in the presence of medium alone, Nl OMV, Nm OMV, or flu in the presence or absence of irradiated TMNC added at a 1:1 ratio. Proliferation was measured by [³H]Tdr incorporation and sampled on days 3–7. One representative experiment of 13 (donor aged 10 years) is shown.

The timing and magnitude of this response is more characteristic of a noncognate mitogenic kinetic (32) than T cell proliferation to a cognate Ag (16, 31). It is consistently present and not confined to a single age group, occurring in cells derived from children and adults (Fig. 1G). There was no statistically significant difference in the peak proliferative response to Nl OMV on days 2–3 in individuals ≥6 years (median 50914 CCPM; range 0–74640 CCPM) compared with children ≤5 years (median 28913 CCPM; range 7653–69471.27 CCPM). Furthermore, this mitogenic response was induced by OMV produced from multiple strains of Nl belonging to a variety of clonal lineages (ST-613, ST-595, ST-1494, and ST-640; data not shown) and was not observed to Nm OMV (Fig. 1G). Due to the high frequency of molecules present in bacteria that are able to mediate polyclonal B cell proliferation (33), we postulated that this early peak was due to B cell rather than T cell proliferation. Depletion of CD19<sup>+</sup> B cells abrogated the early response to Nl OMV, confirming that it is B cell-dependent (Fig. 1A–D and H). Depletion of CD19<sup>+</sup> cells also revealed a smaller proliferative response to Nl OMV that typically peaked later at days 4–9, which is characteristic of cognate T cell proliferation (16, 31) (Fig. 1C). The proliferative kinetics to Nm OMV and flu were unaltered by B cell depletion. Subsequent experiments have revealed that B cells are the proliferating cell population that are responsible for the early peak at days 2–3 in response to Nl OMV and that this response is independent of T cell help (our unpublished observations). As a result of these findings, CD19<sup>+</sup> cells were routinely depleted from all further T cell assays (Figs. 2, 3, 5, and 6) to allow cognate T cell proliferative responses to be distinguished from noncognate cellular proliferation.

To characterize the mucosal memory responses to these URT colonizers, we then depleted CD45RA<sup>+</sup> naive T cells from the B cell-depleted TMNC pool. This eliminated the response of proliferating naive T cells, allowing CD45RO<sup>+</sup> memory T cell responses that have been primed by Ag encountered in vivo to be distinguished in vitro. In initial experiments the magnitude of T cell responses was lower upon depletion of CD19<sup>+</sup> and CD45RA<sup>+</sup> cells compared with depletion of CD19<sup>+</sup> cells alone (Fig. 1, C and E). This may have been due to the depletion of naive T cells that were proliferating earlier in the time course. However, CD45RA is also expressed on a subset of tonsillar dendritic cells, depletion of which may have created suboptimal conditions for the presentation of Ag to T cells. Indeed, the proportion of HLA-DR<sup>+</sup> cells in the TMNC population was reduced after CD45RA<sup>+</sup> cell depletion (data not shown). To overcome any deficit in the level of Ag presentation, irradiated autologous TMNC were added to cell cultures (Fig. 2). Addition of irradiated autologous TMNC augmented the magnitude of the memory response without changing the overall kinetic. Thus, in later experiments, irradiated autologous TMNC were added to cultures where indicated.

Nasopharyngeal sampling and bacterial cultures were not taken in this investigation because they cannot provide a comprehensive colonization history when taken at a single point in time. However, long-term carriage studies have estimated that 59% of children have been colonized at least once by Nl by the age of 4 years, compared with only 7% that have been colonized by Nm (18). Peak proliferative responses to Nl OMV, Nm OMV, and flu were therefore compared between young children ≤5 years, median 4 years, n = 13) that may have been colonized by either Neisseria species (Fig. 3). As reported previously (16), peak responses to Nm were low in young children (median 263 CCPM) in contrast to more potent flu responses (median 4853 CCPM). Peak memory T cell responses to Nl were also low in this age group (median 1223 CCPM), suggesting that nasopharyngeal colonization by NI is not associated with the induction of mucosal T cell memory.

