FINAL REPORT

Title: A PHASE II, OPEN LABEL, RANDOMISED, TWO CENTRE STUDY TO EVALUATE THE IMPORTANCE OF NATURALLY INDUCED IMMUNE REGULATION ON THE MUCOSAL IMMUNE RESPONSE TO MENINGOCOCCAL SEROGROUP B OUTER MEMBRANE VESICLE (OMV) VACCINE WHEN ADMINISTRATED INTRAMUSCOLARLY TO ADULTS & ADOLESCENTS (SysVac01-C60P2)

Short title: The impact of Group B meningococcal outer-membrane vesicle vaccine on the regulation of naturally acquired *Neisseria meningitidis*-specific cellular immunity

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Introduction

Neisseria meningitidis (Nm) is a natural coloniser of the human upper respiratory tract (URT) with carriage rates in healthy individuals ranging from 5-40%. In most cases carriage is asymptomatic, however on rare occasions Nm can successfully invade the host to cause septicaemia and meningitis. Disease is highest in infants and young children who have yet to develop natural immunity to Nm. Although nasopharyngeal colonisation is the first step in infection, it is also critical for priming naturally acquired immunity, leading to long-term immune protection against this pathogen.

Natural protective immunity against invasive meningococcal disease is thought to be principally antibody-mediated, due to a strong correlation between the development of complement-fixing serum bactericidal antibody (SBA) and disease resistance. However, T cells with specificity for meningococcal antigens have been identified both in the periphery and palatine tonsils of older children and adults; indicating a potential role for T cells in the protective immune response. Mucosal T cell responses towards Nm are predominantly Th1-biased, thereby enhancing B cell production of complement-fixing and opsonizing antibodies, and granulocyte phagocytic function. Whilst immunity at the mucosal surface is likely to play an important role in controlling bacterial colonisation, we have demonstrated the emergence of putative Nm-specific Tregs at the mucosal surface in older children and adults who are likely to have had repeated episodes of colonisation. These Tregs were not found in the blood and may be important in controlling localised inflammation and tissue damage, but may also hinder effective immune clearance and prolong colonisation.

Vaccination changes the nature of the mucosal immune response, selectively re-programming pre-existing naturally acquired immunity with a loss of Th1 bias. MenB OMV vaccine also boosts systemic T cell responses to Nm antigens, but whether in the context of pre-existing natural immunity, systemic challenge induces Treg thus potentially disrupting natural regulatory networks has not been determined. In this Phase ll, open label, randomised, two centre study, we have therefore characterised the phenotype and function of T-cell regulation induced by natural exposure, and explored the possibility that parenteral vaccination with meningococcal OMV induces systemic Treg.

Results

Participants and clinical samples

9 adults were recruited to a MenB clinical trial (Eudract number 2008-001927-74). Each study participant received a total of 2 doses of the meningococcal group B vaccine (MeNZB ™; Novartis Vaccines and Diagnostics SRL) with an interval of 6 weeks by intramuscular injection in the arm. Peripheral blood was collected prior to and after two doses of MeNZB ™. NZ MenB OMV vaccine is based on a New Zealand strain (B 4: P1.7b,4) and contains outer membrane vesicles (OMV) adsorbed to aluminium hydroxide. Palatine tonsils and where possible, a citrated 50ml blood sample were also obtained from otherwise healthy, unvaccinated adults (18-40yr) undergoing routine tonsillectomy for a variety of conditions including recurrent tonsillitis, hypertrophy and sleep apnoea. The collection of samples and the research complies with relevant guidelines and institutional practices (Central and South Bristol Research Ethics Committee; E4388 and North Somerset and South Bristol Research Ethics Committee; 08/H0106/85). All activities were conducted with informed consent and in accordance with relevant local and national guidelines. Immunosuppressed individuals or those with active infection were excluded from the study. None of the participants had a history of meningococcal disease or had received a MenB vaccine previously. In addition, Buffy coat lymphocytes, fractionated from blood donations were obtained from the national blood service (Filton, UK). Citrated blood was used for peripheral blood T-cell assays. Tonsillar mononuclear cells (TMNCs) were obtained by density gradient centrifugation.

Meningococcal-specific regulatory activity resides within the CD25+ fraction of TMNCs

When the majority of CD25 highly-expressing cells, predominantly co-expressing CD4. There was a significant increase in the magnitude of proliferation observed in response to MenB OMV (H44/76) in CD25-depleted cultures compared to whole TMNC cultures ($p<0.05$; $n= 6$; median 25yrs, range 15-36yrs). Proliferation was again suppressed upon re-addition of the CD25+ fraction. In contrast, this effect was not seen in cultures from the same patients, stimulated with influenza antigen, where the presence or absence of the CD25+ fraction did not significantly alter responses. This data confirms the presence of a MenB-specific CD25hi Treg population within TMNCs and shows that there is no loss of function in these cells upon magnetic separation and re-addition to CD25- cultures.

