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CD4^+CD25^+ Regulatory Cells Contribute to the Regulation of Colonic Th2 Granulomatous Pathology Caused by Schistosome Infection

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Abstract

Eggs of the helminth Schistosoma mansoni accumulate in the colon following infection and generate Th2-biased inflammatory granulomas which become down-modulated in size as the infection proceeds to chronicity. However, although CD4^+CD25^+Foxp3^+regulatory T cells (Tregs) are known to suppress Th1-mediated colitis, it is not clear whether they control Th2-associated pathologies of the large intestine which characterise several helminth infections. Here we used a novel 3D-multiphoton confocal microscopy approach to visualise and quantify changes in the size and composition of colonic granulomas at the acute and chronic phases of S. mansoni infection. We observed decreased granuloma size, as well as reductions in the abundance of DsRed^+ T cells and collagen deposition at 14 weeks (chronic) compared to 8 weeks (acute) post-infection. Th2 cytokine production (i.e. IL-4, IL-5) in the colonic tissue and draining mesenteric lymph node (mLN) decreased during the chronic phase of infection, whilst levels of TGF-β1 increased, coincident with reduced mLN proliferative responses, granuloma size and fibrosis. The proportion of CD4^+CD25^+Foxp3^+Tregs: CD4^+ cells in the mLN increased during chronic disease, while within colonic granulomas there was an approximate 4-fold increase. The proportion of CD4^+CD25^+Foxp3^+Tregs: in the mLN that were CD103^+ and CCR5^+ also increased indicating an enhanced potential to home to intestinal sites. CD4^+CD25^+ cells suppressed antigen-specific Th2 mLN cell proliferation in vitro, while their removal during chronic disease resulted in significantly larger granulomas, partial reversal of Th2 hypo-responsiveness and an increase in the number of eosinophils in colonic granulomas. Finally, transfer of schistosome infection-expanded CD4^+CD25^+Tregs down-modulated the development of colonic granulomas, including collagen deposition. Therefore, CD4^+CD25^+Foxp3^+Tregs appear to control Th2 colonic granulomas during chronic infection, and are likely to play a role in containing pathology during intestinal schistosomiasis.

Introduction

Schistosomiasis is an important parasitic helminth disease afflicting more than 200 million people, causing approximately 280 thousand deaths annually, with a further estimated 700 million at risk of infection [1,2]. In the case of Schistosoma mansoni, infections are typically chronic (>10 years) and the majority (>90%) give rise to an intestinal form of disease [3] caused by the deposition of parasitic eggs in the intestinal mesenteries (mainly of the colon and terminal ileum) and the subsequent development of Th2-associated granulomatous infiltrates rich in macrophages and eosinophils [4]. Such infections lead to diarrhoea, pseudopolyposis, microulceration, bleeding and fibrosis [5]. Recent reappraisal of Disability-Associated Life Years (DALYs) attributable to schistosomiasis, where more subtle disease manifestations such as intestinal schistosomiasis have been included, raises the disease burden caused by this infection as much as 40-fold, putting schistosomiasis on a par with malaria as a global public health problem [6]. Variation in granuloma size in the colon between patients is positively associated with peripheral blood mononuclear cell (PBMC) reactivity to soluble egg antigens (SEA) [7]. Thus, changes in lymphocyte responsiveness appear to be related to the size of granulomas in the intestine and by implication, the severity of pathologies in patients with intestinal disease.

In order to investigate the phenomenon of Th2-associated colonic inflammation and possible mechanisms underlying its regulation, we utilized a murine model of infection with S. mansoni which provides a well accepted permissive experimental host. In the murine model, myeloid antigen presenting cells, including dendritic cells [8,9], and basophils [10], are primed to induce potent anti-egg Th2 CD4^+ lymphocyte responses. Th2 activation appears necessary to protect the host from lethal hepatic and intestinal damage during acute infection [11] and to keep Th1 inflammatory immunopathology in check [12]. However, survival to the chronic stage of infection, representative of human disease, is dependent on modulation of the Th2 granulomatous response in order to subvert IL-4/IL-13-driven morbidity [13]. ‘Naturally occurring’ [i]Tregs bearing the IL-2 receptor α chain molecule
Author Summary

Schistosomiasis is an important parasitic helminth disease afflicting more than 200 million people worldwide. Infections are typically chronic and in the case of Schistosoma mansoni and S. japonicum the majority give rise to an intestinal form of disease caused by the deposition of parasite eggs in the colon and terminal ileum. The eggs cause Th2-associated inflammatory immune granulomas to form, which as the disease develops, are down-regulated by cells of the immune system. However, the mechanisms which underpin the down-regulation of granulomas in the large intestine are not known. In order to investigate the phenomenon of Th2-associated colonic inflammation, we utilized a murine model of infection with S. mansoni and compared immune responses at the acute and chronic phases of infection. We show that a type of regulatory T helper lymphocyte (CD4+CD25+FoxP3+) contributes to regulation of colonic inflammation. These cells modulate anti-egg Th2 responses within the mesenteric lymph nodes and granulomatous pro-fibrotic Th2 responses within the colon. Our study highlights the importance of CD4+CD25+FoxP3+ as a source of regulatory pressure on granuloma formation in the colon and by implication humans with chronic intestinal schistosomiasis.

Materials and Methods

Ethics statement

All experiments were carried out in accordance with UK Animal’s Scientific Procedures Act 1986 and with the approval of The University of York Ethics Committee.

