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Exploring host-immune-microbial interactions during intestinal schistosomiasis

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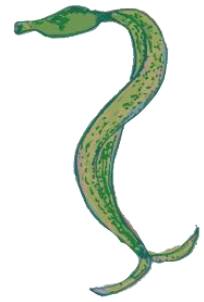
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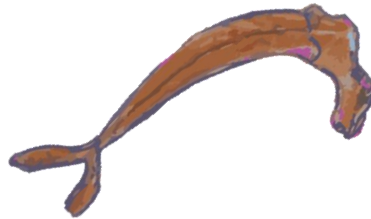


CHAPTER 5

TYPE I INTERFERONS PROVIDE ADDITIVE SIGNALS FOR MURINE
REGULATORY B CELL INDUCTION BY *SCHISTOSOMA MANSONI* EGGS

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Type I interferons provide additive signals for murine regulatory B cell induction by Schistosoma mansoni eggs

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ABSTRACT

The helminth *Schistosoma mansoni* (*S. mansoni*) induces a network of regulatory immune cells, including interleukin (IL)-10-producing regulatory B (Breg) cells. However, the signals required for the development and activation of Breg cells are not well characterized. Recent reports suggest that helminths induce type I interferons (IFN-I), and that IFN-I drives the development of Breg cells in humans. We therefore assessed the role of IFN-I in the induction of Breg cells by *S. mansoni*. Mice chronically infected with *S. mansoni* or intravenously injected with *S. mansoni* soluble egg antigen (SEA) developed a systemic IFN-I signature. Recombinant IFN α enhanced IL-10 production by Breg cells stimulated with *S. mansoni* soluble egg antigen (SEA) *in vitro*, while not activating Breg cells by itself. IFN-I signalling also supported *ex vivo* IL-10 production by SEA-primed Breg cells but was dispensable for activation of *S. mansoni* egg-induced Breg cells *in vivo*. These data indicate that while IFN-I can serve as a co-activator for Breg cell IL-10 production, they are unlikely to participate in the development of Breg cells in response to *S. mansoni* eggs.

Keywords

Type I interferons; regulatory B cells; *Schistosoma mansoni*; chronic infection; interleukin (IL)-10

INTRODUCTION

The helminth *Schistosoma mansoni* induces a network of regulatory immune cells during the chronic phase of infection¹. The induction of B cells with regulatory properties, so called regulatory B (Breg) cells, by *S. mansoni* has been studied extensively²⁻⁵. Breg cells as part of the regulatory network play an important role in limiting immunopathology and attenuate responses to bystander Ags such as allergens⁶. Breg cell-induction, as observed during chronic infection, can be replicated by *in vitro* stimulations with soluble egg antigens (SEA)^{7,8} and the even single, egg-derived molecule IPSE/alpha-1⁸ in the absence of infection. While it is currently unclear which receptors and pathways *S. mansoni*-derived molecules engage, factors consistently reported to be important for Breg cell development and activation are stimulation through the BCR⁹⁻¹², CD40^{9,13-16} and the toll-like receptors (TLR) TLR2/4¹⁷⁻¹⁹, TLR7²⁰ and TLR9¹⁷. Moreover, different cytokines including IL-21²¹, IL-35^{22,23}, BAFF^{24,25}, APRIL²⁶ and type I interferons (IFN-I)²⁷ have been described to support Breg cell development.

IFN-I are a large family of cytokines, containing 14 IFN α subtypes and a single IFN β , central in the immune response to viral infections²⁸. Induced, amongst others, by ligation of pattern recognition receptors (PRRs) of immune and non-immune cells, IFN-I act in an auto- and paracrine manner to induce an antiviral state, but can also interfere with innate and adaptive immune responses^{29,30}. IFN-I can enhance Ag presentation and chemokine production in innate cells, promote effector T cell responses and induce B cell antibody production in viral infection (reviewed in ³⁰). The role of IFN-I in bacterial, fungal and intracellular parasitic (mainly *Leishmania*, *Plasmodium* and *Trypanosoma* spp.) infections is complex, with possible beneficial and detrimental outcomes for the host (reviewed in ²⁸). Only recently, reports have highlighted the potential of helminths or their products to induce IFN-I in mouse models. Infection with the gastrointestinal helminth *Heligmosomoides (H.) polygyrus* has been shown to induce IFN-I signalling in gut and lung in a microbiota-dependent manner, protecting mice from RSV infection ³¹. *S. mansoni* eggs and soluble egg antigens (SEA) have been shown to induce an IFN-I signature both in splenic DCs and in *in vitro* differentiated bone marrow DCs (BMDCs)^{32,33}, and *Nippostrongylus (N.) brasiliensis* induces IFN-I in skin DCs³⁴. A more generalized expression of IFN-stimulated genes (ISGs) in response to *S. mansoni* products has so far only been shown by Webb et al. for whole lung tissue following i.p. sensitization and i.v. challenge with *S. mansoni* eggs³³.