In older children, peak memory T cell responses to all Ags were significantly increased (NI, median 9367 CCPM, p < 0.01; Nm, median 2276 CCPM, p < 0.05; flu, median 22404 CCPM, p < 0.05). There was no age-related difference in baseline Tdr incorporation (data not shown). This increase in proliferation of the memory pool to Nm is consistent with increasing carriage rates and with previous data (16). Conversely, NI T cell memory was detected at an age when carriage studies have demonstrated that NI colonization is declining. The reason for the lag between peak carriage of NI and the acquisition of memory is not certain. We speculate that colonizing NI fails to prime the adaptive immune system in early childhood and propose that the increased response in older childhood may be due to cross-reactive immunity primed by subsequent colonization events by other Neisseria species.
B cell memory to Nl OMV is absent during the peak age of asymptomatic Nl carriage in young children

The failure to detect T cell memory to Nl in early childhood was surprising because this is the period in which asymptomatic nasopharyngeal carriage of this organism is most frequent (17, 18, 22). It is possible that memory T cells from young children are less responsive to antigenic stimulation in vitro than cells from older children, or that there may be an increased rate of reversion from a cell surface CD45RO+ to CD45RA+ phenotype, as has been shown previously (34, 35). Therefore, to confirm the absence of immunological memory to Nl in the mucosa of young children, we also assessed B cell memory in the PT. This was done using a protocol developed by Crotty et al. (29), which employs a mixture of B cell mitogens to amplify the number of memory B cells within a mononuclear cell population and drive their differentiation into plasma cells in vitro. This allows the number of Ag-specific IgM+ and IgA+ memory B cells from the PT to be quantified by ELISPOT assay. IgM+ memory B cells cannot be differentiated from naïve B cells using this method and therefore Ag-specific IgM+ ASC were not assessed. As a means of compensating for any background spots or non-Ag-specific reactivity within the assays, numbers of anti-OMV spots were compared with those against a control Ag, KLH, to which individuals would not have mounted immune responses (Fig. 4). In agreement with the memory T cell data, there was no difference between the numbers of ASC to Nl OMV and KLH in young children (≤5 years), suggesting an absence of B cell memory to Nl in this age group (Fig. 4, A and B). IgG+ spots generated against Nl OMV were significantly greater than to KLH in older children (6–15 years) and adults (≥16 years), p < 0.05, but remained at low levels (Fig. 4, C and E). These results confirm that during the peak period of nasopharyngeal carriage, Nl does not prime the development of immunological memory in the URT mucosa.

There was no difference between the numbers of ASC generated against Nm OMV and KLH in younger (≤5 years) or older children (6–15 years), suggesting a paucity of mucosal B cell memory to Nm in these age groups (Fig. 4, A–D). In agreement with previously observed T cell responses (16), the number of IgG+ spots to Nm OMV (median, 48,34) was significantly greater than to KLH (median 5) in adults (p < 0.01) (Fig. 4E), coinciding with the peak period of nasopharyngeal carriage (12). Also in agreement with the memory T cell responses detected in this study, the numbers of memory B cells to flu were greater than to KLH in people of all ages.

Ni-specific Treg activity is absent in early childhood and does not suppress the effector T cell response to Ni OMV

We have previously demonstrated the presence of mucosal CD4+CD25+ Treg activity in the PT that suppresses the effector T cell response to Nm OMV but not flu (19). We speculated that Treg may be responsible for suppressing the effector response to Nl in early childhood, thus explaining the apparent absence of specific immunological memory at this age. To test this hypothesis, proliferative cultures were established using TMNC populations from children that had been depleted of CD19+ B cells alone or both CD19+ and CD25+ cells. To verify that Treg activity can be detected in cultures depleted of B cells, we depleted CD19+ and CD25+ cells from several older children (12–15 years) (Fig. 5). In agreement with previous observations (19), an increase in proliferation to Nm OMV was detected in cultures depleted of both CD19+ and CD25+ cells compared with depletion of CD19+ cells alone, demonstrating the presence of Treg activity (Fig. 5A). Conversely, in children ≤5 years we found no increase in proliferation in response to Nl OMV or flu after depletion of CD25+ cells (Fig. 6). These results indicate that the absence of immunological memory to Nl in young children is not due to active suppression of effector responses by CD4+CD25+ Treg. Interestingly, there was a low but statistically significant increase in the proliferative response to Nm OMV after depletion of CD19+ and CD25+ cells (median 3583 CPM) compared with depletion of CD19+ cells alone (median 2092 CPM), p < 0.05, indicating the presence of Nm-specific Treg activity in young children which are unlikely to have previously been colonized by Nm (Fig. 6). This is in contrast to our previous investigation (19) in which no Treg activity was observed in this age group and suggests that Nm either activates Treg cells in naïve individuals, or that previous colonization by another species primes the induction of cross-reactive Treg to Nm but not Nl. The reason for the disparity between our results presented in this study and results obtained previously is unclear, but it is possible that depletion of CD19+ cells increased the sensitivity of the assay in this investigation.
Ni OMV but not Nm OMV induce the production of polyclonal Ig that resembles “natural” Ab