Experimental infection and parasitological readout

C57BL/6 (B6) and hCD2-VA-DsRed-B6 mice were maintained within the University of York under specific pathogen-free conditions. hCD2-VA-DSRed-B6 mice, were a gift of D. Kioussis and A. Patel (National Institute for Medical Research, London) and express fluorescent DSRed T cells (>90% CD3+) to facilitate in situ detection of T cells by multiphoton microscopy (see below). Eight to ten-week female mice were infected percutaneously via the lateral tail vein. Total mLNs cells (2×10⁶/ml), sorted CD4+CD25+FoxP3+ (90% purity) were injected intraperitoneally to infected mice at 9, 11, and 13 weeks. Treg cell purification, adoptive transfer and in vitro culture

Treg, from the mLNs were purified by depletion of non-CD4+ cells followed by isolation of CD25+ cells using antibodies conjugated to magnetic beads (Miltenyi Biotec). For adoptive transfer, 2.5×10⁶ CD4+CD25+FoxP3+ (>90% purity) were injected via the lateral tail vein. Total mLNs cells (2×10⁶/ml), sorted CD4+CD25+FoxP3+ (90% purity) were injected into mice cultured in complete RPMI-1640 medium (containing 10% FCS, 50 μg/ml penicillin/streptomycin), in combination with naïve mLNs CD4+CD25- cells (0.1×10⁶/ml) as a source of APC. Cells were stimulated with plate-bound anti-CD3 mAb (1 μg; Becton Dickinson), or SEA (50 μg/ml) [16]. Cells were cultured for 72 h and supernatants retained for cytokine analysis. Proliferation was measured from 72 to 96 h by ³H-thymidine incorporation and scintillation counting.

Cytokine and collagen quantifications

ELISAs were used to quantify IL-4, IL-5 and IFNγ [17], while IL-10 and IL-13 were measured by Cytoset (Invitrogen) or DuoSet (R&D Systems) kits respectively. A TGFB-sensitive, mink lung epithelial cell bio-assay (MLEC transfectected with firefly lucerase; gift from Daniel Rikin, NY Medical Center) was used to determine levels of bio-active TGFB1 [18]. As the bio-assay was not compatible with tissue extracts, a TGFB1 ELISA (R&D Systems) was employed. In order to determine cytokine levels in the colon, frozen tissues were first homogenised in proprietary
Quantitative Real Time PCR

Total colonic mRNA was used to generate cDNA using Superscript III DNA polymerase (Invitrogen) and foxp3 transcript analysed by qRT-PCR (ABI PRISM 7000; Applied Biosystems) using Taqman probes (Sigma-Aldrich). The relative expression of foxp3 was normalised to values obtained for ε2. Primer pairs and probes were: foxp3 5'-GCAAGTGTCCTGACATGTA, 5'-CGACGCCCTCAGTCTCATGGT, Probe 5'-CAACAGTGGACCGTAGATGA, 5'-GCAGTGTGGACCGTAGATGA, 5'-ATGTCCCAGCACTGGCTACT, Probe 5'-ACAAGTGCTC-9TCTTCAGCCTCCTGGTGAACAC. The expression of each gene was normalised to expression of the internal control gene β2microglobulin.

Flow Cytometry

Cells were blocked with anti-CD16/CD32 (eBioscience) at 0.5 μg / 1×10⁶ cells, then labelled with anti-CD4-Pacific Blue, anti-CD25-APC (PC-61), anti-CD103-PE (all eBioscience), anti-CD25-FITC (7D4), anti-CTLA-4-FITC, or anti-CCR5-biotin (BD Bioscience) for 30 minutes. Biotinylated antibodies were sequentially detected with streptavidin-PE-Cy7 (eBioscience). For intracellular staining of foxp3, cells were fixed in 1% formalin, re-suspended in permeabilisation buffer (Becton Dickinson) prior to labelling with anti-FoxP3-PE or -AF647 (eBioscience). Cells were analysed using a Cyan flow cytometer with Summit software (Beckman Coulter).

Statistical analyses

Significant differences between two experimental groups were determined by unpaired Student’s T test, and between three or more groups by 1-way ANOVA with Tukey post-hoc tests using Prism software (GraphPad). Because colonic foxp3 counts were skewed, analysis was undertaken after Log10 transformation. All data are representative of a minimum of two independent experiments. Significance is indicated ***P<0.001, **P<0.01, *P<0.05. Significance values are shown on the figures with line connectors between the appropriate groups. Where statistical significance was not achieved (P>0.05), figures are intentionally left blank.

Results

Colonic granuloma size and anti-egg Th2 responses decline with chronicity of infection

Egg deposition and anti-egg granulomatous responses in proximal colons were examined over a time-course of infection in B6 mice. The numbers of eggs increased during infection from 292±212.8 (day 42) to 2339±863.5 (day 98) (Fig. 1A). Mean areas of isolated colonic granulomas declined from the acute to chronic time point visualised by H&E staining (Fig. 1B & C), supporting previous observations [16,19]. Estimates of volumes of granulomas isolated from enzymatically digested colons (Fig. 1D) corroborated histological observation and showed a significant decrease in size between the acute and chronic stage of infection. In addition, the decrease in granuloma size at the chronic stage was accompanied by a decrease in collagen, indicative of fibrosis, as shown by Van-Geison stained sections (Fig. 1E).