B cells express the IFN α / β receptor (IFNAR) and respond to IFN-I³⁵⁻³⁷. B cell responses to IFN-I are most extensively studied in autoimmunity. In systemic lupus erythematosus (SLE), IFN-I are considered to promote the activation of autoreactive B cells, maturation into plasmablasts and autoantibody production, contributing to disease pathology³⁸. Menon et al. add important knowledge to the picture by showing that plasmacytoid DCs (pDCs) drive the formation of IL-10-producing Breg cells by IFN α production and CD40 ligation in healthy individuals, but fail to do so in SLE patients. While Breg cell-derived IL-10 normally provides an important feedback loop that limits IFN α production, SLE patients have hyperactivated pDCs that fail to induce Breg cells, possibly due to Breg cells being less responsive to supra-optimal concentrations of IFN α ³⁹. In patients with certain types of multiple sclerosis (MS) IFN β therapy is a treatment option commonly applied. It has been reported that IFN β therapy not only increased IL-10 production by monocytes and T cells^{40,41}, but also B cells and plasmablasts⁴².

Whereas Breg cells can be induced by *S. mansoni*-derived Ags *in vitro*, this is less potent than the induction of Breg cells during chronic infection, and the induction of Breg cells by IPSE/alpha-1 has only been demonstrated *in vitro*⁸. Helminth infections trigger a multitude of different immune responses in the host *in vivo*, and it is likely that additional signals, in addition to helminth molecules, are required for optimal Breg cell induction. Here, we sought to address whether IFN-I are central to the induction of Breg cells by *S. mansoni*. We show that both *S. mansoni* infections and intravenous injections with SEA induce a systemic IFN-I signature *in vivo*. Recombinant IFN α enhanced B cell IL-10 production in response to SEA and SEA+aCD40 *in vitro*, while blocking antibodies against IFNAR alpha chain (IFNAR1) reduced the *ex vivo* IL-10 production by *in vivo*-primed B cells. However, B cell induction in response to egg administration *in vivo* was not affected in IFNAR^{-/-} mice. Collectively, these data show that IFN-I provide additive signals for Breg cell induction by *S. mansoni* *in vitro*, but are not crucial for *S. mansoni*-induced Breg cells *in vivo*.

MATERIAL AND METHODS

Animals

Female C57BL/6 mice (Harlan) were housed under SPF conditions in the animal facility of the Leiden University Medical Center (Leiden, The Netherlands). *Ifnar1*^{-/-} mice on an C56BL/6 background were housed at the University of Manchester. All animals were used for experiments at 6-12 weeks of age. All animal studies were performed in accordance with either the Animal Experiments Ethical Committee of the Leiden University Medical Centre or under a license granted by the home office (UK) in accordance with local guidelines.

Preparation of SEA and Eggs

S. mansoni eggs were isolated from trypsinized livers or guts of hamsters after 50 days of infection, washed in RPMI medium supplemented with penicillin (300U/mL), streptomycin (300µg/mL) and amphotericin B (300µg/mL) and stored at -80°C until use. SEA was prepared as previously described⁴⁷. Protein concentration was determined by BCA. SEA preparations were routinely tested for endotoxin contamination by Limulus Amoebocyte Lysate (LAL) assay or TLR4-transfected HEK reporter cell lines.

