Exploring host-immune-microbial interactions during intestinal schistosomiasis

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CHAPTER 6

PATENT *SCHISTOSOMA MANSONI* INFECTIONS ENDORSE DISTINCT REGULATORY CELL NETWORKS, WITH EVIDENCE FOR MICROBIAL ASSISTED ALLEVIATION OF ALLERGIC AIRWAY INFLAMMATION

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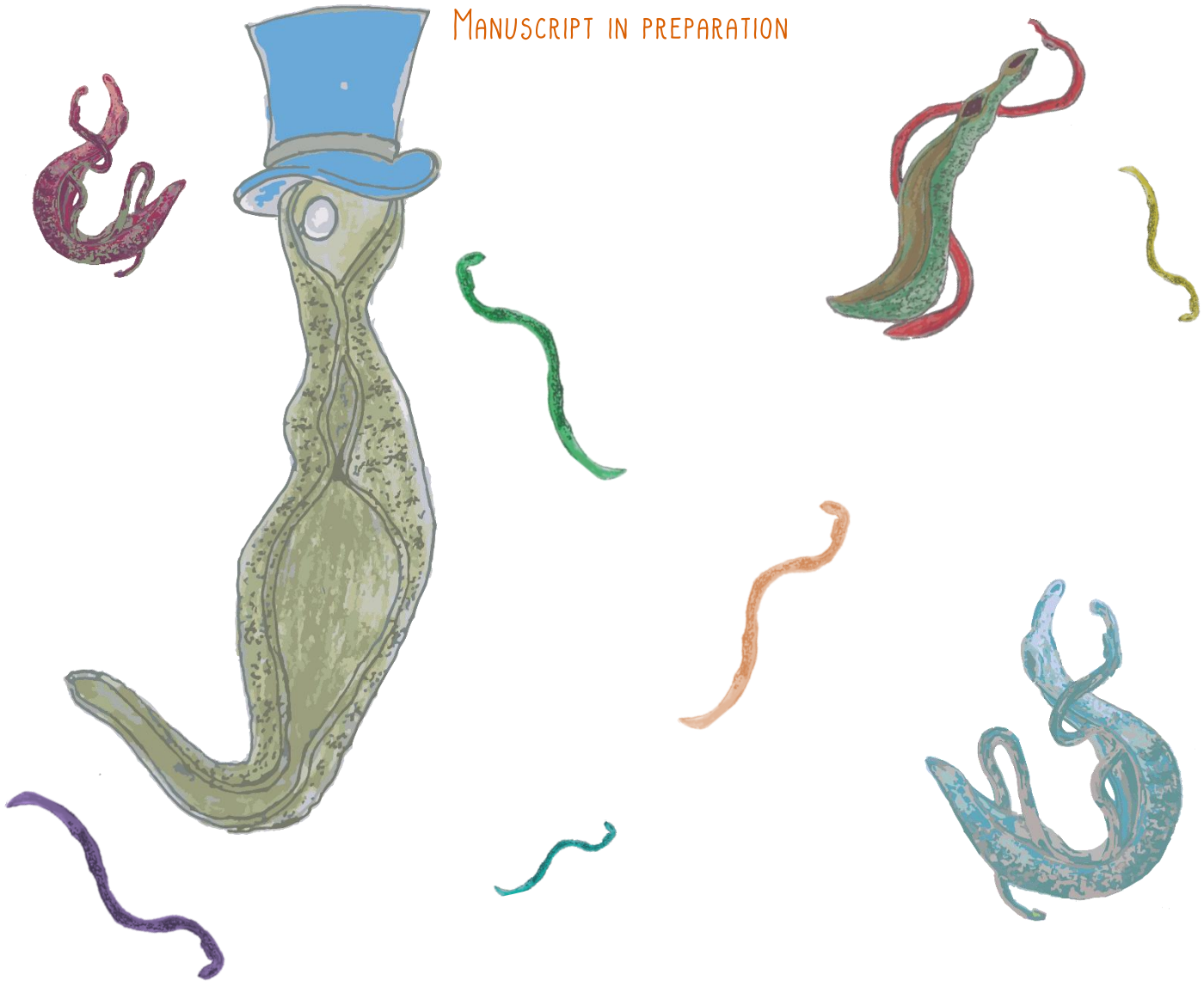
MANUSCRIPT IN PREPARATION



Patent Schistosoma mansoni infections endorse distinct regulatory cell networks, with evidence for microbial assisted alleviation of allergic airway inflammation

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ABSTRACT

Chronic schistosome infections drive systemic activation of the regulatory arm of the immune system, including Regulatory B cell (Breg) and T cell (Treg) populations that curb and restrain excessive inflammatory responses, partially through the provision of anti-inflammatory IL-10. Murine schistosome-induced Breg and Treg cells have repeatedly proven proficient down-regulators of allergic airway inflammation (AAI) and suppressors of T effector responses. However, the signals and life stages dictating their expansion are far from understood. Comparing conventional mixed-sex (egg production) and male worm only (no egg production) *Schistosoma mansoni* infections, we show both patent and non-patent infections to elicit the generation of phenotypically distinct splenic regulatory cell networks. However, at chronic stages of infection, B cells and $CD4^+$T cells from egg producing infections displayed a heightened propensity to produce IL-10 compared to non-patent infections. As we recently observed substantial differences in intestinal microbiota composition between chronic single-sex and mixed-sex infections, we next questioned the involvement of their associated microbiotas in regulatory cell induction and modulation of AAI. In comparison to allergic mice recolonised with naïve microbiotas, the transfer of single-sex and mixed-sex microbiotas lead to a significant reducing in cellular infiltration to the airways, with mixed-sex microbiotas imprinting a distinctive M1 phenotype on pulmonary macrophages and skewing towards mild Th1 $CD4^+$ responses. Although no large alterations were observed in pulmonary regulatory populations, schistosome microbiota recipients displayed an enhanced frequency of splenic $Foxp3^+$ and CTLA-4^+CD4^+ T cells. Importantly, when transferred into non allergic IL-10 reporter mice, schistosome microbiotas evoked subtle elevations in splenic and mesenteric IL-10. These data shed further light on the contribution of worm and egg derived signals in the promotion of schistosome-mediated regulation and suggest that the regulatory capacity of *Schistosoma* parasites is partially mediated through changes in the microbiota.

Keywords

Schistosoma mansoni, microbiota, Breg, Treg, chronic infection, allergic airway inflammation.

Abbreviations

Adult worm Antigen (AWA) Allergic airway inflammation (AAI); Alternative activation (AA); B Cell receptor (BCR); Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4); Extracellular secretory products (ES products); Follicular (FO); Glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR); Interleukin (IL); Latency associated peptide (LAP); Marginal Zone (MZ); Mesenteric lymph nodes (MLNs), Regulatory B cell (Breg); Regulatory T cell (Treg); Schistosome egg antigen

(SEA); Short chain fatty acids (SCFAs); Transitional Type 1 (T1); Transitional Type 2 (T2); transitional type 2 marginal zone precursors (T2-MZPs); T Helper (Th); Toll like receptor (TLR); Trimethylamine (TMA); Trimethylamine oxide (TMAO);

INTRODUCTION

The mammalian immune system has evolved in tandem with environmental microbes and parasites and is thus reliant on their encounter for adequate immune education and development[1]. Indeed, allergy and autoimmunity are rising within westernised populations, and have shown inversely correlated with helminth infections, including schistosomiasis[1,2]. In human infections, *Schistosoma* parasites appear to lessen the severity of asthma[3] and allergic skin reactivity[4,5], with evidence for the induction of strong regulatory networks[6]. Experimentally, schistosome-mediated protection against models of allergic airway inflammation (AAI) is facilitated by the actions of discrete regulatory cell populations, including Regulatory T cells (Tregs)[7–9] and B cells (Bregs)[9–12], that typically emerge during infection chronicity[11–13]. A current research ambition is to learn from these parasites, identify the mechanisms underlying Breg and Treg induction, and strategically implement this understanding for the treatment of hyper-inflammatory conditions such as allergy.

Tregs are essential mediators in the control of helminth mediated pathology and are thus commonly targeted by helminths to enhance their longevity and limit attack from the host immune system[14]. Treg populations are typically hallmarked by the master transcription factor Foxp3^{+}[15], as well as putative markers such as the IL-2α receptor (CD25)[16], glucocorticoid-induced tumour necrosis factor receptor family related protein (GITR)[17], and the inhibitor receptor Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)[18,19]. In schistosomiasis, both CD25^{+} and CTLA-4^{+}CD4^{+} T cells are important providers of the immunosuppressive cytokine IL-10 and are capable of modulating Th2-dominated granulomatous inflammation[20–24] and subverting potentially lethal Interleukin-4 (IL-4) /IL-13 driven pathology[25]. In allergic disease, Tregs have pivotal roles in maintaining tolerance to innocuous Ags[26,27]. The transfer of helminth-induced Tregs into uninfected sensitised hosts downregulates AAI[28], and Treg abolishment, by means of anti-CD25 antibodies[8] or genetic ablation[7], reverts schistosome mediated AAI suppression and aggravates allergic responses. Moreover, regulatory cell populations are known to work in synergy, with one allergic system showing *S. mansoni* induced Bregs to confer protection against allergy through activation of their Treg counterparts[9]. In fact, while IL-10 is critical to the downmodulation of schistosome evoked inflammation[25,29,30], schistosome-induced Tregs can work independently of IL-10 production[22], with the majority of IL-10 coming from a non CD4+ T cell source[23], including B cells[31,32].

Bregs expand during experimental and natural infections with *Schistosoma* species[12,32–35]; which may represent an immunoevasive strategy contributing to long-term parasite survival[36]. In murine infections, Bregs are enriched splenic marginal zone (MZ)[12,32] and pulmonary compartment[32], and protect AAI through means of IL-10 production and/or Treg expansion[9–12,35]. Similarly, *S. haematobium* infected individuals have an elevated percentage of IL-10 producing peripheral B cells in comparison to uninfected persons, with IL-10 levels returning to baseline following anti-helminthic administration[11]. Importantly, these schistosome-driven Bregs demonstrate an enhanced capacity to restrain T effector responses, induce Tregs, and are characterised by regulatory associated markers such as CD1d or the surface latency associated peptide (LAP)/ TGF-β1[31,32]. Unlike Tregs, there is no lineage-specific marker to define Breg functionality[37]**.** Instead, Bregs adopt a wide variety of phenotypes depending on the inflammatory threat encountered and are standardly identified via their provision IL-10, albeit other IL-10 independent effector mechanisms do exist[10,38–42]. In terms of instructive signalling, engagement with inflammatory cytokines [37,43,44], CD40[45] and the B cell receptor (BCR) are considered crucial for their appearance and IL-10 production. Toll-like receptor (TLRs) ligands [46,47] support this instruction, with described roles bacteria and/or their metabolites[48–51], and direct interaction with *Schistosoma* products[52]. On the basis that Breg cells are not lineage specific but emerge in response to discrete inflammatory cues[37], closer interrogation of chronic inflammatory scenarios may provide novel insight into the molecular determinants controlling the Breg differential program and IL-10 transcription.