By multiphoton imaging of proximal colon derived from infected hCD2-VaDsRed-B.6 mice revealed further quantitative information on temporal granuloma modulation in situ (Fig. 1F, Videos S1 & S2) and analysis of 3D images showed granuloma volumes were significantly decreased at the chronic stage (Fig. 1G). Furthermore, numbers of granuloma-associated DsRed⁺ lymphocytes (Fig. 1H; >90% CD3⁺T lymphocytes, data not shown), and granuloma-associated type-I collagen deposition, revealed as second harmonic imaging (blue), was significantly also reduced (Fig. 1H). Although a significant increase in the recently synthesised (salt-soluble) collagen pool within the colon was apparent by the chronic phase, modulation of the egg-driven fibrotic response was demonstrable when adjusted for the increased numbers of eggs (as a surrogate for numbers of granulomas) in chronic infected colons (Fig. 1J).

At the acute stage, anti-CD3 mAb and SEA-specific proliferation of mLN cells were significantly elevated and biased towards secreting Th2-type cytokines (Fig. 2A). However, by the chronic stage, SEA-induced cell proliferation and production of IL-4, IL-5 and IL-13 were significantly reduced (Fig. 2B). While the secretion of IL-10 in response to SEA was significantly lower during chronic compared to acute infection the production of bio-active TGFβ1 to SEA at the chronic stage was significantly elevated compared to naive stage (Fig. 2B).

It was not possible to obtain sufficient numbers of viable lymphocytes via enzymatic digestion of granulomatous colons due to the fibrotic nature of these intestinal granulomas but levels of IL-4 and IL-5 in whole colonic extracts were elevated at the acute phase (Fig. 2C), suggesting that tissue inflammatory responses in infected colons mirrored the Th2 response in the mLN. Surprisingly, levels of IL-10 significantly decreased in infected colonic tissue (Fig. 2C) but levels of bio-active colonic TGFβ1 were elevated during chronic disease. When adjusted for numbers of deposited eggs, production of colonic IL-4 and IL-5 was significantly diminished at the chronic phase (Fig. 2D). Thus, whilst local cytokine responses to egg deposition has both shared and distinct facets to those of the mLN, measurements indicate that colonic Th2 responses establish during the onset of egg deposition and subsequently diminish as chronicity proceeds.

Increased numbers of CD4⁺FoxP3⁺Treg cells occur in the mLN and colonic granulomas as chronicity proceeds

The proportion of CD4⁺ FoxP3⁺Treg in the mLN as a proportion of total CD4⁺ cells, as determined by flow cytometry of cell suspensions, increased from 13.1±2.0% in naive mice to 16.0±0.4% during acute infection (P<0.001), and increased further to 20.6±0.2% during chronic infection (P<0.001; Fig. 3A). Absolute numbers of both mLN CD4⁺ effector and CD4⁺FoxP3⁺Treg cells increased during acute disease from naive levels, and remained significantly elevated during chronic infection (Fig. 3A). The increase was confirmed by enumeration of FoxP3 Treg in stained sections of mLN from naive mice and those with acute and chronic infection (Fig. 3B & C). In contrast, absolute numbers or proportions of CD4⁺FoxP3⁺Treg in the spleens did not expand (12.8±0.6% cf. 13.9±0.8% cf. 14.8±2.1%, Fig. 3A).

A pronounced increase in the proportion of CD4⁺FoxP3⁺Treg within colonic granulomas at the chronic phase of infection, from 2.9±0.6% to 18.8±0.7% was revealed by enumeration of double positive versus single positive cells in anti-CD4 / anti-FoxP3 immunostained cryosections of colonic tissue (Fig. 3D & E). This profound (>10 fold) proportional elevation in FoxP3⁺ cells compared with total number of T lymphocytes in gut tissue was corroborated by qRT-PCR of FoxP3 transcript normalised to CD3 transcript (Fig. 3F). Thus, during enteric S. mansoni infection, relative and absolute expansion in the numbers of CD4⁺FoxP3⁺ cells occurs preferentially within gut-associated lymphoid tissue (GALT). Furthermore, relative increases of CD4⁺FoxP3⁺ cells within colonic granulomas are apparent during chronic disease.

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CD103, the αE molecule of the αEβ7 mucosal integrin involved in homing of T cells to intestinal sites [20], increased on mLN CD4+FoxP3+Tregs at the chronic stage compared to naïve mice (31.0±2.3% cf. 62.4±3.1%, P<0.001, Fig. 3G). CCR5 is also involved in T cell homing to intestinal inflammatory sites [21], and significantly, the proportion of CCR5+CD4+FoxP3+Tregs in the

![Figure 1. Colonic granuloma size and fibrosis is reduced in the chronic phase of schistosome infection. A). Accumulation of eggs/gram of colon tissue (n = 4 mice/time point); mean eggs (± SEM). B) Representative photomicrographs of the colon at the acute (8 wks) or chronic (14 wks) stage of infection stained with H&E. Scale bars are 200 μm; egg denoted 'Sm'. C) Granuloma area at the acute or chronic stage; bars are mean/animal (n = 4) from three separate histological sections. D) Granuloma volumes calculated from isolated granulomas at the acute or chronic stage calculated from three separate granulomas per individual animal. E) Cross sections of colon at the acute (8 wks) or chronic (14 wks) stage of infection stained with haematoxylin/Van Geison (collagen fibres = pink). All scale bars are 200 μm. Parasite egg is denoted 'Sm'. F) 3D images of multiphoton confocal stacks of colonic tissue from infected hCD2-VaDsRed-B.6 mice sampled in situ during acute or chronic infection. DsRed fluorescent cells are 90% CD3+; blue fluorescence is second harmonic generation of type I collagen; green/yellow auto-fluorescence are schistosome eggs. Grid squares are 63.9 μm². Quantification of multiphoton confocal stacks from hCD2-VaDsRed-B.6 mice: G) granuloma half-volumes, H) DsRed+ cell counts and I) collagen half-volumes. Data calculated from four separate granulomas from individual animals (n = 4). J) Salt-soluble collagen from colonic tissues of naïve, acute, and chronic mice (n = 4). Data is mean (±SEM) collagen conc/mg of tissue (left), or adjusted for numbers of eggs/mg (right). doi:10.1371/journal.pntd.0001269.g001