S.mansoni infections and in vivo injections

For the high dose infection model, mice were percutaneously infected with approximately 180 cercariae and serum collected on day 49 after infection. For the evaluation of splenic ISG expression in SEA/IFNα treated mice, mice were intravenously injected with 50ug SEA in PBS or intraperitoneally injected with PBS or IFNα (20x10³ units). Splenocytes were harvested 12 hours after injection, snap-frozen and stored -80°C for later analysis. For the egg challenge model in IFNAR^{-/-} mice, mice received two intraperitoneal injections (day 0 and day 7) of 5000 *S.mansoni* eggs diluted in sterile PBS. Mice were sacrificed 7 days after the last injection.

Splenocyte and B cell isolation

Spleens were homogenized by passage through a 70µM cell strainer (BD Biosciences) and erythrocytes depleted from the single cell suspension by lysis. B cells were purified from splenocytes by anti-CD19 MicroBeads (Miltenyi Biotech) following the manufacturer's instructions. For cell sorting experiments, MACs-isolated CD19⁺ B cells were sorted by flow cytometry into FO B cells (CD23⁺CD21^{low}) and MZ B cells (CD23⁻CD21^{hi}).

In vitro stimulation

Splenic CD19⁺ B cells, MZ B cells and FO B cells (1.5×10^6 /mL) were cultured in medium (RPMI 1640 GlutaMAX; Thermo Fisher Scientific) supplemented with 5% heat-inactivated fetal calf serum (FCS; Greiner Bio-One) 2-mercaptoethanol (5×10^{-5} M), penicillin (100U/mL) and streptomycin (100µg/mL; all Sigma-Aldrich). Cells were stimulated with the following stimuli as indicated in the figures: SEA (20µg/mL), aCD40 (clone 1C10; 0.5µg/mL; Biolegend), recombinant IFN α (Biolegend), CpG ODN 1826 (class B; 0.2-1µM; Invivogen), aCD40L blocking antibody (clone MR1; 10µg/mL; kind gift from L. Boon, Bioceros), aIFNAR1 blocking antibody (clone MAR1-5A3; 10µg/mL; eBioscience). After 2 days (FO and MZ B cells) or 3 days (total B cells) culture at 37°C, supernatants were harvested for cytokine analysis by ELISA or CBA. For flow cytometric analysis of IL-10, cells were re-stimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) for 4 hours in the presence of Brefeldin A (10 µg/ml; all Sigma-Aldrich).

Flow cytometry

Cells were stained with antibodies against B220 (clone RA3-6B2), CD21 (clone 7G6), CD23 (clone B3B4) and IL-10 (clone JESS-16E3). Dead cells were stained with live/dead fixable aqua dead cell stain kit (ThermoScientific). Fc γ R-binding inhibitor (2.4G2, kind gift of L. Boon, Bioceros) was added to all stainings. Flow cytometry was performed on a FACS Canto II using FACSDiva software (BD Biosciences) followed by data analysis using FlowJo.

ELISA and CBA

The concentration of IL-6 and IL-10 in cell-free culture supernatants was assessed by OptEIA ELISA kits (BD Biosciences) (Total B cells) or BD cytometric bead array (CBA) Flex-set kits (BD Biosciences) (MZ and FO B cells). The concentration of cytokines in serum of chronically infected mice was also assessed by CBA Flex-set kits, except for IFN α 3 and IFN β which were measured by ELISA (PBL).

RNA extraction and qPCR analysis

RNA from frozen splenocytes was extracted using TriPure isolation reagent (Roche) and translated to cDNA using SuperScript™ III Reverse Transcriptase and Oligo (dT; Life Technologies). Quantitative PCR was performed using SYBR Green Master Mix (Applied Biosystems) using a Biorad CFX96 Real-time system C1000 thermal cycle. Expression levels were normalized to *Gadph*. The following primers were used:

RPLP0: 5'- TCTGGAGGGTGTCCGCAACG-3' 5'- GCCAGGACGCGCTTGTACCC-3'; *MX1*: 5'- TTCAAGGATCACTCATACTTCAGC-3' 5'-GGGAGGTGAGCTCCTCAGT-3'; *Oas1a*: 5'- GCTGCCAGCCTTTGATGT-3' 5'-TGGCATAGATTGTGGGATCA-3';