The identification of Breg and Treg generating factors is made more complex by the intricacy of the schistosome life-cycle. Notably, with schistosomes undergoing multiple transformative and tissue-migratory stages within their host, it is difficult to ascertain whether regulatory cell generation is purely parasite driven, and/or a result of host-derived reparative or inflammatory mediators[14]. It is also possible that microbial derived signals play a participatory role, with schistosome infections shown to drive intestinal dysbiosis[53–56] (*Costain et al., manuscript in preparation*) and antibiotic administration influencing the instigation of schistosome-specific immune responses[57]. Importantly, multiple reports have shown microbial members (of their derived products) to promote Breg[48,50,51,58] and Treg[59,60] generation, and helminth-derived microbiotas to modulate the severity of airway[61] and intestinal inflammation[54,62,63]. Taken together, these observations warrant closer inspection of the role of schistosome associated microbiotas in regulation of host immunity.

In the current study we provide further clarity on the signals driving splenic regulatory cell expansion during murine schistosomiasis. By virtue of infections with egg producing or non-egg producing *S.*

mansoni parasites, we demonstrate that patent schistosomiasis endorses the expansion of phenotypically distinct splenic B cell and T cell networks, coinciding with heightened production of IL-10. While non-patent infections failed to enhance the propensity for $CD4^+$ T cells to produce IL-10, B cell IL-10 levels were elevated in these worms only infections as well suggesting that worm derived molecules, in the absence of eggs or their associated inflammation, can support Breg programming. Interestingly, the transplant of schistosome-associated microbiota into antibiotic treated recipients led to subtle increase in splenic and mesenteric IL-10, as well as a shift to Th1-like allergen-specific responses in the lung, and downmodulation of HDM-evoked allergic airway inflammation. Together, these data strongly suggest schistosomes to not only modulate host immunity directly via their own secretions or molecules, but also through changes in the microbiota composition.

MATERIALS AND METHODS

Animals

Age-matched C57BL/6 mice (Envigo) and IL-10GFP Vertx reporter mice (raised in house) on a C57BL/6 background were housed under SPF conditions at the Leiden University Medical Centre (LUMC, Leiden, The Netherlands) or the University of Manchester (Manchester UK). For germ-free (GF) experiments, GF mice were bred in isolators at the University of Manchester Gnotobiotic Facility. All animal experiments were conducted in accordance with either the Animal Experiments Ethical Committee of the Leiden University Medical Centre or under a project license granted by the Home Office UK and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

Schistosome infection

Biomphalaria glabrata snails infected with a Puerto Rican strain of *S. mansoni* were maintained at LUMC or obtained from K. Hoffman (Aberystwyth University, UK). Mice were percutaneously infected with 40 male and female parasites (mixed-sex infection), or 40 male parasites (single-sex infection), with parasite sex determined by purpose-made multiplex PCR, targeting schistosome-sex specific sequences[64]. Mice were culled at 4, 7, 9, 12 or 14 weeks post infection. Infections in mixed-sex infected mice were confirmed by macroscopic evaluation of livers, followed by digestion of the whole intestine in 4% KOH (24hr, 37°c) for egg counts. Evaluation of circulating cathodic antigen (CCA) levels in mice urine allowed for confirmation of single-sex infections[65]. For GF schistosome infections, GF mice were infected with 180 male and female *S. mansoni* cercariae with infections

lasting 7 weeks. To minimise exposure to the environment, infections were conducted under strict sterile condition, and mice were fed an autoclaved diet and water.

Antibiotic treatment and faecal transplant

For depletion of intestinal bacteria communities, mice were treated with metronidazole, ampicillin, gentamycin, neomycin (all 1mg/ml; Sigma) and vancomycin (0.5mg/ml; Alfa Aesar) in drinking water for 2 weeks, with replenishment every 3-4 days[66]. 2 days after the removal of Abx from the drinking water, mice were recolonised by oral gavage with faeces from naïve or schistosome infected mice, with faeces taken at week 14 of infection. 2 days post primary colonisation, mice received an additional 'booster' gavage to promote bacterial recolonisation[67]. Mice were euthanised 3 weeks post the first faecal gavage or taken further for induction of HDM evoked allergic airway inflammation.

House dust mite evoked allergic airway inflammation

Mice were anesthetised with isoflurane and sensitised intranasally with 100µg of HDM (Greer Laboratories) dissolved in 30µl of sterile PBS. 1 week following HDM sensitisation, mice were challenged for 4 consecutive days with 10µg of HDM in 30µl volumes under isoflurane anaesthetic. Mice were culled 2 days after the final allergen challenge, with collection of BAL, lung and mediastinal LNs.

Isolation of immune cells

Immune cells were isolated from the spleen, lung, bronchiolar lavage fluid (BAL) and mesenteric lymph nodes (MLNs). Spleens and MLNs were homogenized by passage through a 70µM cell strainer, with spleens undergoing an additional incubation with RBC lysis for erythrocytes depletion. Cells were manually counted by haemocytometer and resuspended in FACs buffer (PBS supplemented with 2mM EDTA (Sigma) and 2% FBS) or in complete RPMI 1640 GlutaMAX media (Invitrogen) containing 5% FBS, 5×10^{-5} M 2-Mercaptoethanol (Sigma) and antibiotics (100 U/mL of penicillin and 100 µg/mL of streptomycin; Invitrogen). Cells were then taken further for cell culture, flow cytometry or B cell isolation. For HDM based experiments, BAL cells were obtained by flushing the lungs 2 times with FACs buffer. Lungs were sliced and digested at 37 °C for 40 minutes with 0.15mg/ml Liberase TL (Roche) and 0.05µg/ml DNase I type VI (Sigma) in HBSS (Sigma). Digestion was stopped by the addition of FACs buffer followed by resuspension and passage of cells though a 70µm cell strainer. Lung samples underwent an additional RBC lysis step before counting by haemocytometer and resuspension in X-VIVO™ (Lonza) or FACs buffer.

B cell isolation and in vitro stimulation

Total B cells were purified from splenocytes by anti-CD19 Microbeads (Miltenyi Biotech) following manufacturer's instructions. Total B cells (6×10^6/mL) were cultured for 72h at 37 °C in complete RPMI 1640 GlutaMAX with or without SEA (20µg/mL), anti-CD40 (0.5µg/mL), adult worm antigen (AWA) (20 µg/mL) and worm Extracellular secretory (ES) products (20 µg/mL). Following culture, supernatants were harvested for cytokine analysis by ELISA. For flow cytometry based detection of IL-10, cells stimulated with PMA (100 ng/mL) and ionomycin (1µg/mL) in the presence of Brefeldin A (10µg/mL; all Sigma–Aldrich) for the final 4 hours of incubation.

Flow cytometry

Cells of equivalent numbers were washed twice in PBS, before staining with live/dead fixable aqua dead cell stain kit (1:400; Thermo Scientific) or Zombie UV dye (1:2000, BioLegend) for 10 minutes at room temperature. Cells were subsequently stained with B cell or T cell specific antibodies (Table 1) with FcγR-binding inhibitor added to all samples. For staining panels involving the analysis of intracellular makers, samples were further fixed and permeabilized with BD Cytofix/Cytoperm™ (BD) or eBioscience™ Foxp3 / Transcription Factor Fixation/ Permeabilization for 1h at 4 °C, before staining with specified intracellular markers.

ELISA

Concentrations of IL-10 and IL-6 in cell-free culture supernatants was assessed by OptEIA ELISA kits (BD) according to manufacturer's instructions.

Company	Target	Clone		Company	Target	Clone
Ebioscience	B220	RA-6B2+A2:D39		Invitrogen	CD11b	M1/70
BD	CD19	Ebio(ID3)		Invitrogen	CD11c	N418
Biolegend	CD1d	1B1		Biolegend	CD62L	MeL-14
BD	CD21	7G6		Biolegend	CD64	x54-5/7.1
BD	CD23	B3B4		Biolegend	F4/80	BM8
Biolegend	CD24	M1/69		Biolegend	Ly6C	HK1.4
BD	CD5	53-7.3		Biolegend	MHC-II	M5/114.15.2
BD	CD86	B7-2		Biolegend	PDCA-1	927
BD	CD9	MZ2		BD	Siglec-F	E50-2440
BD	Galectin-1	RG9-35		Ebioscience	Ter-119	TER-119
Biolegend	CD4	RM4-5		Biolegend	XCR1	ZET
Biolegend	CD8	53-6.7		Biolegend	CD25	PC61
Biolegend	IFNγ	XMG1.2		Invitrogen	CD3	17A2
Biolegend	IL-10	JES5-16E3		Biolegend	CD4	RM4-5
Ebioscience	IL-13	ebio13A		Ebioscience	CD44	Im7
Biolegend	IL-17	TC11-1810.1		BD	CTLA-4	UC10-4B9
Biolegend	IL-4	11B11		Ebioscience	Gata-3	TWAJ
Ebioscience	IL-5	TRFK.5		Biolegend	GITR	DTA-1
Invitrogen	TCRb	H57-597		Ebioscience	RORyt	B2D
Invitrogen	Foxp3	FJK-16s		Biolegend	T-bet	4Bio

Table 1. Flow cytometry antibody list

Serum metabolomics

Serum samples were randomized, thawed at room temperature, briefly vortexed then centrifuged for 20 minutes at 13,000 x g in a centrifuge cooled to 4° C. In an Eppendorf tube, 30 µl of the serum supernatant was combined with 30 µl of a buffer solution (pH 7.4, 100% D2O) containing 0.075 M Na2HPO4, 2 mM NaN3 (an antimicrobial) and 0.08% of the internal standard 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP). The mixture was transferred to 1.7 mm glass NMR tubes. A quality control sample was generated by pooling from each sample and combining with buffer as described above.

One dimensional 1H NMR spectra were acquired on a Bruker Advance III HD 600 MHz NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), equipped with a SampleJet system and a cooling rack of refrigerated tubes at 6° C. A relaxation edited spin-echo using the 1D-Carr-Purcell-Meiboom-Gill (CPMG) presat pulse sequence was applied to all samples, analysed at a temperature of 300 K. For each sample, 128 transients were collected in 64K frequency domain points with a spectral window set to 20 ppm.

Automated processing of the spectra was performed using TopSpin 3.6 (Bruker Corporation, Germany) including spectral calibration, phase and baseline correction. The resulting raw NMR spectra were imported into MATLAB (Version 2018a, Mathworks Inc). After digitization of the spectra, redundant peaks (TSP, H2O) were removed. The spectra were manually aligned to quality control samples and reference peaks using Recursive Segment-Wise Peak Alignment 1. For the SIC profiles, the data was normalized using a probabilistic quotient, to account for variability in sample dilutions 2. Metabolite identification was performed using an in-house metabolite library, cross-referencing with the Human Metabolome Database (https://hmdb.ca/) and Statistical Total Correlation Spectroscopy (STOCSY) 3. The relative concentrations of all age associated metabolites were calculated from the spectral data using trapezoidal numerical integration.

Statistics

Statistical analyses were performed using GraphPad Prism 9 software. Data are shown as mean values ± S.E.M. Where applicable, experimental groups were analysed by unpaired t-test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test as appropriate. Significant differences were defined at $P < 0.05$.

Distinct Treg networks emerge during patent and non-patent schistosomiasis

Phenotypically distinct Treg populations emerge within the liver[22–24], colon[21] and spleen[20] of chronically infected animals, with both worm[68,69] and egg-derived products[70,71] demonstrating Treg inducing capacity in *in vitro* settings or through challenge models. However, to our knowledge, it has yet to be investigated whether worm-alone signals, in the absence of egg driven inflammation, may support Treg expansion during active infection.

