We have shown that Schistosoma mansoni egg soluble antigen (SEA) prevents diabetes in the nonobese diabetic (NOD) mouse inducing functional changes in antigen presenting cells (APCs) and expanding T helper (Th) 2 and regulatory T cell (Treg) responses. A Th2 response to S. mansoni infection or its antigens is key to both the establishment of tolerance and successful reproduction in the host. More recently we demonstrated that SEA treatment upregulates bioactive TGF\(\beta\) on T cells with consequent expansion of Foxp3\(^{+}\) Tregs, and these cells might be important in SEA-mediated diabetes prevention together with Th2 cells. In this study we profile further the phenotypic changes that SEA induces on APCs, with particular attention to cytokine expression and markers of macrophage alternative activation. Our studies suggest that TGF\(\beta\) from T cells is important not just for Treg expansion but also for the successful Th2 response to SEA, and therefore, for diabetes prevention in the NOD mouse.

1. Introduction

Helminths employ a range of immunomodulatory strategies to modulate the host immune response and utilize it to extend their longevity in the host and facilitate transmission. Characteristic of infection with helminths such as Schistosoma mansoni is the induction of a Th2 response. Two products released from eggs have recently been shown to play important roles in the immune deviation induced by S. mansoni infection [1, 2]. The NOD mouse provides a good animal model of the human autoimmune disease, Type 1 diabetes [3]. We have previously shown that S. mansoni infection or exposure either to the helminth eggs or soluble extracts of worms (SWA) or eggs (SEA) can prevent diabetes onset in this mouse [4–6].

As Type 1 diabetes is a Th1-mediated autoimmune disease [7], any skewing of the response towards Th2 would result in diabetes prevention. Other responses that have the potential to impact on diabetes would be the induction of immunoregulatory cytokines such as IL-10 and TGF\(\beta\) and regulatory T cells (Tregs) [8, 9]. We and others have shown that both SEA and SWA have profound effects on cells of the innate immune system including dendritic cells (DCs) and NKT cells [5, 10, 11]. In the case of bone marrow derived DCs, it has been shown that exposure to S. mansoni antigens results in DC retention of a more immature phenotype while inducing them to mediate a Th2 response in vivo [5, 10]. Immature DCs have been associated with ability to induce a Th2 response as well as immune tolerance through induction of Tregs [12]. Although there was little evidence of phenotypic change in SEA exposed bone marrow derived murine DCs, recent studies showed increased expression of mannose receptor (MR), DEC-205, and DC-SIGN as well as induction of IL-10 [6]. SEA has, furthermore, been shown to induce Foxp3 expressing Tregs in a TGF\(\beta\) dependent manner [6]. Most studies in mice have been carried out using bone marrow derived APCs and it could be argued that it would be more relevant to examine the effect of S. mansoni antigens on isolated splenic DCs as well as in vivo on macrophage (M\(\Phi\)) populations. In this manuscript we have addressed in detail the effect of SEA on functional and phenotypic changes in DCs and M\(\Phi\)s and the impact that this might have on the development of different T cell subpopulations including Tregs.
2. Materials and Methods

2.1. Mice. Female NOD/Tac mice were housed and barrier bred in the Pathology Department, University of Cambridge animal facilities (Cambridge, UK) and used between 4–12 weeks of age. All work was conducted under UK Home Office project license regulations after approval by the Ethical Review Committee of the University of Cambridge.

2.2. Preparation of SEA and SWA. Preparation of SEA and SWA was described previously [13]. Briefly, for SEA, eggs were harvested from the livers of outbred infected mice, which were treated to prevent granuloma formation. Livers were homogenized through sieves and the eggs were collected, washed, and sonicated in PBS on ice, prior to centrifugation to separate the saline soluble fraction. SWA was prepared from adult worms recovered from the portal venous vasculature. Both antigens were sterile filtered and endotoxin removed to below <1 EU/mg using Polymixin B agarose beads (Sigma).