Ifit3 5'-TGAAGTCTCAGCCCACA-3' 5'-TCCCGGTTGACCTCACTC-3';

Stat11 5'-GTGCCTCTGGAATGATGGGT-3' 5'-GAAGTCAGGTTACCTCCGT-3';

Ifi30 5'-GAACATGGTGGAGGCCTGTC-3' 5'-TGGCGCACTCCATGATACTC-3';

Ifit1 5'-TCTAAACAGGGCCTTGACAG-3' 5'-GCAGAGCCCTTTTGGATAATGT-3'

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.02). All data are presented as mean $\pm$ standard error of the mean (SEM). P-values < 0.05 were considered statistically significant.

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Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

RESULTS

S. mansoni infections and SEA injections induce a systemic IFN signature *in vivo*

We first sought to assess whether chronic *S. mansoni* infection induces a systemic IFN-I signature. High-dose infection with 180 *S. mansoni* cercariae significantly increased the serum concentration of IFN α 3 in the majority of animals (Figure 1A), while lower doses of 20-80 cercariae did not (Suppl. Figure 1). Systemic levels of IL-5 and IL-12/23p40 were similarly increased, while IFN β , IL-10, and IL-17 were only elevated in a minority of animals (Figure 1A). The production of IFN-I subtypes is often difficult to assess, as they are frequently produced at low levels and transiently, or consumed by neighbouring cells following production, which might explain the high dose of infection necessary to reliably detect IFN-I in the serum. Irrespective, the significant increase in serum IFN-I following high-dose infection supports the notion that *S. mansoni* induces a systemic IFN-I signature.

Next, to investigate the contribution of egg-products to IFN-I induction, we examined the expression of interferon stimulated genes (ISGs) in the splenocytes of mice intravenously injected with SEA after 12 hr (Figure 1B). SEA-treated mice demonstrated a clear IFN-I signature, with significant upregulation shown for the *Mx1* and *Oas1a*, and a trend towards enhanced expression for *Ifit3* and *Ifit1*. Additionally, SEA-treatment induced *Oas1a* expression to a similar level to that observed for IFN α -treated control mice (Supplementary Figure 2). Collectively, these data suggest that egg-exposure alone, in the absence of worms, is sufficient to drive IFN-I responses.