![Diagram A]

![Diagram B]

![Diagram C]

![Diagram D]

![Diagram E]

![Diagram F]

![Diagram G]

![Diagram H]

![Diagram I]

![Diagram J]
mLN increased markedly (9.6 ± 2.3% vs 19.3 ± 2.6%; Fig. 3G). Thus, increases in the number of mLN CD4+FoxP3+ Tregs expressing CD103 and CCR5 suggests these cells have enhanced potential to be recruited and retained within the colonic infection site during chronic infection.

The majority of Schistosome-expanded CD4+CD25+ mLN cells are FoxP3+ and suppress antigen-specific CD4+ proliferative responses in vitro

More than 75% of CD4+CD25+ mLN cells co-expressed FoxP3, regardless of the stage of infection confirming that the majority of CD4+CD25+ cells can be classified as CD4+FoxP3+Tregs (Fig. 4A). In addition, while CD4+CD25+ effector cells from mice with an acute infection exhibited a strong proliferative response in vitro to SEA, CD4+CD25+ Tregs, taken at the chronic stage of infection did not proliferate (Fig. 4B). Moreover, following depletion of CD4+CD25+ Tregs or after their re-addition in a 1:2 Treg / effector T cell ratio, we observed that while the Treg conferred a significant degree of suppression on the anti-SEA response, chronic CD4+ CD25− effector T cells remained hyporesponsive, or anergic, compared with their acute-stage counterparts (Fig. 4D). Taken together, these in vitro assays provide evidence that schistosome-expanded CD4+CD25+ Tregs suppress the pre-dominant Th2 anti-SEA response. However, compared with Tregs from naive mice, they are not enhanced in their ability to suppress antigen-specific CD4+ proliferation.

Anti-CD25mAb treatment impairs the regulation of colonic granuloma size

Depletion of CD25+ cells is a common technique to experimentally induce Treg deficiency [14,22,23,24,25,26]. Whilst not all CD25+ cells are FoxP3+ Tregs (75%–80% in our infection...
Tregs increase at the chronic phase in both the mLN and colonic granulomas. A) Representative flow cytograms showing the frequencies of labelled CD4+ and FoxP3+ cells in suspensions of the mLN and spleen. Values in italics are quadrant percentages. Values in bold, upper right-hand quadrant, are mean (±SEM) CD4+FoxP3+ cells as a % of total CD4+ cells (relevant quadrants outlined in bold). Histograms show mean (± SEM) total CD4+ (open) and CD4+FoxP3+ (closed), mLN or spleen cell or numbers (n = 3 mice). Significant differences compared with naive cell numbers are indicated. Bar indicates significant difference between CD4+FoxP3+ mLN numbers at the chronic compared with acute stage. B) Representative images of CD4+ (green) and FoxP3+ (red) cells in labelled cryosections of mLN and, C) proportion of CD4+FoxP3+ / CD4+ cells/ field of view (2 fields of view / animal, n = 3) Scale = 14 μm. D) Representative images of CD4+ and FoxP3+ cells in colonic granulomas, with high power insert also shown. E) Frequencies of labelled CD4+FoxP3+ / CD4+ colonic granuloma cells / field of view (2 fields of view / animal, n = 3). F) qRT-PCR analysis of FoxP3 mRNA in colonic tissue plotted as arbitrary units of FoxP3 normalised to CD3 transcript in colonic tissue. Bars are mean FoxP3 A.U. transcript per group. G) Flow plots of FoxP3 and CD103, and FoxP3 and CCR5 expression, gated on CD4+ mLN cells of naive or chronic mice (n = 4). Values in italics are quadrant percentages. Values in bold, upper right-hand quadrant, are mean (±SEM) CD4+FoxP3+CCR5+ cells, or CD4+FoxP3+CCR5+ cells as a % of total CD4+FoxP3+ cells (relevant quadrants outlined in bold).