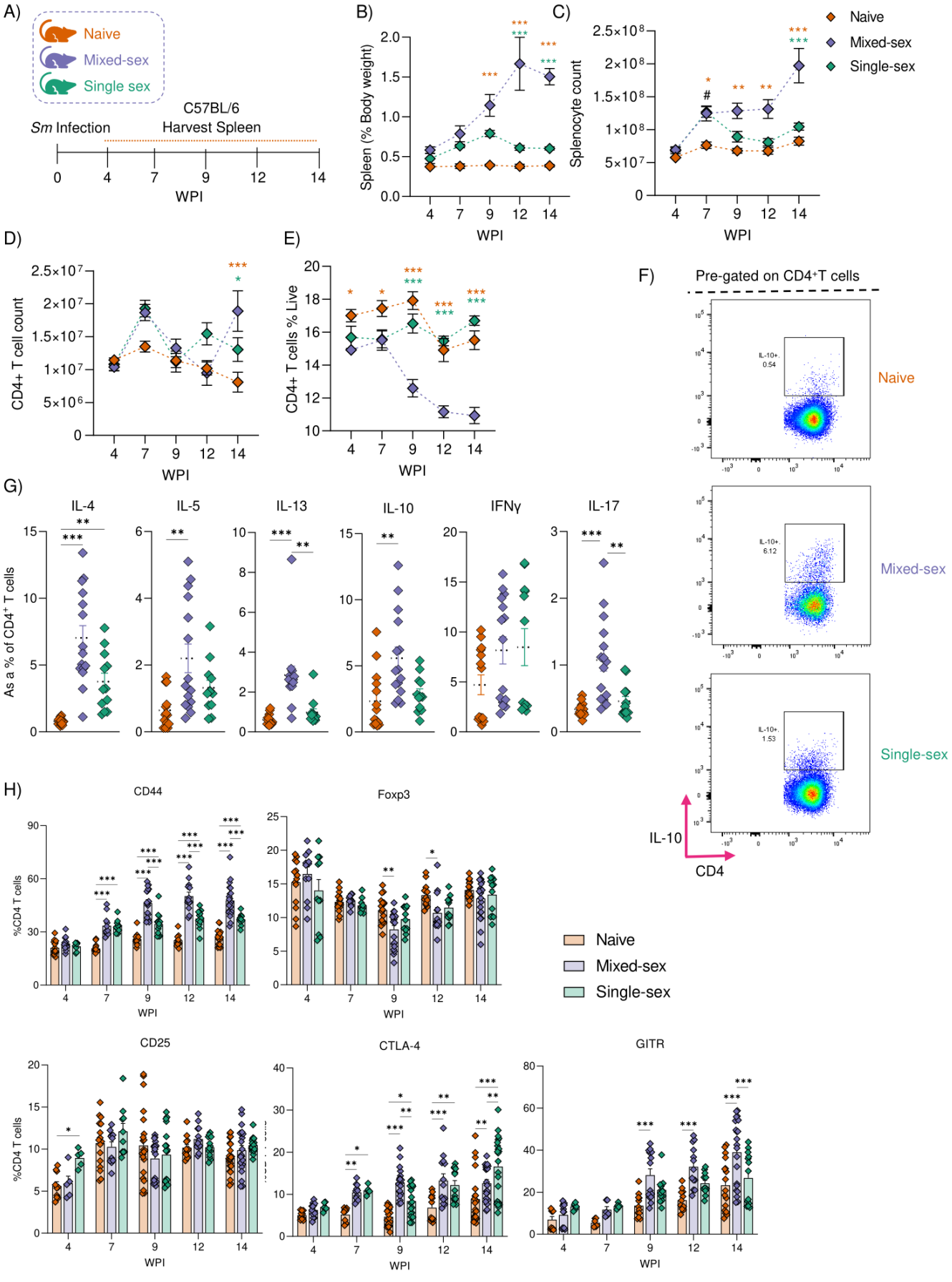
In attempt to define the schistosome-derived signals that support Treg induction, we monitored splenic Treg frequency in mice conventionally infected with 40 male and female schistosome cercariae (mixed-sex model), or male cercariae alone (single-sex model) in which no eggs are produced, with infections lasting 4, 7, 9, 12 or 14 weeks in duration (Figure 1A). Firstly, looking at broad splenic responses, only conventional mixed-sex infections were associated with splenomegaly, as defined as the increase in spleen weight relative to overall body weight (Figure 1B). These increases began at 9 weeks post infection, persisted into chronicity, and enlarged overtime. Expectedly, these increases in spleen size translated into enhanced splenocyte numbers (Figure 1C) and equivocal CD4+ T cell counts between groups (Figure 1D), despite a decrease in total $CD4^+$ T cell proportions in mixed-sex infected mice (Figure 1E). Focusing on week 14, a time-point at which Treg numbers are known to expand during schistosomiasis[20,22,23], only mixed-sex infected mice were typified by greater expression of T cell IL-10 following *ex vivo* PMA/iono stimulation (Figure 1F&G). Mixed-sex infected $CD4^+$ T cells also expressed greater levels of IL-17 and Th2 associated cytokines, IL-5 and IL-13 than naïve or single-sex infected mice in response to PMA/iono (Figure 1G). Whilst IL-4 expression was upregulated in both single-sex and mixed-sex CD4+ T cells (Figure 1G).

We next tested the frequencies of different Treg populations over the course of single-sex and mixed-sex schistosomiasis (Figure 1H). The activation and Treg associated marker CD44[72] was significantly enhanced in infected mice groups from week 7 onwards, with the more prominent upregulation in egg producing infections from week 9. Corroborating previous reports showing little evidence for Foxp3 induction during acute or chronic schistosomiasis[20], Foxp3 expression remained consistent across all groups, with the exception of weeks 9 and 12 in which a decrease in $Foxp3^+CD4^+$T cells was observed in single-sex and mixed-sex mice groups. Very interestingly, despite multiple studies showing $CD25^+CD4^+$ T cells to play important roles in the regulation of schistosomiasis immunopathology[23,25], this does not necessarily seem to coincide with an increase

in their frequency here. In line with a previously reported role for CTLA-4$^+$ T cell populations in the regulation of schistosomiasis pathology[20] we observed a marked increase in CTLA-4 expression in both single and mixed-sex infections. Interestingly, at week 14 CTLA-4 was more prominently expressed on single-sex CD4$^+$ T cells

than those from mixed-sex mice. Finally, upregulation of GITR was limited to mixed-sex infected mice, with significant up-regulation observed in mixed-sex infected mice from week 9 when compared to naïve controls or mice infected with male cercariae only.

Figure 1. Definition of splenic Treg networks during schistosomiasis. (A) C57BL/6 WT mice were infected with ~40 male and female S. mansoni cercariae (mixed-sex infection) or ~40 male S. mansoni cercariae (single-sex infection). Spleens were harvested from naïve or infected mice at weeks 4, 7, 9 12 or 14 of infection. (B) Spleen weights as a proportion of total body weight. (B) The total number of leukocytes recovered from the spleens of naïve or infected mice. (C) CD4+ T cells as a % of total liver cells. (D) Total numbers of CD4 T cells. (F) Representative flow plots for IL-10 secretion, pre-gating on live CD45+TCRβ+CD4+ cells. (G) Cytokine secretion from PMA ionomycin stimulated splenocytes cells at week 14 of infection. (H) The proportion of CD44+, Foxp3+,CD25+,CTLA-4+ and GITR+ CD4+ T cells across the course of infection, presented as a % of total CD4+ T cells. Results are mean +/- SEM from two (F&G) or three (B,C,D,E&H) experiments pooled (n=12-24 mice per group per time-point). Significant differences were determined by one-way (G) or two-way ANOVA (B,C,D,E&H) followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B-E) Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between single-sex and naïve mice are indicated by #


A)
Naive
Mixed-sex
Single sex
C57BL/6
Sm Infection
Harvest Spleen
0
4
7
9
12
14
WPI
B)
Spleen (% Body weight)
C)
Splenocyte count
Naive
Mixed-sex
Single-sex
D)
CD4+ T cell count
E)
CD4+ T cells % Live
F)
Pre-gated on CD4+T cells
IL-10+
0.54
6.12
1.53
Naive
Mixed-sex
Single-sex
IL-10
CD4
G)
IL-4
IL-5
IL-13
IL-10
IFNγ
IL-17
As a % of CD4+ T cells
H)
CD44
Foxp3
%CD4 T cells
Naive
Mixed-sex
Single-sex
CD25
CTLA-4
GITR

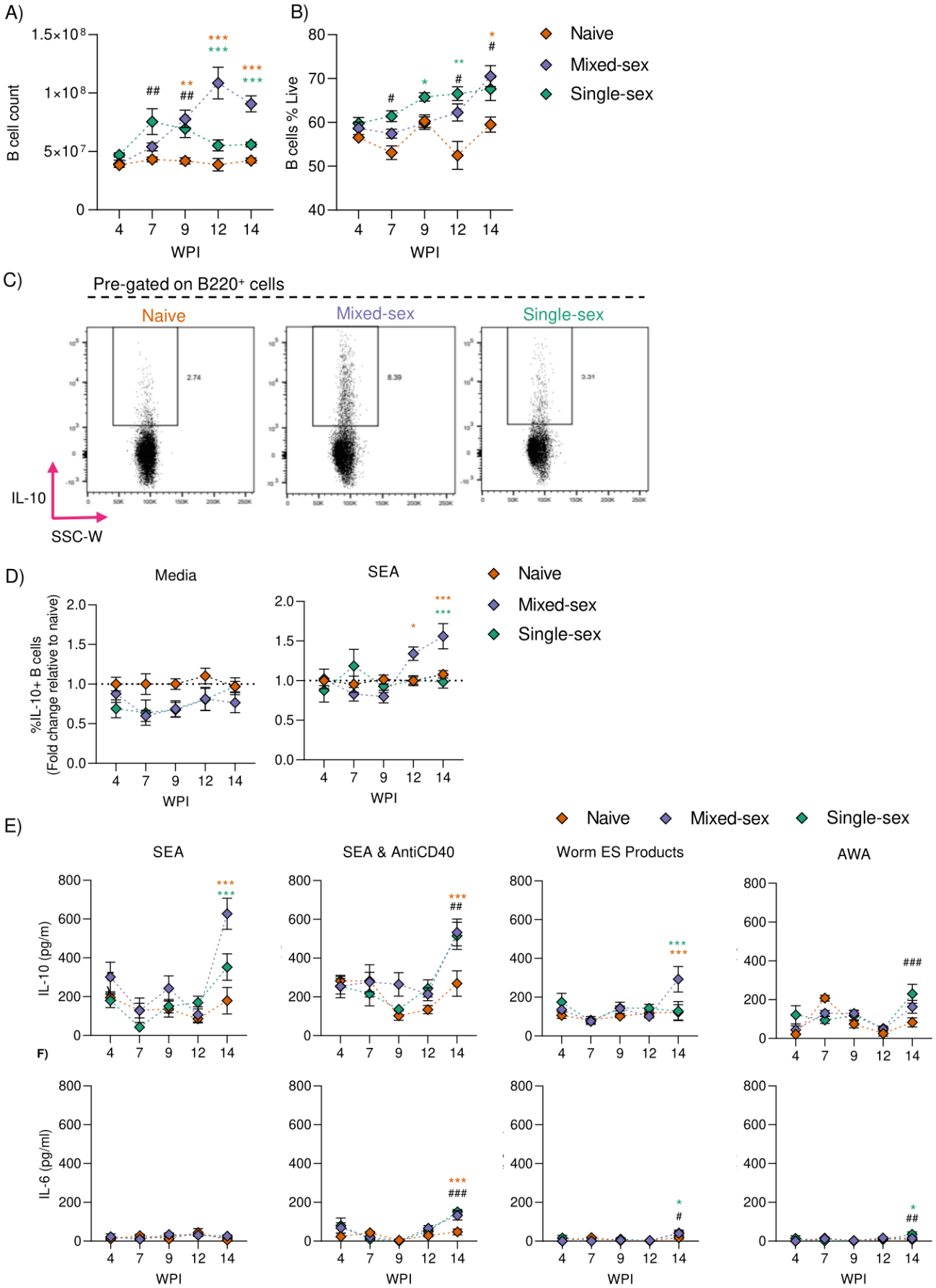
Regulatory B cell expansion during patent and non-patent infection