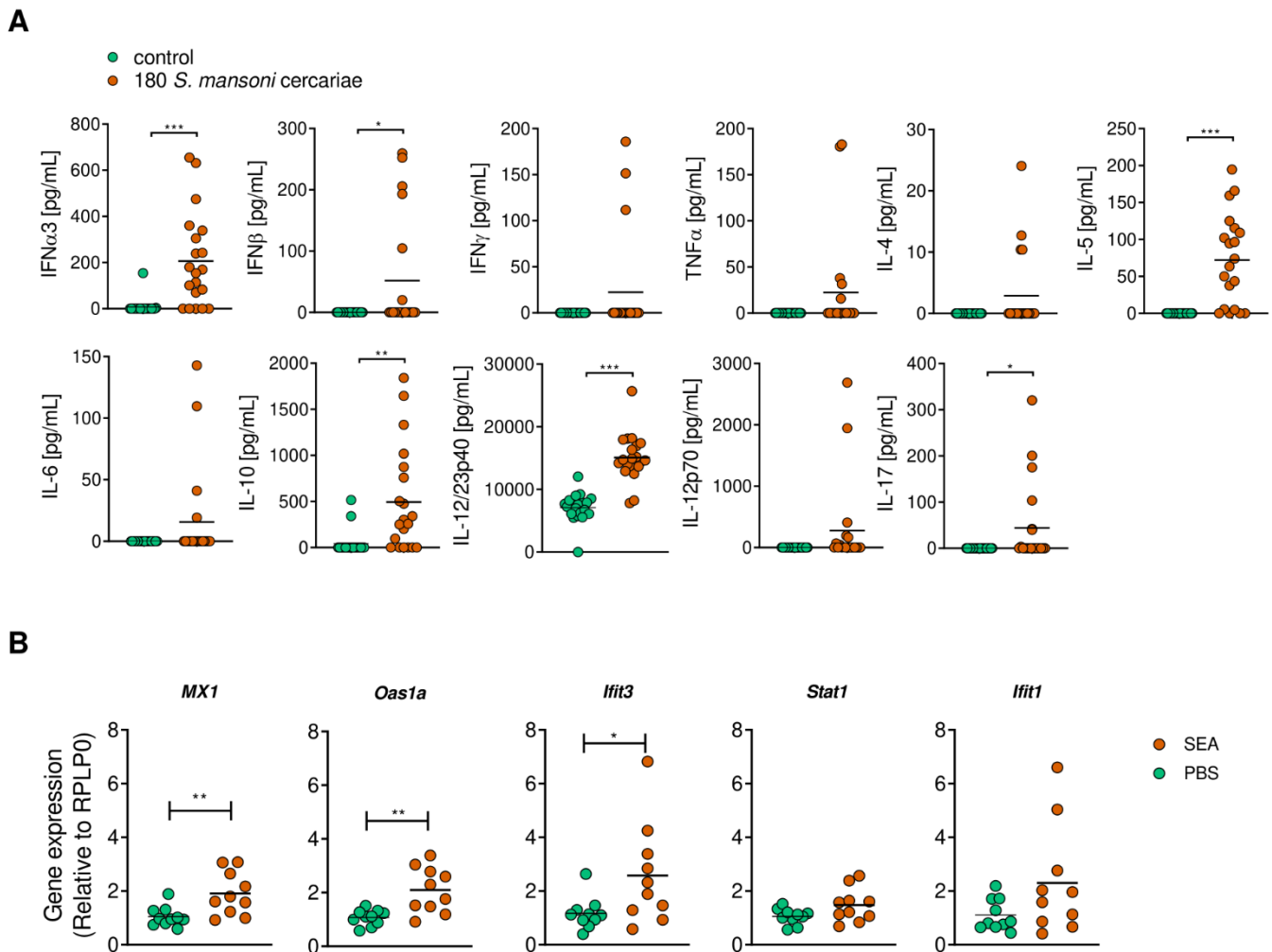


Figure 1: *S. mansoni* infections and SEA injections induce a systemic type I IFN signature. (A) Mice were infected with 180 *S. mansoni* cercariae and serum samples taken at d49 of infection for assessment of cytokine levels by ELISA/CBA. Pooled data from 2 experiments, n=20/group. (B) Splenocytes from SEA injected mice were harvested 12 hours post injection. The mRNA expression of interferon responsive genes (ISGs) was evaluated by qPCR (normalised against RPLP0). Data from 2 experiments n=2-8 per group. Significant differences were determined by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Recombinant IFN α enhances SEA/aCD40-induced B cell IL-10 production *in vitro*

We have previously demonstrated that SEA induces B cell IL-10 production and that CD40 ligation enhances SEA-induced Breg cell development⁸, while others have reported a synergistic effect of IFN α and CD40 ligation on the development of IL-10-producing human B cells³⁹. We therefore tested

the effect of simultaneous stimulation of splenic B cells with SEA, anti-CD40 and recombinant IFN α *in vitro*. After 3 days of culture, the concentration of IL-10 in culture supernatants of SEA-stimulated B cells increased with increasing doses of IFN α , whereas IFN α alone had no effect (Figure 2A; Supplementary Figure 3B for gating scheme). The strongest induction of B cell IL-10 production could be observed when cells were co-stimulated with SEA and anti-CD40 compared to SEA alone (Figure 2A). IFN α at concentrations of 10^3 - 10^4 U/mL (equivalent to circa 15-150ng/mL) significantly enhanced IL-10 production in response to SEA and SEA+anti-CD40, whereas IL-10 production seemed to plateau at 10^5 U/mL IFN α (Figure 2A). IL-10 production after co-stimulation with IFN α increased up to 4-fold compared to the control condition without addition of IFN α . IFN α also enhanced IL-6 production, a pro-inflammatory cytokine known to be produced by B cells, in response to SEA and anti-CD40, albeit to a lesser extent (Figure 2A). This indicated a pattern of cytokine expression characteristic for Breg cells. Conversely, the percentage of IL-10-producing B cells after 3 days of stimulation with SEA or SEA+anti-CD40 in the presence of IFN α did not increase (Figure 2B), which suggests that the peak of the stimulatory activity of IFN α occurs early and has passed, possibly due to a decline in the IFN α concentration in culture supernatant due to consumption, when the intracellular staining was performed after 3 days of culture. As a control, we also stimulated B cells with CpG ODN1826 (class B) and IFN α . Already a low concentration of 10^3 U/mL IFN α strongly amplified the CpG ODN1826-induced cytokine production (Supplementary Figure 4A) and percentage of IL-10-producing B cells (Supplementary Figure 4B). These data show that IFN α provides additional signals for the induction of B cell IL-10 production in cells activated with known Breg cell-inducing stimuli SEA or CpG ODN1826.