Our cellular proliferation data suggests that while there is an absence of T cell-mediated mucosal immunity to Ni during the peak period of carriage, this commensal may not be ignored by the immune system. As demonstrated previously (Fig. 1, A, C, G, and H), Ni OMV induce a potent proliferative response in TMNC that is CD19⁺/H11001 B cell-dependent and is absent in response to Nm OMV. Subsequent investigations have demonstrated that this is due to T cell-independent B cell proliferation (unpublished data). To understand the potential functional significance of this response in the interaction between colonizing Ni and the human host, the production of total Ig from TMNC following 2–3 days stimulation with OMV was assessed by ELISPOT assay (Fig. 7). CD3⁺/H11001 T cells were depleted from TMNC before Ag challenge to enrich the population for B cells and characterize T cell-independent responses only. In addition to inducing proliferation, Ni OMV induced the differentiation of a significantly greater number of total IgM⁺ ASC in young children ≤5 years, p < 0.01 (Fig. 7A) and older individuals ≥6 years, p < 0.001 (Fig. 7B) than stimulation with Nm OMV. This occurred in individuals of all ages. Ni OMV also induced a significant increase in the number of total IgG⁺ ASC than Nm OMV in older individuals (Fig. 7B) but not young children (Fig. 7A). The reason for this disparity in the IgG response is unclear, but may be due to the larger pool of IgG⁺ memory B cells present in the PT of older individuals as a result of Ag encounter, than in younger individuals. There was no difference in the number of total IgA⁺ ASC between cells stimulated with Ni OMV and...
and flu (H1N1, 7-fold, Nm OMV for 2–3 days). The number of Nm OMV and KLH-specific IgM was quantified (Fig. 8). Stimulation with Nl OMV induced the differentiation of a significantly greater number of IgM ASC for each group. Groups were compared using the Wilcoxon Signed-Ranks test.

To further explore the function of this Ig response and distinguish cognate BCR engagement from polyclonal stimulation, the Ag specificity of IgM and IgG ASC to a broad range of Ags was quantified (Fig. 8). Stimulation with NI OMV induced the differentiation of a significantly greater number of IgM ASC that bound to BSA (~6-fold, p < 0.01), NI OMV (~6-fold, p < 0.01), and flu (~7-fold, p < 0.05) than did stimulation with Nm OMV (Fig. 8A). The number of Nm OMV and KLH-specific IgM ASC was also increased in response to stimulation with NI OMV compared with stimulation with Nm OMV, but this increase did not reach statistical significance. These data demonstrate that NI OMV induce a nonspecific polyclonal B cell response that produces IgM with broad Ag specificity. Conversely, there was no difference between the numbers of Ag-specific IgG ASC in response to stimulation with NI OMV than with Nm OMV, which were close to background levels (Fig. 8B). Absence of significant binding to NI OMV suggests that the differentiation of IgG ASC observed in Fig. 7B is not a cognate response. We therefore predict that the IgG response is also polyclonal. However, due to the fewer number of total IgG ASC than IgM ASC that differentiated in response to stimulation with NI OMV (Fig. 7), the sensitivity of the ELISPOT assay may be too low to detect polyclonal Ag-binding in the IgG B cell pool.

Discussion

How the adaptive immune system manages the complex environment of the URT, where even closely related commensal bacteria such as Nm and Nl have markedly different pathogenic potentials, is uncertain. We have previously shown that human mucosal T cell immunity to Nm Ags increases with age (16). This acquisition of antimeningococcal immune memory coincides with an increased frequency of asymptomatic Nm carriage, which typically peaks in early adulthood (12). In contrast, in the present study, we show that during early childhood, which is the peak period of NI nasopharyngeal colonization (17, 18, 22), mucosal T cell memory to Nl Ags remains low. Although responses were above background levels, they were much lower than the strong responses observed to Nm in early adulthood (16). This was unexpected because Nl carriage in young children is associated with reduced carriage of Nm (36) and reduced frequency of invasive meningococcal disease (37) and has been suggested to prime cross-reactive immunity to Nm (18, 37). Indeed, NI and Nm express cross-reactive B cell epitopes (38) and immunization of mice with live NI or OMV protects them against challenge with live Nm (39, 40). We found that mucosal B cell memory to NI was also low, confirming that NI does not prime a memory response in the URT mucosa of young children. We therefore suggest that contrary to a widely held paradigm (18, 41), the naturally acquired mucosal immune response to the meningococcus is not primed by colonization with NI. However, it remains possible that other commensal Neisseria species (42) that colonize the nasopharynx subsequent to NI may play a role.