2.3. Cell Culture and Reagents. Cells were cultured in Iscove’s modified Dulbecco’s medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 50 μM 2-mercaptoethanol (All Sigma), and 5%–10% fetal calf serum (FCS) (Gibco). Anti-CD3 (aCD3) (2C11) and aCD28 (37.51) were obtained from BD Pharmingen. LPS from Salmonella enterica (Sigma) and the TLR1/2 agonist Pam3CysK4 (Invivogen) were used to stimulate DCs. Anti-TGFβ (1D11.16) was precipitated from hybridoma supernatants and dialyzed against PBS before use.

2.4. TGFβ Bioassay. The MLE/PAI cell line is derived from mink lung epithelial cells and contains freely luciferase under PAI-1 promoter control. MLE cells were cultured with 50 μL of tissue culture supernatants or a double-dilution standard curve derived from recombinant human TGFβ1 (R&D Systems) for 18 hours. Cells were then lysed and luciferase activity was measured using Promega’s Luciferase Reporter System and a BetaLux scintillation counter (Wallac).

2.5. Flow Cytometry. Cells were stained with appropriate combinations of the following antibodies:

- CD4 (RM4-5)-PCP-Cy5.5, CD25 (PC61)-PE, CD44 (IM7)-FITC, CD11b (M1/70)-PCP-Cy5.5, CD11c (HL3)-FITC or APC, galectin-3 (M3/38)-Alexa647, Foxp3 (FJK-16s)-Alexa647, CD205/DEC-205 (NDL-145)-FITC, CD206/MR (MR5D3)-FITC, PD-L1 (M1H5)-PE, CD1d (1B1)-FITC, SIGN-R1 (22D1)-Alexa647, IL-4 (11B11)-PE, IL-17 (TC11-18H10)-PE, IL-10 (JES5-16A3)-APC, IL-13 (eBio13A)-Alexa647, and IFNγ (XMG1.2)-PE-Cy7. Appropriate isotype control antibodies were from the same manufacturer as for the specific antibody. Biotinylated polyclonal rat anti-mouse galectin-1 (R&D Systems) was used in conjunction with streptavidin-PE (BD Biosciences). Intracellular Foxp3 staining was according to the manufacturer’s instructions (anti-mouse/rat Foxp3 staining set; eBioscience). For intracellular cytokine staining, cells were stimulated for 5 hours with 0.5 μg/mL PdBu, and ionomycin and cytokine secretion was blocked using 1 μg/mL brefeldin A (all Sigma). Cells were fixed promptly in 1% paraformaldehyde PBS and permeabilized with 0.5% saponin (Sigma) in staining buffer. For live cell discrimination, 7-aminoactinomycin D (BD Biosciences) was used. For all staining, Fc receptor ligation was blocked using anti-FcYr 2.4G2 supernatant grown in house. Cells were acquired using a BD FACScalibur, a BD FACScan (BD Biosciences), or a CyAn-ADP (Beckman Coulter) and analyzed using FlowJo (TreeStar) software.

2.6. Cell Purification. Naïve T cells (>98% pure) were sorted from splenic cell suspensions on the basis of CD4+CD45modCD25− using a MoFlo (Beckman Coulter). Splenic dendritic cells (>90% pure) were purified by immunomagnetic selection on an autoMACS Pro (Miltenyi Biotec). Briefly, spleens were digested using Liberase CI (Roche) and passed through a 70 μm strainer to obtain a single-cell suspension. Nonspecific binding was blocked using 2.4G2 anti-FcYr supernatant, and DCs were positively selected using pan-DC microbeads (Miltenyi Biotec). For T cell polarization, immature DCs were differentiated from bone marrow precursors using GM-CSF as previously described [5]. Peritoneal exudate cells, PECs (~50% CD11bhi Mφs), were flushed from the peritoneal cavity by lavage with cold PBS. Pancreatic and hepatic leukocytes populations were isolated from collagenase digested samples by centrifugation through a 33% Percoll (GE Healthcare) gradient as previously described (EJI).