Figure 3. Frequencies of CD4+FoxP3+ Tregs increase at the chronic phase in both the mLN and colonic granulomas. A) Representative flow cytograms showing the frequencies of labelled CD4+ and FoxP3+ cells in suspensions of the mLN and spleen. Values in italics are quadrant percentages. Values in bold, upper right-hand quadrant, are mean (±SEM) CD4+FoxP3+ cells as a % of total CD4+ cells (relevant quadrants outlined in bold). Histograms show mean (± SEM) total CD4+ (open) and CD4+FoxP3+ (closed), mLN or spleen cell or numbers (n = 3 mice). Significant differences compared with naive cell numbers are indicated. Bar indicates significant difference between CD4+FoxP3+ mLN numbers at the chronic compared with acute stage. B) Representative images of CD4+ (green) and FoxP3+ (red) cells in labelled cryosections of mLN and, C) proportion of CD4+FoxP3+ / CD4+ cells/ field of view (2 fields of view / animal, n = 3) Scale = 14 μm. D) Representative images of CD4+ and FoxP3+ cells in colonic granulomas, with high power insert also shown. E) Frequencies of labelled CD4+FoxP3+ / CD4+ colonic granuloma cells / field of view (2 fields of view / animal, n = 3). F) qRT-PCR analysis of FoxP3 mRNA in colonic tissue plotted as arbitrary units of FoxP3 normalised to CD3 transcript in colonic tissue. Bars are mean FoxP3 A.U. transcript per group. G) Flow plots of FoxP3 and CD103, and FoxP3 and CCR5 expression, gated on CD4+ mLN cells of naive or chronic mice (n = 4). Values in italics are quadrant percentages. Values in bold, upper right-hand quadrant, are mean (±SEM) CD4+FoxP3+CCR5+ cells, or CD4+FoxP3+CCR5+ cells as a % of total CD4+FoxP3+ cells (relevant quadrants outlined in bold).

Figure 4. CD4+CD25+ mLN cells suppress antigen-specific CD4+ Th2 responses in vitro. A) Flow plots of mLN cell suspensions (n = 3 mice) labelled with anti-CD25 and anti-FoxP3, gated on CD4 expression. Values in italics are quadrant percentages. Values in bold, upper right-hand quadrant, are mean (±SEM) CD4+FoxP3+ cells as a % of total CD4+ cells (relevant quadrants outlined in bold). B) Antigen-specific proliferation of CD4+CD25+ effector cells from acute mice and CD4+CD25+ Tregs from chronic mice, or co-cultured together in a 2:1 ratio. C) Co-culture of CD4+CD25+ effector cells derived from naive mice with CD4+CD25+ Tregs derived from naive, acute or chronic mice. D) CD4+CD25+ effector cells from mice with an acute or chronic infection, either depleted of CD4+CD25+ cells, or with CD4+CD25+ cell ‘add-back’ co-cultures from the same stage of infection at a 2:1 ratio. All histograms are mean (± SEM) cpm 3H-thymidine incorporation.

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the number of eggs in tissues or excreted from CD25+ cell recipients were not significantly different compared with controls (Table S1). This showed that immune cell transfers did not significantly affect adult worm development, fecundity, or egg transmission. Recipients of CD4+CD25+ cells had significantly smaller granuloma area and collagen deposits but Ds-Red+T cell numbers within the colonic granulomas were not significantly altered (Fig. 6B &C; Videos S3 & S4). Recipients also had decreased levels of recently synthesised collagen and IL-4 in colonic extracts (Fig.6D) although simultaneous down-regulation of Th2 mLN responses were not observable in recipient mice (data not shown). Lower numbers of transferred cells (1x10^6) did not significantly alter acute-stage enteric granuloma formation (data not shown). Thus, CD4+CD25+ Tregs cells, which expand within the mLN of chronically-infected mice, exert a suppressive effect on the development of acute-phase Th2 inflammation.

**Discussion**

Our data provides both in vitro and in vivo evidence that intestinal-associated CD4+CD25+FoxP3+Tregs expand during chronic, schistosome-induced colitic inflammation. They mediate significant levels of antigen-specific Th2 suppression in vivo including reduced IL-4 production, eosinophil recruitment, collagen production, and an overall reduction in the size of egg-induced granulomas in the large intestine. Thus, our data demonstrates experimentally, that CD4+CD25+Tregs are capable of modulating Th2 inflammation and fibrosis associated with intestinal disorders.

Whilst an expansion of gut-associated CD4+FoxP3+Tregs was observed following schistosome infection, this could be a product of proliferating, naturally occurring (n)Tregs in response to auto-antigens (e.g. arising from disrupted intestinal barrier), or comprise an induced population of FoxP3+Tregs recognising schistosome antigens. However, in a syngenic adoptive transfer model, numbers of nTregs did not expand relative to CD4+ effector cells within the mLN [24], suggesting that nTreg expansion does not fully account for the heightened ratio of FoxP3+CD4+:CD4+ cells during chronic infection. The development of schistosome infection-induced Tregs is likely to be favoured by the constitutive production of intestinal signals such as TGFβ and retinoic acid [29,30]. Intriguingly, SEA induces TGFβ1 secretion [31] and is critical for the development of auto-immune suppressing FoxP3+ Tregs following in vivo SEA injection [32]. Since we observed
heightened bioactive TGFβ1 from mLN cells and within the colon at the chronic phase of infection, we speculate that TGFβ1 might have a role in the induction of gut-homing CD4⁺FoxP3⁺ Tregs from naïve precursors. Both proportions and absolute numbers of CD4⁺FoxP3⁺ Tregs significantly increased within the mLN during acute infection, when anti-egg Th2 responses are at their peak. Although inhibition of Treg induction by Th2 differentiation programs has been reported [33], our data would suggest that Th2 differentiation is insufficient to block outgrowth of a regulatory T cell phenotype during acute intestinal schistosomiasis.