Several studies demonstrate an expansion of splenic IL-10 producing Bregs during chronic stages of murine schistosomiasis[12,23,31,32,35] with evidence as well for Breg induction in infections with male worms alone[35], but this was in different mouse strains (BALB/C vs C57BL/6). Here, we monitored Breg frequency across the course of conventional mixed-sex or single-sex infections in C57BL/6 mice, allowing better comparisons. Coinciding with enhanced splenomegaly (Figure 1B) and splenocyte numbers (Figure 1C), splenic B cell counts were increased in mixed-sex infected mice during chronic weeks of infection in comparison to single-sex infected mice and uninfected controls (Figure 2A). However, when looking at relative B cell proportions, their frequency remained comparable to naïve and mixed-sex infected mice until week 12 of infection, whereby enhanced B cell frequency was observed in mixed-sex mice (Figure 2C). On the contrary, the proportion of B cells in single-sex infected mice remained significantly elevated in single-sex infected mice from week 9 onwards in comparison to both naïve and mixed-sex infected groups.

To assess the propensity of B cells to produce IL-10, splenic B cells were isolated across the course of infection and stimulated for 72 hrs in the presence of schistosome egg antigen (SEA) and analysed for intracellular IL-10 by flow cytometry (Figure 2C&D). Unlike their single-sex or naïve counterparts, B cells from mixed-sex infected mice demonstrated a startling increase in IL-10 production at week 14 post infection, with evidence for enhanced IL-10 production in comparison to naïve B cells at week 12. To corroborate these findings and further define which parasite derived molecules or stimulation conditions may promote B cell IL-10, or oppositely the more pro-inflammatory IL-6, we analysed the supernatants of 3 day cultured B cells, whom had been exposed to SEA with or without anti-CD40, worm extracellular secretions (ES) or adult worm antigens (AWA) (Figure 2E). In response to SEA and worm ES products, the pattern for IL-10 by ELISA was near identical to that observed by flow cytometry, with mixed-sex B cells significantly increasing IL-10 production at week 14. However, upon stimulation with SEA and antiCD40, B cells from single-sex infected mice demonstrated the same propensity to produce IL-10 as mixed-sex B cells, though with the important exception that anti-CD40 + SEA also induce IL-6 production in B cells as compared SEA alone, which did not induce IL-6 production in B cells. For AWA only single-sex B cells showed heightened IL-10 and IL-6 secretion at week 14, but only half of amounts detected with SEA or SEA + anti-CD40. Together, these data highlight the presence of Breg cell differentiation in both worm only and egg-producing infections though the potency of the Breg cells – at least in terms of IL-10 production – seems less in worm only-infected mice. Furthermore, given the large increase in spleen size (Figure

1B) and B cell numbers (Figure 2A) in mixed-sex infections at week 14, one can infer that the total levels of IL-10 during conventional egg producing infections to be considerably greater than that of naïve or single-sex infected mice. In this stage, it is unclear why B cells from mixed-sex infected mice respond differently to worm-derived products (worm ES and/or AWA) as compared to single-sex infected mice, as they both have been exposed to adult worms throughout the infection. It cannot be excluded that separate Breg populations have developed in mixed-sex infected mice that primarily respond to eggs, showing the much stronger response there. Together, these data highlight the induction of Breg cell populations in both single-sex and mixed-sex infections to evoke a Breg phenotype and elucidate differences in IL-10 secretion in response to either worm or egg-derived signals in the presence or absence of costimulatory pathways.

Figure 2. Signal specific expansion of IL-10 producing B cells during S. mansoni infections. The total number (A) and proportion (B) of CD19+ B cells in the spleens of naïve, mixed-sex and single-sex infected mice. (C-E) CD19+ B cells were isolated from the spleens of naïve and infected mice and stimulated in vitro for 3 days with SEA (20 µg/mL), anti-CD40(0.5µg/mL), adult worm antigen (AWA) (20 µg/mL) and worm extracellular secretory (ES) products (20 µg/mL). (C) Representative flow plots for IL-10 secretion in isolated B cells at week 14 of infection. (D) Percentage of IL-10+ B cells as assessed by flow cytometry and presented as fold change relative to naïve. SEA and media conditions only. (E) B cell supernatants were analysed for IL-10 and IL-6 concentrations by ELISA. Data are from one single experiment (E&F; AWA and worm ES products), or from two (E&F) or three (A-D) experiments pooled (n= 6-24 per time point). Data are presented as mean+/-SEM. Significant differences are indicated by *p<0.05, **p<0.01, ***p<0.001 and determined by two-way ANOVA followed by Tukey's post hoc test. Significant differences between naïve vs mixed-sex infected mice, and single-sex vs mixed-sex infected mice are indicted by orange (*) and green asterisks (*) respectively. Significant differences between single-sex and naïve mice are indicated by #.


A)
B)
B cell count
B cells % Live
WPI
Naive
Mixed-sex
Single-sex
C)
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Naive
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Single-sex
IL-10
SSC-W
D)
Media
SEA
%IL-10+ B cells
(Fold change relative to naive)
E)
SEA
SEA & AntiCD40
Worm ES Products
AWA
IL-10 (pg/m)
IL-6 (pg/ml)
F)

Signature B cell phenotypes in single-sex and mixed-sex infections

After having established that splenic B cells from schistosome infected mice have a greater propensity to produce IL-10, our attention turned to phenotyping the B cell subsets responsible. A variety of Breg subsets have been described in mice and men, with their exact phenotypes and immunosuppressive activities differing depending on the inflammatory threat encountered[37,73]. In previous studies, we demonstrated that MZ B cells**,** as opposed to FO B cells, are responsible for the majority of B cell IL-10 during chronic infection stages[11]. To accommodate for the wider range of Breg populations described[37,43,73], we broadened our gating scheme allowing for the demarcation of Transitional type 1 (T1), Type 2 (T2) and transitional type 2 marginal zone precursors (T2-MZPs) (Supplementary Figure 1 A&B).

Schistosome infections dramatically altered the splenic B cell profiles, as visualised by and representative flow plots (Supplementary Figure 2A) and summarised in statistical tables (Supplementary Figure 2B). Whilst FO and T1 proportions were fairly comparable between infection groups and time-points, we observed dramatic enrichment for T2 and T2_MZP B cell subsets within mixed-sex infected mice, with single-sex infections also accompanied by increased T2 frequency at weeks 7, 9 and 14 (Supplementary Figure 1C). These increases coincided with an overall reduction in MZ_MZP and MZ frequency within infected mice groups. CD1d and CD5 are commonly used to capture the murine B Cell IL-10 compartment[6,37,73,74]. Proportionally, CD5 expression was shown significantly higher on mixed-sex and single-sex T2 B cells than naïve T2 B cells, with no discernible differences in expression amongst other B cell subsets (Supplementary Figure 1D). Mixed-sex T2_MZP B cells expressed higher levels of $CD1d^{+}$ than naïve or single-sex T2_MZPs.

Although attempts were made to evaluate the IL-10 production and Treg induction capacity of individual B cell subsets, technical issues precluded firm conclusions to be drawn (Data not shown).

Evidence for microbial instructed IL-10 production and regulation of splenic Treg populations

The obvious explanation behind the differential Breg and Treg signatures in single-sex and mixed-sex infections is the presence or absence of eggs, their complex mixture of Ags and the vigorous inflammation and damage that accompanies their tissue penetration and entrapment. In addition, our group previously reported reduced barrier integrity during patent schistosomiasis with evidence for enhanced bacterial translocation (*Costain et al., manuscript in preparation*). As such we reasoned that regulatory cell expansion may (additionally) be supported by intestinally-derived signals, that leak into systemic circulation as a result of egg transit. ^{1}H nuclear magnetic resonance (NMR) spectroscopy–based metabolic profiling was performed on the sera of infected mice groups

to evaluate the relative metabolic profiles (Figure 3). Several metabolites were shown to differ amongst groups, including higher amounts of the bacteria related product Trimethylamine (TMA) in chronically infected mixed-sex mice. Although other experimental helminth infections were accompanied by greater levels of short-chain fatty acids (SCFA; e.g acetate, butyrate and propionate)[62,75], we observed no differences in butyrate levels between infection groups throughout the infection period. Several amino acids were decreased in the circulation of infected mice, with both mixed-sex and single-sex infected mice showing decreased levels of isoleucine, leucine and valine levels between weeks 9 and 12.

Next, given the considerable differences between mixed-sex and single-sex intestinal microbiotas and metabolomes (*Costain et al., manuscript in preparation*), we next aimed to directly assess the