2.7. RNA Isolation and Real-Time RT-PCR. Total RNA was extracted using an RNeasy Mini kit, converted to cDNA using a Reverse Transcription kit, and quantified in real time using SYBR green fast PCR (all Qiagen). cDNA was analyzed in duplicate reactions with amplification of target gene and housekeeping gene (hypoxanthine phosphoribosyl transferase 1, Hprt1) transcripts performed on the same reaction plate on a 7500 fast real-time PCR system (Applied Biosystems). Proprietary Quantitect Primer Assays for all genes were purchased from Qiagen. Data are presented after normalization to the threshold cycle (CT) for Hprt1, either as CTgen1 − CTHprt1 or as Hprt − RE = 1000 × 2(CTgen1−CTHprt1).

2.8. Statistics. Statistical analyses were performed using GraphPad Prism 4 software. Tests performed and calculated two-tailed P values are indicated in the individual figure legends. For nonparametric datasets, the Mann Whitney unpaired t test was employed.

3. Results

3.1. SEA Induces Functional and Phenotypic Changes on Dendritic Cells, Creating the Ideal Conditions for Th2 and Treg Cell Expansion. SEA triggers important changes in dendritic cell (DC) function, which are key in initiating modulation of the host immune system [14–19]. In the absence of additional TLR stimuli, we have shown that small consistent phenotypic changes occur when DCs are stimulated in vitro
with SEA [6]. This is particularly seen with C-type lectin receptors (CLRs). These receptors such as DEC-205, SIGN-R1, MR, and galectins 1 and 3, expressed on the surface of APCs, have been shown to recognize glycans present in schistosome antigens and are important for adaptive responses induced by the parasite [20–25]. Here we confirm that primary splenic DCs stimulated in vitro with SEA also upregulate the surface expression of galectins 1 and 3, and SIGN-R1, thus possibly increasing their ability to bind schistosome self-glycans (Figure 1(a)). Using bone marrow generated DCs, we have shown that the IL-10/IL-12 axis can be tilted by SEA [5, 6]. In Figures 1(b) and 1(c) we show that purified spleen CD11c+ DCs stimulated in vitro with SEA in the presence of TLR ligands upregulate the secretion of bioactive TGFβ whereas IL-12 (p40 and p35) mRNA expression is downmodulated.

Interestingly, SEA in association of Pam3CysK4 (a TLR1/2 ligand) induces the secretion of bioactive TGFβ, but this effect is not seen in combination with LPS (TLR4) or FSL-1 (TLR2/6, Figure 1(b)). No significant changes were found in IL-10 mRNA expression from purified splenic DCs (Figure 1(c)).

3.2. SEA Alternatively Activates MΦs, which May Favor Treg and Th2 Cell Expansion In Vivo. We examined the effect of i.p. injected SEA on cellular phenotypes and function in the peritoneal cavity of NOD female mice. Examination of cytokine expression at the RNA level revealed that, while IL-4 mRNA was not detected in the control peritoneal cells (PECs), there was a substantial expression in PECs from SEA-treated mice, together with increased expression of IL-2, IL-6, IL-10, and TGFβ (Figure 2(a)). Interestingly, IL-12p35
Figure 2: Schistosome antigens alternatively activate MΦs in vivo. (a) Increased expression of anti-inflammatory cytokines and mediators in the peritoneal cavity following SEA treatment of NOD mice are illustrated. (b) Induction of arginase and Fizz-1, markers of MΦ alternative activation in PECs is given. (c) Alternative activation in response to S. mansoni antigens shows a distinct pattern from that of the classical activation seen in response to LPS. Six-week-old female NOD mice were injected i.p. with 50 μg SEA or 10 μg LPS on day 0 and day 7, and PECs were collected for FACS and RT-qPCR analysis on day 10. Data shown are the responses of six mice per group for (a) and (b) or one mouse per condition for (c). Statistical analysis by Mann Whitney U test, two-tailed.
expression was not affected by SEA while IL-12p40 was upregulated (Figure 2(a)). Expression of other subunits of the IL-12 family (EBI3, p28 for IL-27 and p19 for IL-23) was not affected by SEA treatment (data not shown). The significant upregulation of TGFβ suggests the presence of alternatively activated macrophages (aaMΦs) in PECs from SEA-treated mice and the increased expression of integrin β8 (Figure 2(a)) indicates an increased capacity to activate TGFβ at the point of the immunological synapse [26]. Further support for the presence of aaMΦs is the upregulated expression of arginase-1 and 2 as well as Fizz-1 (Figure 2(b)). By comparison, treatment of NOD mice with LPS increased iNOS expression but, unlike with SEA, the full range of markers of alternative activation was not seen (Figure 2(c)). Analysis of surface marker expression on PECs identified potentially relevant costimulatory molecules that might indicate the presence of aaMΦs as well as CLRs involved in the binding of SEA. After SEA injection there is an increased percentage of cells expressing MR on peritoneal MΦs (pMΦs) with no significant change in mean fluorescent intensity (MFI) (Figure 3(a)). This increased surface expression was mirrored by an augmented receptor mRNA
3.3. SEA Induces IL-4, IL-10, and IFN-γ Tregs and Th2 cells.