Mucosal T cell memory to NI Ags was increased in older children, a population where NI carriage is less common. There are several possible explanations for this lag in the acquisition of memory to NI. Firstly, the immune system may be functionally immature in the younger age group. Although this remains a possible contributing factor, we consider this to be unlikely to be the complete explanation because although the immune system is still developing in neonates, it is considered to be fully developed by 2 years of age (43). In addition, both B cell and T cell memory responses to flu were detectable in our younger subjects. These responses were primed by natural exposure to the influenza virus as immunization against influenza is not routine in the United Kingdom. Furthermore, humoral responses to the URT colonizer Streptococcus pneumoniae have previously been detected in the periphery (44) and mucosa (45, 46) of children <5 years of age. Similar to NI, colonization by S. pneumoniae peaks at 2 years of age (47), suggesting that the mucosal immune system is responsive in this age group. Secondly, it is possible that not all children investigated had been previously colonized by NI. However, multiple carriage studies conducted at different times and in different populations including U.K. populations close to the site of this investigation have demonstrated that carriage of NI is prevalent in young children, peaking at 18 mo of age and occurring less frequently in older children and adults (17, 18, 22). Also, if this were the case, we would have expected at least some previously colonized responders within this cohort, but saw little T or B cell memory in any of the individuals. However, without knowing the specific colonization history of the individuals sampled in this study, this possibility cannot be excluded with certainty. Thirdly, children in the ≤5 years age group may have been colonized by an NI strain that lacked cross-reactive T cell epitopes with the strain used in this study. We also consider this unlikely because T cell epitopes are located on conserved regions of Neisseria outer membrane proteins (48, 49) and are likely to be cross-reactive between strains. Fourthly, we have previously demonstrated the presence of Nm-specific Treg activity in the PT and so investigated the possibility that Treg suppress the T cell response to NI in young children (19). However, we found no increase in T cell proliferation.

FIGURE 8. Ag specificity of ASC produced in response to T cell-independent stimulation with Neisseria OMV. TMNC were depleted of CD3+ cells and cultured in the presence of medium only, NI OMV, or Nm OMV for 2–3 days. A, IgM+ ASC specific to BSA, Nm OMV, NI OMV, flu, and KLH (n = 8, age range 4–37 years, median 5 years). B, IgG+ ASC specific to NI OMV and Nm OMV (n = 6, age range 3–15 years, median 9.5 years). The number of ASC to medium alone was subtracted from each data point as background. Horizontal bars represent the median number of ASC for each group. Groups were compared using the Wilcoxon Signed-Ranks test.
upon depletion of putative CD4^+CD25^+ Treg. Furthermore, T cell responses to NI OMV are detectable in the PT of young children when naive T cells have not been depleted (Fig. 6), suggesting that NI does not induce peripheral tolerance in the mucosa. Taking these findings together, we speculate that during colonization, NI evades the adaptive immune system, maintaining immunological ignorance. Thus, in this yet untested model of NI commensalism, we propose that the increase in T cell memory to NI in older childhood is due to priming by other Neisseria species including Nm, resulting in cross-reactive immunity.