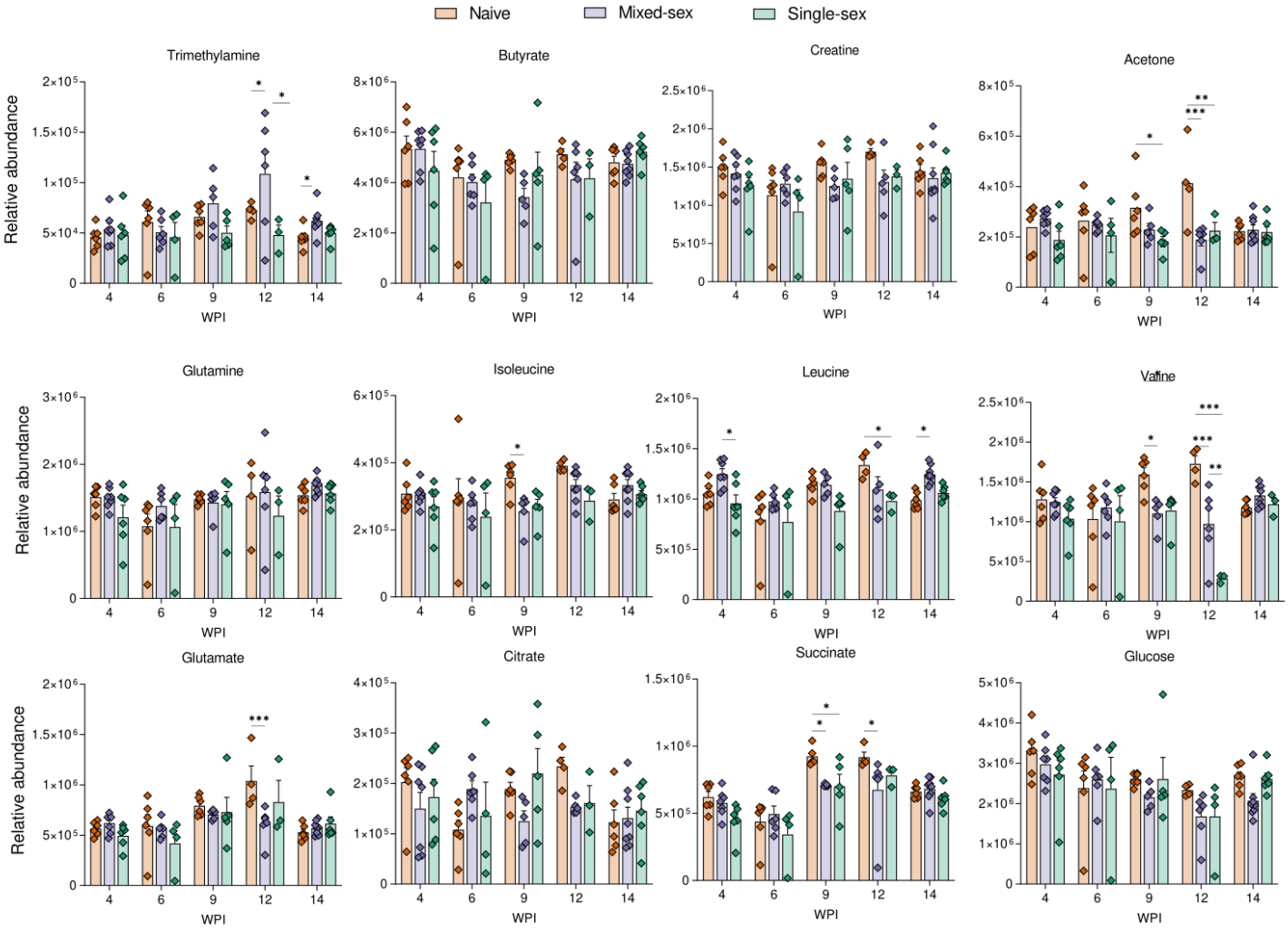

Figure 3. Serum metabolite profiles across infection. Relative abundance of metabolites in the serum of naïve and schistosome infected mice. Results are mean +/- SEM from one single experiment (n= 5-8). Significant differences were determined individual two-way ANOVAs followed by Tukey's post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.

capacity of their associated microbiotas to support the production of T cell and B cell IL-10. Antibiotic-treated Vert-X mice (express GFP under control of the IL-10 promotor) were recolonised for 3 weeks with naïve or chronically infected schistosome-associated microbiotas (Figure 4). Although deemed statistically insignificant, splenic B cells from mixed-sex and single-sex microbiota recipients had an increased tendency to produce IL-10 compared to those from naïve microbiota recipients (Figure 4A). Within the mesenteric lymph nodes (MLNs), B cells and T cells from single-sex microbiota recipients also displayed an enhanced tendency to produce IL-10 compared to other microbiota transfer groups (Figure 4B). No Considerable differences were shown for B cell or T Cell phenotypes otherwise (Data not shown).

To further assess whether microbially derived signals may contribute the generation of schistosome induced regulatory networks GF mice were infected with egg producing schistosomes, and splenic

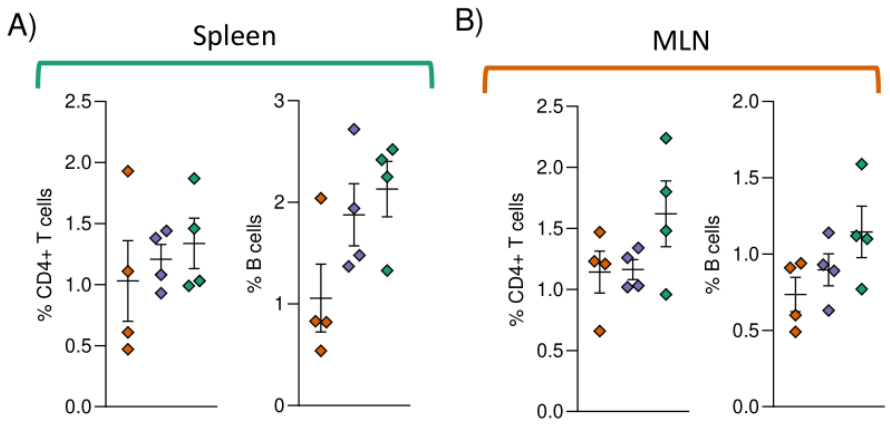

Figure 4. Receipt of schistosome microbiota promotes splenic and mesenteric IL-10. Abx-treated Vertx mice (IL-10 reporters) received two faecal transplants from naïve mice, or 14-week infected mixed-sex or single-sex mice. After 3 weeks recolonisation (A) splenic and (B) mesenteric CD4+T Cell and B cells were analysed for IL-10 expression. Data are from one single experiment (n=4). Data are presented as mean+/-SEM. Significant differences are indicated by *p<0.05, **p<0.01, ***p<0.001 and determined by one-way ANOVA.

CD4+ T cell phenotypes and cytokine secretion was analysed after 7 weeks. $CD4^+$ T cell proportions (Supplementary Figure 3A) and their expression of Th2 (Gata-3), Th17 (Roryt) and Th1 (T-bet) associated transcription factors remained unaltered between SPF and GF mice (Supplementary Figure 3B). However, we observed a significant increase in the expression of CD25 and CTLA-4 in GF infected mice in comparison to their SPF counterparts, with a slight tendency towards heightened Foxp3. Likewise, the potential for IL-10 production was heightened in GF schistosome infected mice, as was IFNγ (Supplementary Figure 3C). Analysis of B cell profiles was not conducted here due the 7 week time point of infection, thus closer inspection at chronic disease is needed. Together, these

data provide a convincing argument for microbial involvement in schistosome-elicited immune responses. Knowing the capacity of schistosomes to alleviate allergic inflammation[9–12,35], these data also provide strong rationale to investigate microbial participation here.

Faeces transfer from schistosome-infected mice modulates the severity off allergic airway inflammation

Several studies have shown helminth-associated microbiotas and experimental schistosome infections to attenuate allergic airway disease[9,11,12,35,76]. To determine whether intestinal bacteria from schistosome infected mice could also influence AAI, we transferred faeces from naïve, single-sex or mixed-sex infected mice to antibiotic-treated SPF mice, and after a 3 week colonisation period, challenged them to house dust mite (HDM) intranasally (Figure 5A). Transfer of both mixed-sex and single-sex schistosome infected microbiotas lessened allergic airway inflammation as evidenced by a reduction in cellular infiltration and a trend towards less eosinophils in the bronchial alveolar lavage (BAL) fluid in comparison to HDM allergic control mice (treated with PBS) (Figure 5B). On closer interrogation of differential cell counts, mice recolonised with single sex faeces showed a stronger reduction in the total number of neutrophils, $CD4^+$ T cells, $CD8^+$ T cells and monocytes in the BAL fluid as compared to mice recolonised with mixed-sex faeces. Interestingly, evaluating inflammatory cells in the lung tissue, revealed that recipients of mixed-sex of single-sex microbiotas were typified by a general increase in total lung $CD45^+$ cell counts, with a significant increase in total CD4+ T cells and a trend towards heightened B cell numbers while other cell types remained unchanged or similar across all HDM-sensitized and challenged mice (Figure 5C). Lung CD4+ T cells from HDM challenged mice did not produce elevated Th2 associated cytokines in response to PMA/iono restimulation in comparison to non HDM controls, despite elevated BAL-eosinophilia. However, as we did not evaluate HDM-specific T cell responses, it might be speculated that putative differences related to Th2 cytokines will be mostly found in the Ag-specific T cell responses (Figure 6A). Interestingly, $CD4^+$ T cells from single-sex and mixed-sex faeces recipient mice showed a trend towards greater IL-10 production in response to PMA/iono stimulation, and $CD4^+$ and $CD8^+$ T cells from mixed-sex faeces recipients showed significantly greater expression of Th1 associated IFNγ, but not IL-17.

HDM-treatment altered the ratio of alveolar to interstitial macrophages, with the proportion of interstitial macrophages significantly increasing in the lungs of HDM-treated mice (Figure 6B), while feacal transplant was partially reversing this phenomenon. Coinciding with the greater IFNγ propensity of CD4+ T cells from mixed-sex microbiota recipients (Figure 6A), alveolar and interstitial

macrophages from mice receiving mixed-sex schistosome faeces were typified by a shift towards classical 'M1'-like macrophage signature (Figure 6C), such as less of the Th2-associated mediator RELMα, and more of MHC-II (alveolar MF) and less of the M2-marker mannose receptor CD206 (interstitial MF). HDM treatment evoked a large alteration in pulmonary CD4+ T cell activation ($CD44^{+}CD62L^{-}$) status and transcription factor expression (Figure 6D). While no differences in lung CD4+ T cell activation or expression of Foxp3, Gata-3 or T-bet were found between HDM treated groups, we observed a trend towards heightened Roryt and CTLA-4 expression in mice receiving schistosome infected faeces (Figure 6D). Finally, HDM-treatment did not evoke significant systemic inflammation, as evidenced by equivocal splenic cell counts across all treatment groups (Supplementary Figure 4A). However, the infiltration of splenic CD4+ T cells (Supplementary Figure 4B), and their expression of Foxp3 (Supplementary Figure 4C) was significantly increased upon single-sex and mixed-sex microbiota transfer. Curiously, upregulation of CTLA-4 was exclusive to splenic CD4+ T cells from single-sex microbiota recipients.

Collectively, these data show the potential of a schistosome associated microbiota, from both egg-producing and non-egg producing chronic infections, to modulate the severity of allergic airway disease. These data hint that schistosomes not only modulate host immunity directly but may also act through changes in gut microbial composition.