An anti-inflammatory signature might be tied to expansion of Tregs and Th2 cells. We have previously shown that Tregs have an important role to play in diabetes prevention [6]. Figure 5(a) shows that following a short in vivo treatment with SEA, Foxp3+ Tregs increase only in SEA, but not SWA-treated mice. Further analysis of the liver of SEA-treated NOD mice revealed a highly significant increase in Foxp3+ Tregs, which is consistent with the fact that Tregs are found in the egg-induced granulomas in the liver and regulate their formation (Figure 6(b)) [31, 32].

These in vivo studies, therefore, support our in vitro studies and emphasize differences between the modes of activity of SEA and SWA.

4. Discussion

There has been considerable interest in defining the ways in which S. mansoni infection perturbs the immune system and induces a Th2 response. As we have previously shown that S. mansoni antigens are able to inhibit the Th1-mediated autoimmune disease Type 1 diabetes, we were particularly interested in clarifying this in NOD mice.

We focused our attention on DCs and MΦs as these cell types play a role in orchestrating the adaptive immune response. Much work has been carried out examining the effects of SEA on in vitro generated DCs, and while this has produced valuable insights, we felt that it is important to analyze the effects on populations of cells which would be exposed to S. mansoni antigens in vivo.

In our studies of splenic DCs we showed that following exposure to SEA in vitro these cells upregulated galectins 1 and 3, SIGN-R1, and DEC-205 following exposure to SEA in vitro. This is interesting because these C-type lectin receptors (CLR) have been shown to recognize glycans present in schistosome antigens and are important for adaptive responses induced by the parasite. Galectins are neutralizing antibody. This effect is particularly evident in the pancreas and not the spleen or PLN (Figure 5(a) and data not shown).

We have also confirmed our findings with an in vitro polarization assay. Using bone marrow generated DCs and naive T cells from NOD mice, we have previously shown that SEA increases Foxp3+ Tregs in vitro and that TGFβ neutralization reverts this effect [6]. Figure 5(b) shows that blocking TGFβ in vitro reduces the generation of IL-10 and IL-4 single- and double-producing CD4+ T cells. Also in vitro we observe that SEA can induce the generation of IL-10/IFNγ double-expressing CD4+ T cells and that TGFβ regulates this population. These preliminary data suggest that the role of TGFβ in adaptive responses to schistosome antigens is quite complex and is not restricted to the generation/expansion of Foxp3+ Tregs.

3.5. Egg But Not Worm S. mansoni Soluble Antigens Induce Foxp3+ Tregs In Vivo and In Vitro. We have previously suggested that Foxp3+ Tregs have an important role to play in SEA immunomodulation and prevention of autoimmunity in the NOD mouse [6]. We found that in vitro only SEA, and not SWA, was able to induce Foxp3 in naive T cells from NOD mice. To examine the effects of these helminth antigens on Tregs in vivo, we injected SEA and SWA into NOD mice.