Regulatory T cells that suppress MenB-specific T cell responses are enriched in the tonsil

We then determined whether MenB-specific regulation was confined to the tonsil, a lymphoid tissue associated with the primary site of carriage in the pharynx, or whether regulation is present in the systemic response. We added back purified CD4+CD25+ Treg into CD25low/negative effector CD4+ T cell (Teff) cultures from adult palatine tonsils or peripheral blood. Using tonsil derived mononuclear cells stimulated with MenB OMV (H44/76) or HI Nm we found that the ratio of 1:2 Treg:Effector T cells significantly suppressed meningococcal-specific effector T cell responsesbut that the ratio of 1:12 did not. In keeping with our previous findings, tonsillar Tregs selectively suppressed mucosal Nm responses but not influenza-specific T cells. Treg suppression assays were then performed using peripheral blood. In contrast to mucosal Tregs, we show that Tregs isolated from peripheral blood when added at ratio of 1:2 were unable to suppress Nm or influenza-specific T cells. These findings establish that mucosal Tregs selectively regulate Nm-specific responses and that regulation is largely compartmentalised within the tonsil.

Phenotypic characterisation of regulatory T cells from the tonsil and peripheral blood

The compartmentalised regulation of meningococcal responses within the mucosa led us to explore whether there were global differences in the frequency or phenotype of Treg populations isolated from tonsils or peripheral blood. As a proportion of total lymphocytes, the frequency of CD4+CD25+ cells was comparable between tonsils (1.8%[±0.5%]) and peripheral blood (1.9%[±0.6%]). These cells were clearly identifiable as Treg cells on the basis of their predominant expression of FoxP3 in the tonsil (90.8% [±1.7%]) and in the blood (86.2% [±5.6%]) and predominately $CD127^{lo}$ expression in the tonsil (92.0% [±4.3%]) and in the blood (87.2% [±7.1%]). A marker of antigen experience (CD45RO) within the CD4+ $CD25^{hi}$, FoxP3+ population in both tissues was then assessed using blood and tonsil samples from the same patients. Use of the β_7 integrin antibody in conjunction with an antibody recognising CD103 (α_E integrin) allowed the identification of $\alpha_4\beta_7$ integrin+ and $\alpha_E\beta_7$+ Treg populations, both of which have been associated with mucosal homing . Although Tregs did not express CD103 we found that a population of $\alpha_4\beta_7$ integrin+ Treg were present in both the tonsil (9.3% [±1.0%]) and the blood (16.5% [±2.1 %]). Further phenotypic characterisation using CCR10 and CD45RO showed that neither tonsillar nor peripheral Tregs expressed CCR10 but that the proportion of CD45RO+ Treg was moderately higher in the tonsil (76.3% [±4.0%]) when compared to blood (62.4% [±2.0%]). There were no major differences in the number or phenotype of the global Treg populations isolated from the systemic and mucosal compartments even though Nm-specific Tregs are confined to the tonsil.

Mucosal and peripheral Nm-specific effector CD4+ T cells are both suppressed by tonsil Treg

To explore the possibility that the mucosal Nm-specific T cell population is more susceptible to Treg activity or indeed that systemic T cells are refractory to Treg, we exposed T effector (Teff) cells from the peripheral blood to tonsil derived Treg, both from the same individual. Collated data from 5 donors, age range 17-28, median 20yr shows that mucosal and peripheral Nm-specific proliferative responses are equally suppressed by mucosal Tregs.

Mucosal Nm-specific Th1 or Th2 cytokines are suppressed by mucosal regulatory T cells

Having shown that Nm-specific proliferation is highly regulated, we went onto explore whether cytokine production was also modulated by mucosal Nm-specific Tregs . In contrast to MenB OMV (H44/76), TMNC produced higher levels of Th1 and Th2 cytokines in response to HI MenB which resulted in a more pronounced suppression by mucosal Tregs. In the presence of mucosal Tregs, MenB-specific IL-2, IFN-γ, TNF-α, IL-5 and the known suppressive cytokine IL-10, were significantly decreased (grey bars), in contrast, a slightly different group of cytokines responding to influenza were also suppressed; TNF-α, IL-4 and IL-10. IL-17 which is largely produced by Th17 cells was produced in response to MenB but not suppressed by Tregs. The majority of Nm-specific IL-17 responses were found in the mucosa rather than peripheral blood lymphocytes.