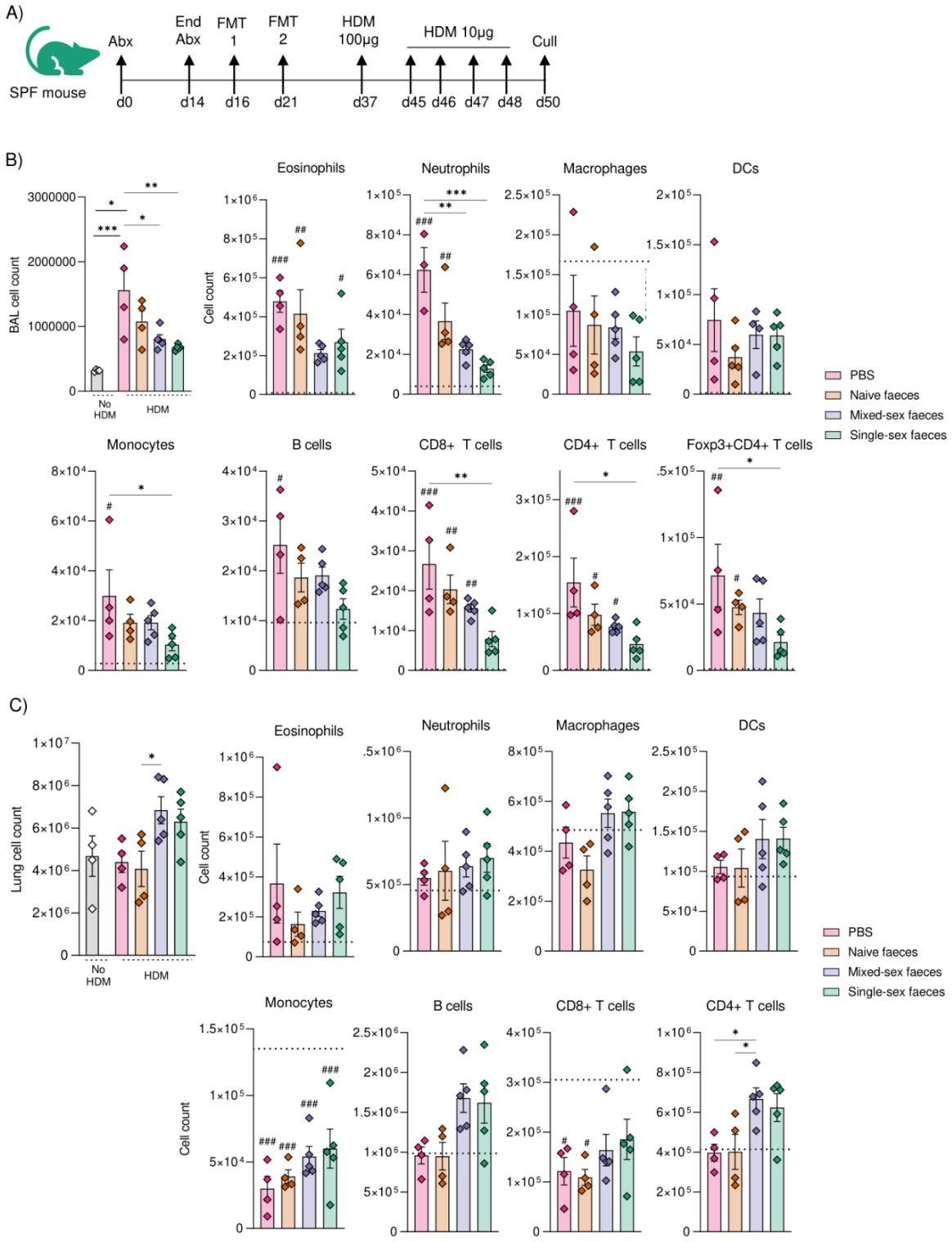

Figure 5. Schistosome-associated microbiota modulates the severity of HDM-evoked allergic airway inflammation. (A) C57BL/6 SPF mice were treated with broad-stream antibiotics (Abx) in drinking water for 2 weeks, followed by two oral gavages with sterile PBS or faeces. Gavages were performed two days apart, with faeces collected from naïve mice or 14 week infected mixed-sex or single-sex infected mice. After a 3 week recolonisation period, mice were subjected to HDM challenge and sensitisation. Mice were sacrificed 2 days after the final HDM challenge, and the severity of airway inflammation was evaluated. Mice not subjected to HDM treatment were gavaged with sterile PBS. Abx; Vancomycin (0.5mg/ml), Ampicillin (1mg/ml), Neomycin (1mg/ml), Metronidazole (1mg/ml) and Gentamycin (1mg/ml). Differential cell counts in the (B) Bronchiolar lavage fluid (BAL) or (C) lung. Dotted line represents mean cell count in no HDM control group. Results are mean +/- SEM from one single experiment (n=4-5 mice per group). Significant differences were determined by one-way ANOVA followed by suitable Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Significant differences between HDM treated and non HDM treated mice are indicated by #.

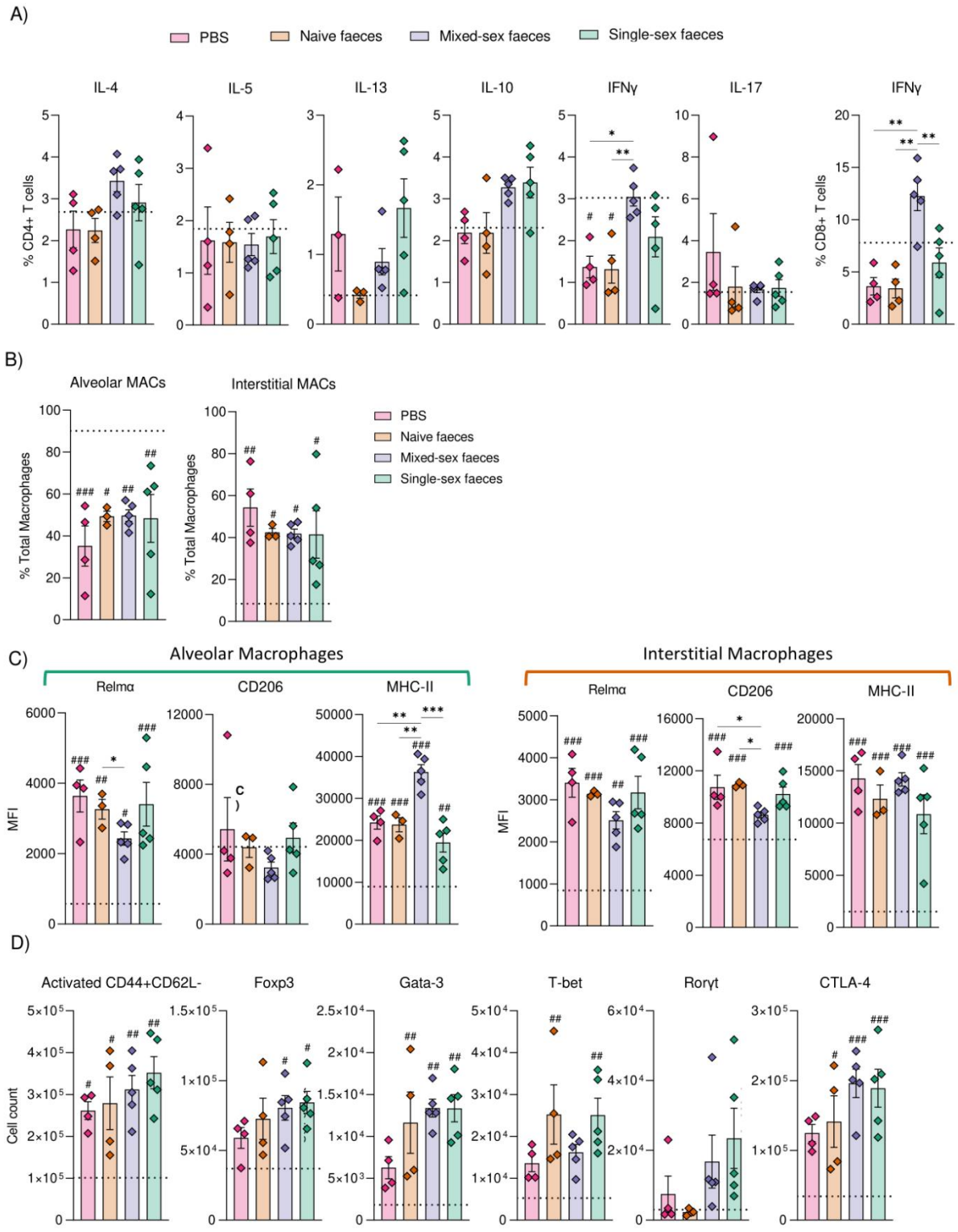

Figure 6. Faeces from schistosome infected mice imprints distinct T cell and macrophage phenotypes in allergic mice. (A) Cytokine secretion from PMA ionomycin stimulated lung cells. (B) The proportion of alveolar and interstitial macrophages as a % of total lung macrophages. (C) The geometric mean fluorescence intensity of RELMα, CD206 and MHC-II on the surface of alveolar and interstitial macrophages. (D) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors and IL-2R (CTLA-4) of pulmonary CD4+ T cells. Dotted line represents mean values in the no HDM control group. Data presented as mean +/- SEM. Data shown are from 1 single experiment (n=3-4 mice per group) Significant differences were determined by one-way ANOVA with Tukey's post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between HDM treated and non HDM treated mice are indicated by #.

Helminths tactfully tamper with host immunological processes through the direct actions of their immunogenic secretions or by driving dysbiosis of the host microbiota[1,62,76]. The potential for schistosome infections to inhibit inflammatory disease is well documented[1,12,31–33,54,77–79], with both experimental[6,9,10,12,35] and epidemiological studies[6] advocating a role for regulatory cells in the control of airway inflammation. Here we reveal the capacity of a schistosome associated microbiota to modulate the severity of AAI (Figures 5&6), with evidence for microbial support in schistosome-associated splenic Breg expansion (Figure 4) and Treg signatures (Supplementary Figure 3).

Infections with male and female egg producing schistosomes or male schistosomes alone evoked the expansion of distinct splenic regulatory networks (Figure 1 & Supplementary 1 &2). Breg generation is nourished by inflammatory environments including the presence apoptotic cells[80], inflammatory cytokines such as Type I IFNs[44], IL-1β[58]and IL-6[58], and receptor ligation with pathogen specific ligands[52,81]. Herein and supporting previous studies[35], we show worm only infections in the absence of egg-evoked inflammation to endorse B cell IL-10 production (Figure 2), further highlighting the known regulatory potential of the worm secretome[68]. However, the potential seems less as compared to patent infection, with less IL-10 secretion, at least with the ex vivo stimulations used here. In addition, as we did not identify the exact B cell subset responsible for B cell IL-10 in mixed-sex and single-sex infections, it is possible that differing B cell subsets harbour Breg capacities in each infection, and that different compounds/ products trigger their functionality and IL-10 production[82]

B cells of mixed-sex and single-sex mice were shown phenotypically unique, with notable expansion of T2_MZP B cells in mixed-sex infected mice only (Supplementary Figures 1 &2). The expansion of T2_MZPs is of considerable interest given their anti-inflammatory and IL-10 production capacities and knowing that microbially-derived components[50,83] and -stimulated cytokines endorse their functionality[58]. For schistosomiasis, a helminth infection accompanied with compromised barrier integrity and evidence of dissemination of luminal products (*Costain et al., Manuscript in preparation*)[84] it is exciting to consider a schistosome-specific microbial element supports T2_MZP proliferation and IL-10 production. Although, we should also consider the involvement of female worm-derived products on Breg generation, knowing that female only infection promote a more immune dampening environment than males[85] . Although we did not address the B cell subset responsible for the majority of IL-10 here, these data highlight the capacity of schistosome-elicited

environments to endorse Breg phenotypes and warrant further investigation into the microbial assistance in their generation.

We demonstrate that *S. mansoni* infections are accompanied by the expansion of splenic $CD4^+$ cells expressing CD44, GITR and CTLA-4, but not CD25 or Foxp3 (Figure 1). The lack of Foxp3 expression corroborates previous work[20] and fits with the narrative that most $CD4^+$ T cell derived IL-10 comes from a non Foxp3+ source[70]. The unaltered expression of CD25 is somewhat surprising given previous reports showing enhanced CD25 expression during both experimental and natural infections[20,21,23,86]. However, given that CD25 has proven unessential in the regulation of murine schistosomiasis [20], it makes sense that we do not see enhanced enumeration of these cell types. CTLA-4 is a crucial negative regulator of T cell activation[18] that is upregulated during *S.mansoni* infection, regulates Type 2 cytokine production and immune cell recruitment[20], and whose gene polymorphisms are suspected to influence schistosomiasis susceptibility[87]. Indeed, patients with severe schistosomiasis fibrosis have lower frequencies of CTLA-4^+ T cells than fibrosis free patients[88]. Our data suggests a role for worm-derived signals and microbial elements in the regulation of CTLA-4, with enhanced expression on splenic $CD4^+$ T cells of single-sex infected mice (Figure 1), albeit we did not observe an increase in CTLA-4 expression on antibiotic-treated Vertx mice recolonised with single-sex faeces (Data not shown). These observations support previous work showing the adult worm Ag PIII to increase the percentage of human $CD4^+$ T-cells expressing CTLA-4[89] and suggests that adult worms instruct CTLA-4 expression as to suppress anticipated egg driven inflammation. Curiously, with the exception of IFNγ, only regulatory associated markers CTLA-4, CD25 and IL-10 were affected by the absence of an intestinal microbiota in GF infections (Supplementary Figure 3), hinting for a key role for the bacteria in modulating regulatory cell networks during schistosomiasis.