To identify which B-cell subset produces IL-10 in response to IFN α stimulation, splenic B cells were sorted into the two main subsets, CD23^{low}CD21⁺ marginal zone (MZ) B cells and CD23^{hi}CD21⁻ follicular (FO) B cells, for subsequent 2-day *in vitro* stimulation with SEA, anti-CD40 and recombinant IFN α (10^4 U/mL) (Supplementary Figure 3B for gating scheme). Unlike their FO counterparts, MZ B cells reacted potently to the addition of IFN α to the culture media (Figure 2C), with IFN α -treated MZ B cells demonstrating a 4-fold increase in IL-10 secretion compared to MZ B cells cultured in media alone. Importantly, the effect of IFN α -stimulation was further potentiated by the addition of SEA or SEA+antiCD40 to the culture media, with the highest production of IL-10 shown for MZ B cells cultured in the presence of IFN α , SEA and antiCD40. In contrast, FO B-cells produced relatively little IL-10 irrespective of IFN α -, SEA or SEA+antiCD40 stimulation. In comparison to unstimulated FO B cells, FO B-cells treated with IFN α -, SEA +/- antiCD40 produced significantly higher amounts of IL-

10. However, these IL-10 levels were considerably lower to that produced by MZ B cells under the same stimulation conditions. Like that observed for total B cells (Figure 2B), the percentage of IL-10 producing MZ B cells remained unaltered by IFN α -stimulation (Figure 2D), supporting the notion that peak time of IFN α stimulatory activity has already been reached. On the other hand, the percentage of IL-10 producing FO B cells even appeared to decrease following IFN α -stimulation (Figure 2D). As for IL-6, only MZ B-cells but not FO B cells showed IL-6 production following IFN α stimulation (Figure 2C). However, similar to that described for total B cells (Figure 2A), these IL-6 levels were considerably lower than that detected for IL-10. Altogether, these data demonstrate a heightened responsiveness of MZ B cells to IFN α and schistosome Ag stimulation, and support the notion that IFN-Is and schistosome Ags synchronously drive regulatory B cell activity *in vitro*.