Figure 6(a) shows that following a short in vivo treatment with S. mansoni antigens Foxp3+ Tregs increase only in SEA, but not SWA-treated mice. Further analysis of the liver of SEA-treated NOD mice revealed a highly significant increase in Foxp3+ Tregs, which is consistent with the fact that Tregs are found in the egg-induced granulomas in the liver and regulate their formation (Figure 6(b)) [31, 32].

These in vivo studies, therefore, support our in vitro studies and emphasize differences between the modes of activity of SEA and SWA.

3.4. TGFβ Is Also Important for SEA Th2-Induced Response In Vivo and In Vitro. We have previously demonstrated that TGFβ is important for the generation and expansion of Foxp3+ Tregs in NOD mice [6]. Our preliminary experiments suggest that the Th2 response induced by SEA can also be regulated by TGFβ. Figure 5(a) shows that the increased percentage of IL-4/IL-13 double-producing CD4+ T cells that we observe in SEA-treated NOD mice can be decreased by in vivo coadministration of SEA together with a TGFβ neutralizing antibody. This effect is particularly evident in the pancreas and not the spleen or PLN (Figure 5(a) and data not shown).

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These in vivo studies, therefore, support our in vitro studies and emphasize differences between the modes of activity of SEA and SWA.
**Figure 4:** The systemic Th2 response to SEA is accompanied by local induction of IL-10 in the pancreas and liver. Increased proportions of CD4+ T cells expressing IL-4 were seen in the spleen (a), the pancreas (b), and the liver (c). In the pancreas and liver, SEA treatment triggered IL-10 production from Th1 cells. (d) Increased mRNA for Th2 and regulatory cytokines in the pancreas of mice treated with schistosome antigens are shown. Six-week-old female NOD mice were injected i.p. with 50 μg SEA on day 0 and day 7, and lymphocytes from spleen, pancreas, and liver were collected for FACS and RT-qPCR analysis on day 10. Data shown are the responses of 5-6 mice per group for (a)–(c) or a pool of three mice per condition for (d). Statistical analysis by Mann Whitney U test, two-tailed.

...of particular interest as they have the potential to alter sensitivity to cytokines and apoptosis [20, 33]. Exposure to SEA upregulates, therefore, the capacity of the DC to respond to further exposure to helminth glycans.

Some differences were observed in the response of DCs and MΦs to SEA. This was seen in the effects of SEA on IL-12. In vitro, we found that splenic DCs downmodulated IL-12p35 and p40 in response to SEA whereas the in vivo response of pMΦs showed an upregulation of the IL-12p40 response with no effect on IL-12p35 (Figure 2(a)). This presents another interesting way in which the Th1 response could be modulated as it has been suggested that excess...
Figure 5: TGFβ modulates the Th2 response to SEA in vitro and in vivo. (a) Treatment of NOD mice with anti-TGFβ reduces the Th2 response to SEA in vivo. Female NOD mice (10-11 weeks of age) were injected i.p. with 50 μg SEA on days 0 and 5, 5 mg anti-TGFβ on days 0, 2, and 5, and cells were taken for analysis on day 10. Data shown are from a single experiment using anti-TGFβ and SEA. (b) Antibody neutralization of TGFβ in vitro diminishes the Th2 response to SEA. Naïve CD4+ T cells (5 × 10^5) were cultured for five days with bmDCs (10^5) and 0.5 μg/ml αCD3 in the presence of SEA (10 μg/ml) and/or anti-TGFβ (20 μg/ml). FACS plots from one of three independent polarization experiments are shown.
Untreated

SEA

SWA

Figure 6: Egg, but not worm, antigens drive Foxp3+ Treg responses. SEA treatment increases the representation of Foxp3+ Tregs in the pancreas (a) and liver (b) of NOD mice. Female NOD mice were treated at eight weeks of age with 50 μg SEA or SWA on day 0 and day 7. Pancreatic infiltrating cells were isolated and stained for intracellular Foxp3 expression on day 10. Statistical analysis by Mann Whitney U test, two-tailed.