The capacity of a schistosome associated microbiota to modulate disease severity has been reported in experimental colitis[54]. Floudas *et al* report an alleviating effect of microbiotas from male only infected mice on colitis progression, whereas conventional dual-sex microbiotas evoked a 'colitogenic' inflammatory profile. The presence of a more 'colitogenic' microbiota in conventional egg-producing infections supports our observation of an M1 phenotype in mixed-sex faecal transfer recipients (Figure 6). However, as these researchers achieved faecal transplant by means of co-housing with schistosome-infected mice for 3 weeks, and microbiota structure is known to alter considerably in this length of time (*Costain et al., Manuscript in Preparation*) we cannot assert whether faecal delivery via oral gavage (as conducted in this study), or faeces uniquely from infection chronicity, would have the same colitogenic effect. Moreover, although out lab recently

reported on the microbial structure of male only and mixed-gender schistosome infections (*Costain et al., Manuscript in preparation*), we did not inspect the microbial composition of faecal transplant recipients here. As many microbial species are lost and are unable to re-establish during the faecal transplant process[90] we are not able to comment on their inflammatory status. Thus, future studies teasing apart the bacterial elements involved in the regulation of inflammation would be of great interest.

The airway macrophages of HDM-treated mixed-sex microbiota recipients, were skewed towards a slight M1 profile (Figure 6). Although too early to draw firm conclusions, this data suggests the presence of an M1 aggravating component within the faeces of mixed-sex infected mice, that promotes an inappropriate inflammatory profile response following allergen exposure. Given the strong Th2 polarising nature of schistosome eggs and their Ags[91,92], we speculate that the polarising agent responsible is likely of microbial origin. However, it is worth noting that schistosome eggs contain a homologue of high-mobility group box 1 (HMGB1) protein, which is a potent stimulator of M1 macrophages[93]. As dietary supplementation with SCFAs has shown to restore macrophage homeostasis following antibiotic treatment and microbiota rexposure[66], it is also possible that the aggravation of an M1 phenotype upon mixed-sex microbiota transfer reflects an absence of certain metabolites or lack of bacterial species that generate them. Moreover, it is conceivable that the consortium of microbes found within a mixed-sex schistosome-associated microbiota are less capable of supporting the regrowth of SCFA-generating bacterial species, that are depleted using this same antibiotic regime[66]. Additionally, while mice receiving mixed-microbiotas showed a general reduction in airway inflammation in response to HDM in comparison to uncolonized mice or those recolonised with naïve faeces, it remains unclear whether the instruction of an M1 phenotype contributes to this phenotype.

Microbial derived components have a well-established role in the calibration of systemic and local immunity[94], with SCFAs (e.g acetate, butyrate and propionate) commonly commended for their immunomodulatory and anti-inflammatory capacities[50,62,76,95]. While our analysis did not detect differences in serum butyrate levels between infected and naïve mice, the pro-inflammatory bacterial-derived metabolite TMA was shown significantly higher in the serum of mice chronically infected with egg producing schistosomes (Figure 3), in support of previous metabonomic studies[96,97]. TMA is microbial derived metabolite, produced from the breakdown of dietary choline and carnitine, and delivered to the liver for conversion into trimethylamine N-oxide (TMAO)[98]. While TMAO is known to promote atherosclerosis, cardiovascular disease, and inflammation[98], the

influence of TMAO metabolism on helminth infection and disease regulation has not been established. Given that TMAO levels are increased in inflammatory bowel disease patients[99], it is possible that enhanced circulating TMAO levels simply reflects the pronounced egg-driven mucosal inflammation. In addition, given that TMA is known to influence macrophage activation[100], and promote platelet activation and thrombosis, it is conceivable that TMA might influence the severity of granulomatous inflammation or promote the attachment and diapedesis of freshly deposited schistosome eggs across the endothelim[101]. With a ubiquitous range of bacterial taxa responsible for TMA production[102], it is currently unknown whether schistosomes actively support the generation of TMA-producing bacteria.

Corroborating our metabolomic analysis (Figure 3), previous metabolic phenotyping during schistosomiasis has revealed disturbances in amino acids, metabolites related to energy (glycolysis, TCA cycle), inflammation and microbial function, but it is unclear how these metabolic profiles arise. Both mixed-sex and single-sex schistosome infections are known to alter microbiota structure[54] (*Costain et al., manuscript in preparation*), indicating that schistosome metabolic profiles are driven by shifts in bacterial colonisation or function. In addition, schistosomes use host-derived metabolites for their own growth and development[103], and metabolic alterations could be indicative of compromised liver function[96]. Finally, similar to that described for *H. polygyrus*[75], it is exciting to consider that schistosomes actively modulate host metabolite profiles as to support specific immune profiles, sustain their own longevity or downmodulate bystander inflammation. Indeed, transfer of schistosome-associated microbiota into Abx recipients suggests for greater IL-10 capacity (Figure 4) and modulation of airway disease (Figures 5&6), but the microbial or metabolic mediators involved required further inspection.

In summary we demonstrate egg producing and non-egg producing schistosome infections to prompt the emergence of distinct splenic regulatory populations, with evidence that schistosomes associated microbiotas promote their instruction and assist in the modulation of unrelated inflammation. Closer interrogation of schistosome microbial crosstalk will likely unravel new and exciting molecules and pathways for the targeting of allergic disease and expansion of regulatory cell types.

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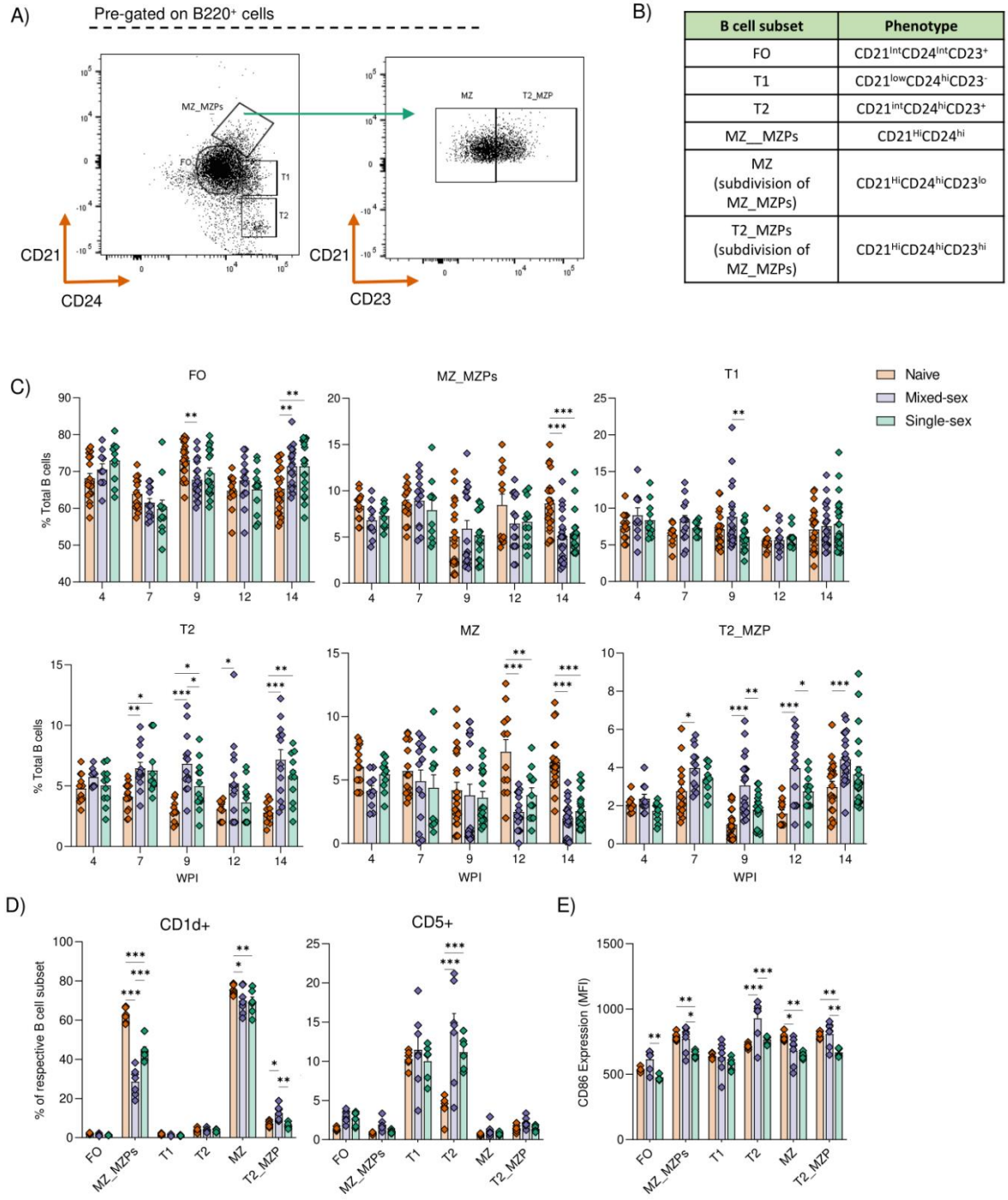
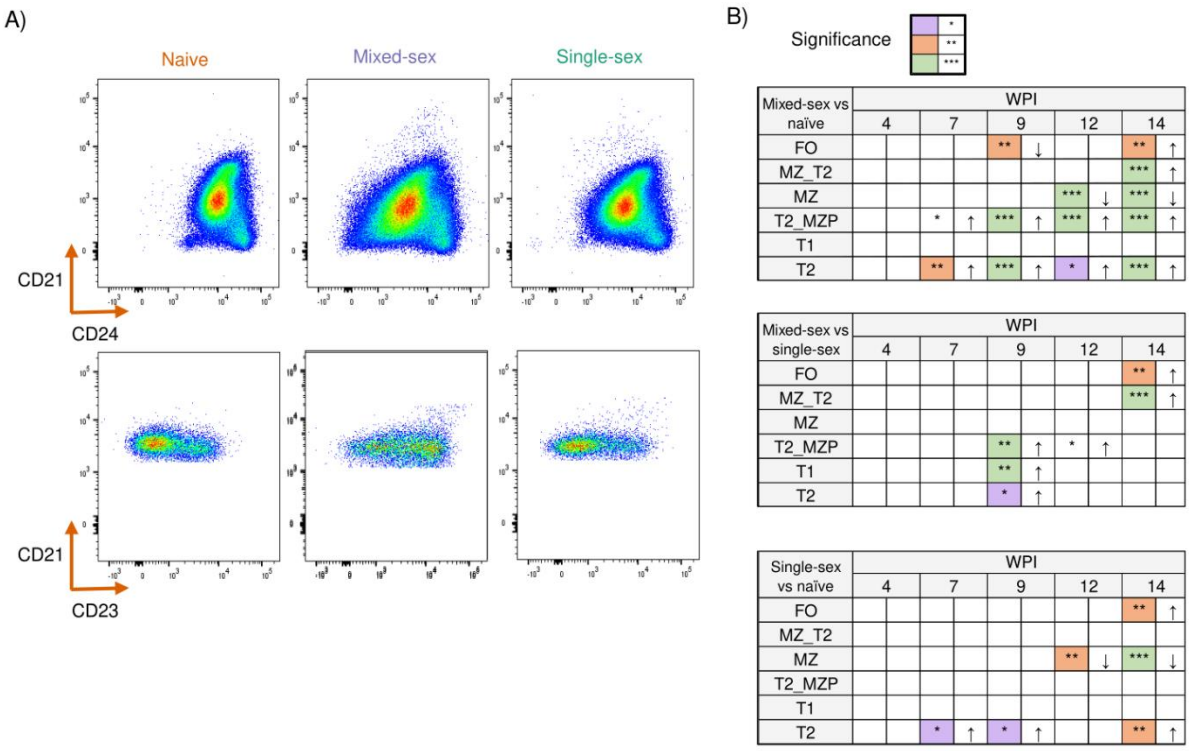