CD4⁺FoxP3⁺ Tregs within the mLN of mice with a chronic infection expressed elevated levels of CD103 and CCR5, both of which are associated with homing to mucosal tissues during inflammation [20,21]. However, infection status did not alter the in vitro suppressive ability on a per-cell basis of CD4⁺FoxP3⁺ Tregs from the mLN, indicating that infection-expanded CD4⁺FoxP3⁺ Tregs and those from naïve mice share a common mechanism of Th2-effector cell suppression. Therefore, it is likely that the increased regulatory activity of CD4⁺FoxP3⁺ Tregs in vivo during chronic infection is a product of either increased numbers trafficking to the site of egg deposition, or greater survival/retention in colonic granulomas. As recipients of schistosome-expanded CD4⁺CD25⁺ Tregs displayed selective suppression of Th2 activity at the enteric infection site versus the mLN, this may reflect a biased homing of Tregs in the colon that provides increased regulatory pressure on the local granulomatous response (i.e., collagen synthesis and eosinophil recruitment). Taken with the observation that ablation of CD4⁺CD25⁺ cells in vivo significantly restores the anti-egg IL-4 and proliferative mLN cell response, these data suggest that intestinal-associated, schistosome infection-expanded FoxP3⁺ Tregs exert layers of Th2 suppression both within gut-draining lymph nodes and within the colonic infection site.

Our data are consistent with recent findings that CD4⁺FoxP3⁺ Tregs constitute a partial component of the modulation of granuloma development in the liver [14,24,34]. We show that depletion of CD4⁺CD25⁺ cells in vivo does not fully reverse the Th2 hypo-responsiveness, nor do chronic enteric granulomas in treated mice fully recover the florid cellularity of the acute phase. The use of transgenic mice deficient for foxp3 [35] could help

Figure 6. Transfer of schistosome-expanded CD4⁺CD25⁺ Tregs modulates the development of acute-stage granulomas. A) Isolated mLN CD4⁺CD25⁺ Tregs from mice with chronic infection used for transfer. B) 3D images of multiphoton confocal stacks of colonic tissue viewed in situ at the acute stage of infection in hCD2-VaDsRed-B.6 control mice, or recipients of infection-expanded CD4⁺CD25⁺ Tregs (2.5x10⁶). Grid squares are 63.9 μm². C) Granuloma area (left), DsRed⁺ cell counts (middle), and collagen half-volume (right) taken from confocal stacks (as above). Data are from 5–6 separate granulomas per mouse. Bars are mean / group (n = 3). D) Soluble collagen and cytokine in colonic extracts from infected control mice, or recipients of CD4⁺CD25⁺ cells. Bars are means / group (n = 3). Data is mean pg/ml (± SEM) per group.
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further investigation of this phenomenon. The development of other regulatory cells such as alternatively-activated macrophages [36], IL-10-secreting non-FoxP3 cells [14], and regulatory B cells [37], may explain the partial role of CD4+FoxP3+ Treg in our system. T cell intrinsic anergy, for instance mediated by GRAIL, signalling [38], could also account for the remaining hypo-responsiveness after depletion of CD4+ CD25+ cells. However, because the micro-environment of the intestines is favourable towards the expansion of CD4+FoxP3+Treg, it is possible that CD4+FoxP3+Treg-mediated suppression of enteric granulomas is more apparent compared with their hepatic counterparts. This could explain why colonic granulomas modulate more rapidly versus hepatic granulomas after the acute stage, and why hepatic granulomas retain a greater size and cellularity than enteric granulomas during chronic disease [19].

During experimental helminth infections, CD4+CD25+Treg-mediated suppression of Th2 effector responses confer a permissive state by stifling effective Th2-mediated worm attrition [23,25,26]. In the case of gut-helminths, this potentially operates permissive state by stifling effective Th2-mediated worm attrition [23,25,26]. In the case of gut-helminths, this potentially operates by regulating Th2 cytokine signalling on smooth muscle contractility and epithelial cell turnover [39,40]. Paradoxically, during S. mansoni infections, while Th2 granulomatous responses protect and aid survival of the host [11,41], intact Th2 responses are also essential to propagate the parasite’s life cycle, as egg transmission from the gut is impained in the absence of T cells [42,43,44], IL-4 [41], or IL-4Rz signalling [45]. Nevertheless, transfer of CD4+CD25+ cells to acute stage recipients does not impinge on egg transmission in spite of a modulatory effect on IL-4 in the colon. In fact, during the chronic stage of infection, where Th2 inflammation in the colon becomes modulated, egg excretion rates are unaffected (our unpublished observations). How intestinal schistosome parasites mediate sustained, chronic egg transmission in the face of marked Th2 hypo-responsiveness remains to be identified.

Enteric helminth infections, or products derived from helminths, are gaining prominence as potential therapies to reverse the effects of inflammatory bowel disease [46]. Some ameliorating capacities may be attributed to antagonism of Th1 processes by induction of IL-4-secreting cells within intestinal tissues rather than by induction of Treg. Indeed, most experimental studies demonstrating the modulatory action of helminth infection used trimetophene sulphate as a haptenizing agent to drive Th1 colitis reminiscent of Crohn’s Disease [46]. Initiation of Th2/NKT colitis by oxazolone in conjunction with Th2-promoting helminth infection exacerbated pathology [47], further supporting that the mechanism of helminth suppression of colitis is based on Th2/Th1 antagonism. Thus, from a clinical perspective, helminth-based therapies might be considered inappropriate for ulcerative colitis or other intestinal disorders with Th2 aetiology. However, our data demonstrates that helminth infection-expanded FoxP3+ Treg clearly regulate coincident pro-fibrotic Th2 processes in the colon. Indeed, distinct molecules released by schistosome eggs deliver triggers that polarize naive CD4+ T cells towards Th2, or a regulatory phenotype [9,10,48]. Potentially, the release of somatic molecules with regulatory potential from degrading eggs that fail to breach colonic tissues could favour Treg expansion during chronic disease in the context of a TGFβ-enriched microenvironment. Exploitation of such regulatory molecules may be of benefit in the treatment of intestinal schistosomiasis or other Th2-based intestinal disorders via the expansion of Treg with bystander potential.