Figure 7: The cellular immune response to S. mansoni egg antigens in NOD mice: alternative activation of MΦs (aaMΦs), maintenance of dendritic cells (DCs) in an immature phenotype, IL-4 release from basophils (BΦs), expansion of classical natural killer T cells (NKT), expansion of T helper 2 (Th2) responses, expansion and activation of T regulatory cells (Tregs), as well as induction of IL-10 production in Th1 cells (Th1) and B cells (B).

IL-12p40 in both a monomeric (p40) and dimeric (p80) forms might antagonize the IL-12 receptor [34], providing a feedback mechanism to downmodulate Th1 responses. With regard to effects of SEA administration in vivo on APC phenotype and function, several were noted that again had the potential to modify a diabetogenic Th1 response. SEA increased PD-L1 expression on pMΦs, with an accompanying increase in message for PD-L1 and 2 in whole PECs (Figure 3(c)). The negative costimulatory molecule programmed death ligand 1 (PD-L1) has been shown to be upregulated on aaMΦs [28], as well as playing a role in diabetes regulation in NOD mice [35]. Effects of i.p. injected SEA were noted in several sites, some of which were particularly relevant to diabetes regulation. After a short course treatment of NOD mice with SEA, the intracellular cytokine expression in CD4+ T cells was profoundly changed in the spleen, pancreas, and liver of prediabetic NOD mice (Figures 4(a), 4(b), and 4(c)). The systemic Th2/Treg response induced by SEA was accompanied by the signature of MΦ alternative activation in the pancreatic infiltrate of prediabetic NOD mice and would be beneficial for diabetes prevention (Figures 4(b) and 4(d)). Together with the expected Th2 [5] and IL-10 Tr1 (CD4+ IL-10+) response, we have identified a population of CD4+ T cells expressing both IL-10 and IFNγ in response to SEA (Figures 4(b) and 4(c)). IL-10/IFNγ double-producing CD4+ T cells were first
identified in murine model of *Leishmania* infection and shown to mediate chronic pathology in the skin lesions with delayed/reduced clearance of infection [36, 37]. IL-10-producing Th1 cells have been proposed as a self-regulating class of Th1 cells, and therefore, this CD4+ T cell population in the pancreas might also mitigate Th1 driven diabetes pathology.

We have previously demonstrated that SEA has direct effects on DCs and T cells and suggest that the simultaneous interaction with both cell types is important for determining the host immune response. These in vitro studies had highlighted a role for TGFβ in the SEA-mediated induction of regulatory T cells; here we provide data suggesting that this immunomodulatory cytokine may also play a role in modulating the Th2 response in vivo as well as in vitro.

5. Conclusion

*S. mansoni* and its soluble antigens can prevent autoimmune diabetes in NOD mice. This paper summarizes and confirms our previous work demonstrating that SEA modulates the immune response through alteration of CLR expression, cytokine production, and Treg expansion in NOD mice. In particular, we show how functional and phenotypic changes on DCs and MΦs, the expansion of different T helper cell subsets (Th2), and Treg cell types (IL-10/IFN-γ double producers and Foxp3+ Tregs) might contribute to diabetes prevention in NOD mice (Figure 7).

**Abbreviations**

SEA: *S. mansoni* soluble egg antigens  
NOD: Nonobese diabetic  
aa: Alternatively activated  
MΦ: Macrophage  
DC: Dendritic cell  
APC: Antigen-presenting cell  
Treg: Regulatory T cell  
Th: T helper  
CLR: C-type lectin receptor  
TLR: Toll-like receptor  
MR: Mannose receptor  
RT-PCR: Reverse transcriptase polymerase chain reaction  
Hprt-RE: Expression relative to hypoxanthine phosphoribosyltransferase*1000*  
PLN: Pancreatic lymph node  
BΦ: Basophil.

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