B cell subset	Phenotype
FO	$CD21^{Int}CD24^{Int}CD23^{+}$
T1	$CD21^{low}CD24^{hi}CD23^{-}$
T2	$CD21^{Int}CD24^{hi}CD23^{+}$
MZ__MZPs	$CD21^{Hi}CD24^{hi}$
MZ (subdivision of MZ_MZPs)	$CD21^{Hi}CD24^{hi}CD23^{lo}$
T2_MZPs (subdivision of MZ_MZPs)	$CD21^{Hi}CD24^{hi}CD23^{hi}$

Supplementary Figure 1. S. mansoni infections elicit distinct splenic B cell profiles. (A) Representative FACs plot of B cell gating. CD19+ B cells were subsetted into Follicular (FO; CD21IntCD24IntCD23), Marginal zone and marginal zone precursors (MZ_MZPs; CD21HiCD24hi) Transitional type 1 (T1;CD21lowCD24hiCD23-) and Transitional type 2 (T2;CD21intCD24hiCD23+). Within the MZ_T2 subset, B cells were further divided into marginal zone (MZ; CD21HiCD24hiCD23lo) and Transitional two-marginal-zone precursor B cells (T2-MZPs; CD21HiCD24hiCD23hi) based on CD expression. (B) Reference table for B cell phenotypes. (C) Percentage of total B cells of FO, MZ_T2, T1, T2, MZ and T2_MZP B cells in naïve or infected mice. (D) The percentage of respective B cell subsets positive for CD1d and CD5. (E) the geometric mean fluorescence intensity of CD86 on the surface of B cell subsets. Results are mean+/- SEM. Data from (D-E) one representative experiment or from (C) three experiments pooled (n=6-24 mice per group). Significant differences were determined by individual one-way ANOVAs (D&E) or two-way ANOVA (C). *p < 0.05, **p < 0.01, ***p < 0.001.

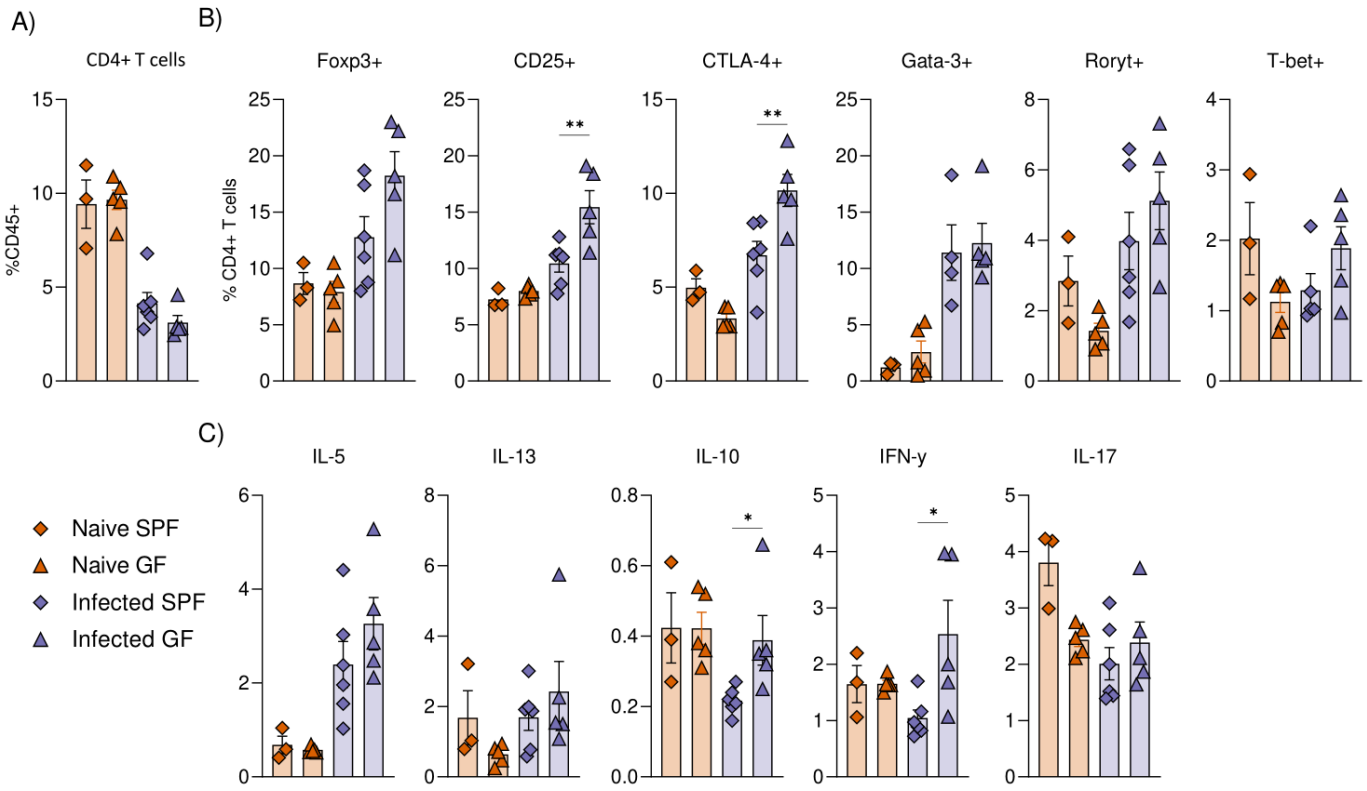


Mixed-sex vs naïve	WPI									
	4		7		9		12		14	
FO					**	↓			**	↑
MZ_T2									***	↑
MZ							***	↓	***	↓
T2_MZP			*	↑	***	↑	***	↑	***	↑
T1										
T2			**	↑	***	↑	*	↑	***	↑

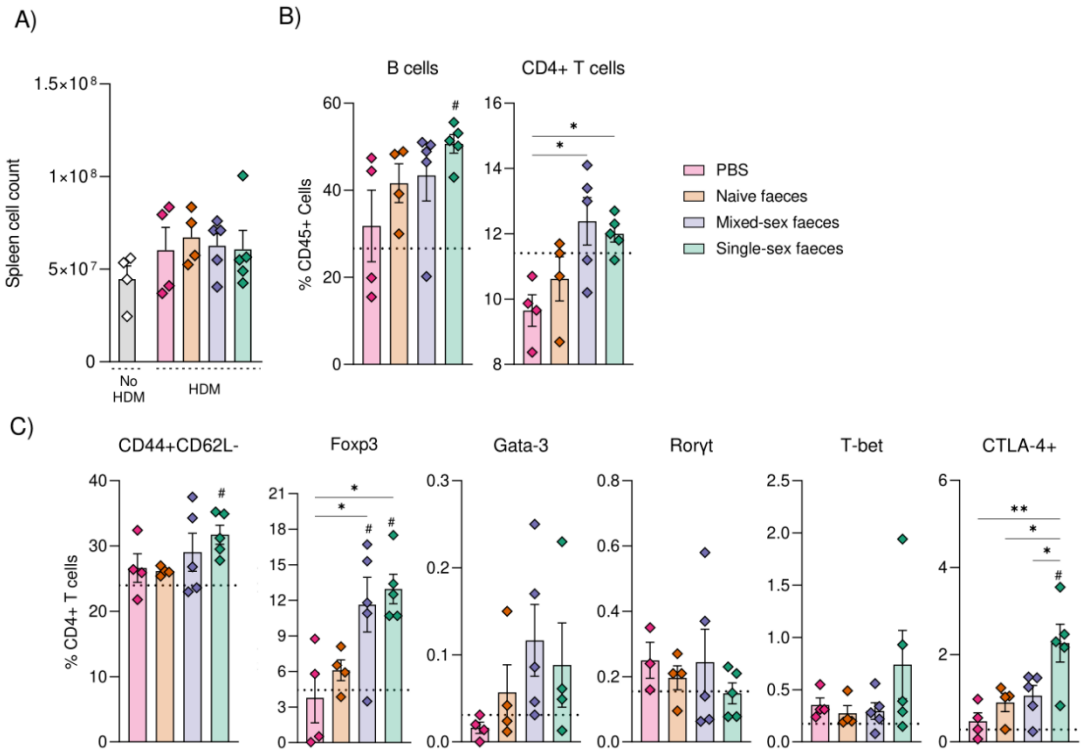
Mixed-sex vs single-sex	WPI									
	4		7		9		12		14	
FO									**	↑
MZ_T2									***	↑
MZ										
T2_MZP					**	↑	*	↑		
T1					**	↑				
T2					*	↑				

Single-sex vs naïve	WPI									
	4		7		9		12		14	
FO									**	↑
MZ_T2										
MZ							**	↓	***	↓
T2_MZP										
T1										
T2			*	↑	*	↑			**	↑

Supplementary Figure 2. B cell phenotypes during schistosomiasis. (A) Representative FACs plots of naïve, mixed-sex and single-sex infected B cells at week 14 of infections. (B) Summary statistics tables showing differences in FO, MZ_T2, MZ, T2_MZP, T1 and T1 proportions (as a % of total B cells) between naïve, mixed-sex and single-sex infected mice. Arrows reflect significant increases or decreases in B cell frequencies, and colours indicate statistical significance.



Supplementary Figure 3. GF or SPF mice were infected with mixed-sex cercariae with infection lasting 7 weeks. (A) Frequency CD4+T cells as a proportion of total CD45+ Cells. (B) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors, as well as CD25 and CTLA-4 in splenic CD4+ T cells. (C) Cytokine secretion from PMA ionomycin stimulated splenocytes cells at week 7 of infection. Results are mean +/- SEM from one single experiment (n=3-6 mice per group). Significant differences were determined by unpaired t test between infected groups. *p < 0.05, **p < 0.01, ***p < 0.001.



Supplementary Figure 4. Splenic alterations following Abx, FMT and HDM challenge. Abx-treated SPF C57BL/6 mice received 2 gavages of PBS (control) or faeces from naïve mice, or those chronically infected (week 14) with single-sex or mixed-sex S.mansoni parasites. After 3 weeks recolonisation, mice were challenged and sensitised to HDM, and culled 2 days after the last HDM challenge for analysis of airway and systemic inflammation. (A) Total spleen counts and (B) the proportion of splenic CD4+ T cells and B cells. (C) The expression of Th1 (T-bet), Th2 (Gata-3), Th17 (Rorγt) and Treg (Foxp3) associated transcription factors and IL-2R (CTLA-4) in splenic CD4+ T cells. Dotted line represents mean values in no HDM control group. Results are mean +/- SEM from one single experiment (n=4-5 mice per group). Significant differences were determined by one-way ANOVA followed Tukey Post hoc testing. *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences between HDM treated and non HDM treated mice are indicated by #.