Supporting Information

Video S1 Acute colonic granuloma shown in Fig 1F. (MOV)

Video S2 Chronic colonic granuloma shown in Fig 1F. (MOV)

Video S3 Control recipient, colonic granuloma shown in Fig. 6B. (MOV)

Video S4 CD4+CD25+ recipient, colonic granuloma shown in Fig. 6B. (MOV)

Table S1 Acute-stage schistosome egg tissue burdens and excretion following adoptive transfer of chronic infection-expanded CD4+CD25+ cells. (DOC)

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Author Contributions

Conceived and designed the experiments: APM GRJ KGH JDT. Performed the experiments: APM GRJ KGH SA RAP JDT. Analyzed the data: APM JDT KGJ GRJ. Contributed reagents/materials/analysis tools: MC. Wrote the paper: JDT APM.

References

1. Chiutsuo L, Lowerre P, Engels D (2004) Schistosomiasis. Nat Rev Microbiol 2: 12–17.
2. Steinmann P, Kreier J, Bos R, Tanner M, Utzinger J (2006) Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis 6: 411–425.
3. Gryseels B, Polman K, Clerinx J, Kestens L (2006) Human schistosomiasis. Lancet 368: 1106–1118.
4. Wilson MS, Menink-Kane MM, Pesc JT, Ramalingan TR, Thompson R, et al. (2007) Immunopathology of schistosomiasis. Immunol Cell Biol 85: 148–154.
5. King CL (2001) Initiation and regulation of disease in schistosomiasis. In: Mahmoud A, ed. Schistosomiasis. London: Imperial College Press. pp 213–264.
6. King CL, Dickman K, Tisch DJ (2005) Reassessment of the cost of chronic helminthic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. Lancet 365: 1561–1569.
7. Rocklin RE, Brown AP, Warren KS, Pelley RP, Houba V, et al. (1980) Factors that modify the cellular-immune response in patients infected by Schistosoma mansoni. J Immunol 125: 1916–1923.
8. Steinfelder S, Andersen JF, Cannons JL, Feng CG, Joshi M, et al. (2009) The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). J Exp Med 206: 1691–1698.
9. Everts B, Perona-Wright G, Smits HH, Holke CH, van der Ham AJ, et al. (2009) Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. J Exp Med 206: 1673–1680.
10. Schramm G, Mohrs K, Wodrich M, Doenhoff MJ, Pearce EJ, et al. (2007) Cutting edge: IPSE/alpha-1, a glycoprotein from Schistosoma mansoni eggs, induces IgE-dependent, antigen-independent IL-4 production by murine basophils in vivo. J Immunol 178: 6023–6027.
11. Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN (2000) Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol 164: 2585–2591.
12. Fallon PG, Dunne DW (1999) Tollization of mice to Schistosoma mansoni egg antigens causes elevated type 1 and diminished type 2 cytokine responses and increased mortality in acute infection. J Immunol 162: 4122–4132.
13. Hoffmann KF, Cheever AW, Wynn TA (2000) IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. J Immunol 164: 6406–6416.

www.plosntds.org 10 August 2011 | Volume 5 | Issue 8 | e1269
14. Zaccone P, Piccirillo CA, Belkaid Y, Prufer J, Mentink-Kane M, et al. (2004) The pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. J Immunol 172: 3157–3166.

15. Taylor JJ, Mohrs M, Pearce EF (2006) Regulatory T cell responses develop in parallel to Th responses and control the magnitude and phenotype of the Th effector population. J Immunol 176: 5839–5847.

16. Weinstock JV, Boros DL (1983) Organ-dependent differences in composition and function observed in hepatic and intestinal granulomas isolated from mice with Schistosoma mansoni. J Immunol 130: 410–422.

17. Hogg KG, Kumkate S, Anderson S, Mountford AP (2003) Interleukin-12 p40 secretion by cutaneous CD11c+ and F4/80+ cells is a major feature of the innate immune response in mice that develop Th1-mediated protective immunity to Schistosoma mansoni. Infect Immun 71: 3563–3571.

18. Abe M, Harpel JG, Metz CN, Nunes I, Lukonoff DJ, et al. (1994) An assay for transforming growth factor-beta using cells transfected with a plasmid encoding a reporter construct, Anal Biochem 216: 276–284.

19. Weinstock JV, Boros DL (1981) Heterogeneity of the granulomatous response in the liver, colon, ileum, and ileal Peyer’s patches to Schistosoma mansoni eggs in murine schistosomiasis mansoni. J Immunol 127: 1906–1909.

20. Izcue A, Coombe JL, Pocier F (2006) Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. Immunol Rev 212: 256–271.

21. Luangsay S, Kasper LH, Minns LA, Mennecet EF, et al. (2003) CCR5 mediates specific migration of Trophoanula gondii-primed CD8 lymphocytes to intestinal epithelial cell. Gastroenterology 125: 491–500.

22. Wilson MS, Taylor MD, Balic A, Finney CA, Lamb JR, et al. (2005) Recruitment of natural CD4+ CD25+ regulatory T cells by infective larvae determines the outcome of filarial infection. Eur J Immunol 39: 1098–1107.