It is unknown why colonizing NI does not prime the mucosal immune system in young childhood when Nm primes a potent response rapidly upon carriage in later life. There are several possibilities to explain this discrepancy. Nm has a polysaccharide capsule which is one of the characteristics that distinguishes it from the other Neisseria species and is essential for survival of meningococci within the bloodstream (50). It is therefore possible that rather than priming a response in the mucosa, increased survival of Nm in the bloodstream following mucosal infection may allow sufficient exposure to the immune system to prime a potent systemic response that tracks back to the URT mucosa. Conversely, due to the lack of serum resistance, NI is rapidly cleared from the bloodstream and may not prime a systemic immune response, resulting in passive ignorance of the bacteria by the host immune system. Alternatively, despite the apparent absence of adaptive immunity to NI in the URT in young children, we have shown that NI OMV actively induce a potent proliferative response in TMNC of both young children and adults that is dependent on the presence of CD19^+ cells and is not seen upon stimulation with Nm OMV. This proliferative response is not strain-specific and is associated with the T cell-independent secretion of polyclonal IgM, which binds NI OMV as well as a range of irrelevant Ags not contained in the bacterial outer membrane. The polyclonal nature of this IgM is characteristic of a T-independent type 1 response and resembles "natural" Ab production (51). "Natural" IgM is constitutively secreted by B1 B cells in mice (51) and CD5-expressing B cells in humans (52) in the absence of Ag stimulation. "Natural" IgA is secreted onto the intestinal epithelium of mice in response to colonization by the commensal flora. It is T cell-independent and at least partially produced by B1 B cells (9). "Natural" IgA is believed to function by coating the colonizing flora without mediating clearing, restricting the size of bacterial populations, and limiting mucosal invasion (53). The response may also maintain systemic ignorance to commensal species of bacteria, as systemic priming and T cell activation is increased toward the flora in mice lacking IgA and B1 B cells, respectively (9). Thus, we propose a model whereby the polyclonal IgM produced in response to NI provides an active mechanism of immune avoidance. We suggest that the IgM binds to colonizing NI on the nasopharyngeal epithelium in vivo, preventing overgrowth of the population and invasion through the epithelial cell layer. This innate IgM may shield NI from the adaptive immune system in the URT, potentially prolonging carriage and facilitating a commensal relationship with the host. If NI penetrates the epithelium, it lacks the thick polysaccharide capsule that protects Nm against Ab binding and complement deposition (50) and would therefore be opsonized for immediate clearance by phagocytes in subepithelial tissues. In contrast to NI, Nm does not induce this mitogenic B cell response. Without the potential shielding effects of innate IgM, colonizing Nm in early adulthood primes an adaptive immune response, inducing cellular memory (16) and T cell-dependent Ig production (13, 14). This immunity is heavily regulated by Treg activity in the mucosa, which may prevent clearance of Nm, prolonging carriage but also predisposing the host to invasive disease (19). Thus, these closely related bacteria interact with the immune system in very different ways.

There are several microbial components located in the outer membrane of NI that may be responsible for mediating polyclonal B cell activation and the production of innate Ig, including LOS and the PorB porin. The most obvious candidate is LOS, which is present in both NI and Nm OMV and activates B cells via TLR4 (54). Nm LOS induces T cell-independent B cell proliferation in mice (55). However, human B cells express only low levels of TLR4 (56) and do not proliferate in response to stimulation with endotoxin (57). NI PorB is closely homologous to Nm PorB (58), which induces T cell-independent B cell proliferation in mice in its native conformation (55) and humans after denaturation (59). It is possible that NI PorB may be mitogenic in the native conformation in humans. Alternatively, other candidates such as B cell superantigens may also be present in the outer membrane of NI. NI OMV contain up to 62 additional proteins that are absent from Nm OMV (60). We are currently investigating the ability of these proteins to mediate polyclonal B cell activation.

The evidence that NI does not prime cross-reactive immunological memory to Nm in early childhood is supported by a recent large seroepidemiological investigation in which Nm-specific IgG was related to disease incidence. Contrary to the inverse relationship between "protective" serum bactericidal activity and disease incidence described in the 1960s by Goldschneider et al. (37), Trotter and colleagues (61) show that disease incidence declines throughout childhood, even in age groups where there is no change in the proportion of children with putative protective serum bactericidal activity. These findings suggest that adaptive immunity may not be responsible for this decline. Instead, we speculate that the production of innate IgM by NI provides some cross-reactive protection against meningococcal infection in the absence of a T cell-dependent immune response (62). In addition, NI may simply out-compete Nm for shared resources in the nasopharyngeal environment of young children, preventing Nm from colonizing, explaining the link between carriage of NI and reduced incidence of invasive meningococcal disease. Our findings also suggest that the decline in NI carriage in children after the age of 2 years is not due to immune-mediated clearance of the bacteria from the nasopharynx but may be due to other factors such as physiological changes of the URT or changes in host behavior during childhood.

In conclusion, although NI and Nm colonize the same location within the URT, we present evidence that suggests they engage with the human mucosal immune system in very different ways. The reason for this divergence in immune response to Nm and NI is unclear, but may have occurred during speciation from a common ancestor and the subsequent adaptation to separate nasopharyngeal niches. Although colonization by NI in early childhood does not result in priming of active antimeningococcal immunity, neither does it induce tolerance. NI induces polyclonal production of IgM, which may offer some protection against invasive meningococcal disease. This low level immune response in turn may also adapt the URT mucosa to the presence of these commensal bacteria. Whether the overall effect is to prolong carriage by harmless Neisseria while rapidly clearing bacteria that penetrate the epithelial barrier, remains to be determined. One potential consequence of this NI-induced innate immunity is the generation of immune ignorance (63), thus prolonging the period of susceptibility to colonization by both pathogenic and commensal Neisseria.

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Disclosures
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References

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