Meningococcal regulation is contact-dependent

As the soluble suppressive cytokine, IL-10, was not increased in the presence of Tregs we investigated whether Nm-specific suppression was exerted via cell to cell contact. Transwells were used to physically separate tonsillar Teff from Tregs; in the lower chamber there were APC, Teff and Tregs whilst APC and Teff were in the upper chamber, both compartments were stimulated with MenB OMV (H44/76) and cell proliferation in the upper chamber was evaluated. Pooled data from 5 adults (age range 17-31, median 21yrs), also showed that inhibition of Teff responses occurred only when Treg were in direct contact (p=.007) and suppression was significantly reduced when Treg were separated by a transwell membrane (p=0.001).

Tregs are not activated by bacterial pathogen-associated molecular patterns but have specificity for the meningococcal NspA protein

We then set out to investigate whether Tregs were primed and activated in a TCR-independent manner by bacterial pathogen-associated molecular patterns (PAMPs) or via TCR recognition of meningococcal proteins. Firstly, CD25+ cells were incubated with neutralising anti-TLR2 and anti-TLR-4 antibodies to block the activation of Treg via TLR before being added back into TMNC cultures stimulated with MenB OMV (H44/76). We show that blocking activation of Treg via TLR did not result in a significant increase in the level of proliferation observed when compared to the isotype control. Having shown that Treg activity is not triggered by anti-bacterial TLR ligation, we explored whether Treg suppression could be activated by meningococcal outer-membrane proteins. Proliferation of whole and CD25 depleted TMNC was assessed in response to PorA- (MenB) OMV, PorB protein and NspA protein. In whole TMNCs, a significant decrease in proliferation in the presence of NspA compared to the CD25 depleted fraction, shows there are Tregs that are primed by NspA capable of regulating Teff responses; $p<0.01$. There was a difference approaching significance in PorA- OMV cultures and taken together, this suggests that there are antigen-specific Treg within the tonsils with specificity for NspA and potentially other proteins present within OMV preparations.

Treg are not detectable in the periphery following vaccination

Next we explored whether exposure to Nm antigens in the blood leads to the generation of Treg and alteration of the balance of pre-existing natural immunity to the meningococcus. Six adults were given two parental doses of MeNZB^{TM} vaccine, and the balance of systemic Nm-specific Treg or Teff populations were compared before and after vaccination. In these naturally exposed individuals, the magnitude of the systemic response to MenB OMV (NZ) increased after vaccination ($p<0.05$), however there was no significant effect of CD25 depletion on the magnitude of the response, either prior to, or following vaccination. Thus in keeping with our previous results, systemic meningococcal responses are not regulated by CD25 cells, and the existing balance of systemic Treg or Teff responses is not skewed by challenge with MenB OMV.

Discussion & Conclusions

We have confirmed the existence of FoxP3^{+}CD127^{lo}CD25^{hi} Tregs with specificity for meningococcal antigens which are compartmentalised to lymphoid tissue close to the site of colonisation (the palatine tonsils) but not in the blood. We have shown that while these Treg express the T cell homing receptor $\alpha_4\beta_7$ integrin, expression was not ubiquitous, suggesting that $\alpha_4\beta_7$ is not essential for retention of Treg within the mucosa. The regulation of effector T cells by Nm-specific Treg was contact-dependent and not associated with an increase in IL-10 production, suggesting that Nm-specific Tregs were not Tr1 cells nor did they use soluble IL-10 to suppress effector T cell function. Tregs suppressed Th1/Th2 cytokine production by mucosal CD4 cells but did not inhibit secretion of IL-17 by Th17 cells. Although natural exposure to Nm results in the development of Treg with Nm specificity within the mucosal compartment, systemic vaccination with MenB OMV did not induce Treg activity. Why naturally acquired MenB-specific regulation was largely restricted to the URT lymphoid tissue rather than the periphery is uncertain. We speculate that the lack of meningococcal-specific Treg in the periphery following systemic vaccination would favour effector responses that control meningococci that enter the blood stream. However, the observation from our co-culture experiments that systemic Nm-specific responses can also be suppressed by mucosal Tregs raises the possibility that recirculating vaccine-induced effector T cells recruited from the periphery to mucosal sites could be significantly regulated. This could have marked effects on the ability to clear meningococcal colonisation. More studies are required to ensure

that Tregs are not primed or generated by vaccination which could significantly alter protective responses.