23. Taylor MD, LeGoff I, Harris A, Malone E, Allen JE, et al. (2005) Removal of regulatory T cell activity reverses hyporesponsiveness and leads to a parallel decrease in vivo. J Immunol 174: 4924–4933.

24. Baumgart M, Tompkins F, Leng J, Hesse M (2006) Naturally occurring CD4+CD25+ regulatory T cells are an essential, IL-10-independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. J Immunol 176: 5374–5387.

25. Taylor MD, van der Werf N, Harris A, Graham AL, Bain O, et al. (2009) Early recruitment of natural CD4+CD25+ Treg cells by infective larvae determines the outcome of schistosomal infection. Eur J Immunol 39: 192–206.

26. D’Elia R, Behnek JM, Bradley JE, Elie KJ (2009) Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival. J Immunol 182: 2340–2348.

27. Couper KN, Blount DG, de Souza JB, Sufia I, Belkaid Y, et al. (2007) Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. J Immunol 178: 4136–4146.

28. Setiady Y, Coccia JA, Park PU (2010) In vivo depletion of CD4+FOXP3+ Treg cells by the PG1 anti-CD25 monoclonal antibody is mediated by FcgammaRIII+ phagocytes. Eur J Immunol 40: 780–786.

29. Chen W, Jin W, Hardegen N, Lei KJ, Li L, et al. (2003) Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198: 1875–1886.

30. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3+ Treg cells via retinoic acid. J Exp Med 204: 1773–1783.

31. Zaccone P, Burton OT, Gibbs S, Miller N, Jones FM, et al. (2010) Immune modulation by Schistosoma mansoni antigens in NOD mice: effects on both innate and adaptive immune systems. J Biomed Biotechnol 2010:795210. pp 795210.

32. Zaccone P, Burton O, Miller N, Jones FM, Dunne DW, et al. (2009) Schistosoma mansoni egg antigens induce Treg that participate in diabetes prevention in NOD mice. Eur J Immunol 39: 1098–1107.

33. Dardalhon V, Awaishi A, Kwon H, Galligos G, Gao W, et al. (2008) IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+-IL-10+ Foxp3+ effector T cells. Nat Immunol 9: 1347–1355.

34. Walsh CM, Smith P, Fallon PG (2007) Role for CTLA-4 but not CD25+ T cells during Schistosoma mansoni infection of mice. Parasite Immunol 29: 293–308.

35. Lahm K, Sparwasser T (2011) In vivo depletion of Foxp3+ Tregs using the DEREG mouse model. Methods Mol Biol 707: 157–172.

36. Pefer JT, Ramalingam TR, Wilson MS, Mentink-Kane MM, Thompson RW, et al. (2009) Rema (remlapha/liz1) suppresses helminth-induced Th2-type immunity. PLoS Pathog 5: e1000393.

37. Amu S, Saunders SP, Kronenberg M, Mangan NA, Azeberger A, et al. (2010) Regulatory B cells prevent and reverse allergic airway inflammation via Foxp3-positive T regulatory cells in a murine model. J Allergy Clin Immunol Mar 19. epublished ahead of print.

38. Taylor JJ, Krzyczek CM, Mohrs M, Pearce EF (2009) Th2 cell hyposensitivity during chronic murine schistosomiasis is cell intrinsic and linked to GRAIL expression. J Clin Invest 119: 1019–1029.

39. Cillo LJ, Humphreys NE, Lane TE, Potten CS, Booth C, et al. (2005) Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science 308: 1463–1465.

40. Zhao A, Urban JR, Anthony RM, Sun R, Sultz J, et al. (2008) Th2 cytokine-induced alterations in intestinal smooth muscle function depend on alternatively activated macrophages. Gastroenterology 135: 217–225, e211.

41. Fallon PG, Richardson EJ, Smith P, Dunne DW (2000) Elevated type 1, diminished type 2 cytokines and impaired antibody response are associated with hepatotoxicity and mortalities during Schistosoma mansoni infection of CD4-depleted mice. Eur J Immunol 30: 470–480.

42. Doenhoff M, Musaallam R, Bain J, McGregor A (1978) Studies on the host-parasite relationship in Schistosoma mansoni-infected mice: the immunological dependence of parasite egg excretion. Immunology 33: 771–778.

43. Cheever AW, Ehues IA, Andrade CA, Cox TM (1993) Biology and pathology of Schistosoma mansoni and Schistosoma japonicum infections in several strains of nude mice. Am J Trop Med Hyg 48: 496–503.

44. Karama DM, Colley DG, Nalbenn BL, Ouma JH, Secor WE (1997) Studies on schistosomiasis in western Kenya: I. Evidence for immune-facilitated excretion of schistosome eggs from patients with Schistosoma mansoni and human immunodeficiency virus coinfections. Am J Trop Med Hyg 56: 515–521.

45. Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, et al. (2004) Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immune pathology. Immunol 20: 629–635.

46. Ryuesser NE, De Winter BY, De Man JG, Loukas A, Herman AG, et al. (2008) Worms and the treatment of inflammatory bowel disease: are molecules the answer? Clin Dev Immunol 567314 2008: 567314.

47. Hunter MM, Wang A, McKay DM (2007) Helminth infection enhances disease in a murine TH2 model of colitis. Gastroenterology 132: 1320–1330.

48. van der Kieij D, Lutz E, Brunsver JF, Kruize YG, Schmitz M, et al. (2002) A novel host-parasite lipid cross-talk. Schistosomal lysophosphatidic acid activates toll-like receptor 2 and affects immune polarization. J Biol Chem 277: 48122–48129.