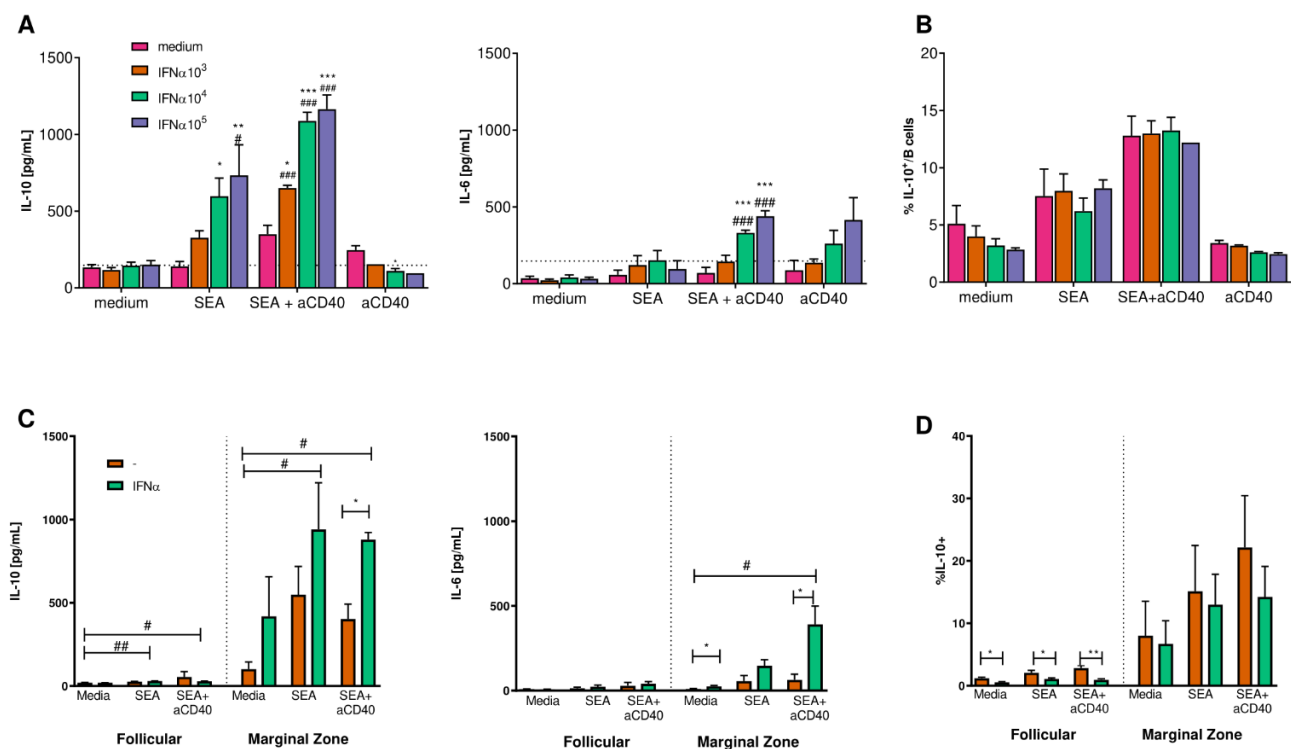


Figure 2: Recombinant IFN α enhances SEA/aCD40-induced B cell IL-10 production. (A-B) B cells were isolated from the spleen of naïve mice and stimulated *in vitro* with SEA (20 μ g/mL), anti-CD40 (0.5 μ g/mL) and IFN α (103-105U/mL) as indicated. After 3 days of culture, supernatants were analysed for IL-10 and IL-6 concentration by ELISA (A), and the percentage of IL-10⁺ B cells assessed by flow cytometry (B). (C- D) Splenic marginal zone and follicular B cells from naïve mice were sorted using flow cytometry and cultured for 2 days in the presence of SEA (20 μ g/mL), anti-CD40 (0.5 μ g/mL) and IFN α (104U/mL). IL-6 and IL-10 production as measured by CBA (C) and frequency of IL-10⁺ cells in each respective subset as determined by flow cytometry (D). Summary of 4 (A) or 3 (B-D) experiments, each data point is the mean of two-four technical replicates. Data are presented as mean $\pm$ SEM. Significant differences are indicated by * p < 0.05, ** p < 0.01, *** p < 0.001 and determined by one-way ANOVA followed by Dunnett's multiple comparisons test. # p < 0.05, ## p < 0.01, ### p < 0.001 indicates significant difference relative to medium only control.

IFNAR1 signalling provides co-signals for IL-10 production by *in vivo* primed B cells

To assess whether IFN-I signalling provides important signals for IL-10 production by *in vivo* primed Breg cells, we treated mice with SEA i.p. and subsequently restimulated total splenocyte cultures *ex vivo* with SEA, in the presence or absence of blocking antibodies against IFNAR1. We also used blocking antibodies against CD40 ligand (CD40L) upon *ex vivo* restimulation to assess the importance of CD40 co-ligation on B cells for IL-10 induction. While blocking CD40L alone, or in combination with blocking IFNAR1, had either no or no additional effect, blocking IFNAR1 signalling significantly reduced the concentration of IL-10 in 2-day culture supernatants (Figure 3A). The production of IL-6 was not affected by either of the blocking agents (Figure 3A), while the percentage of IL-10 producing B cells in culture was mildly but significantly reduced by both blocking agents (Figure 3B). We concluded that signalling via IFNAR1, but not the ligation of CD40, is essential for SEA-induced B cell IL-10 production in this setting.

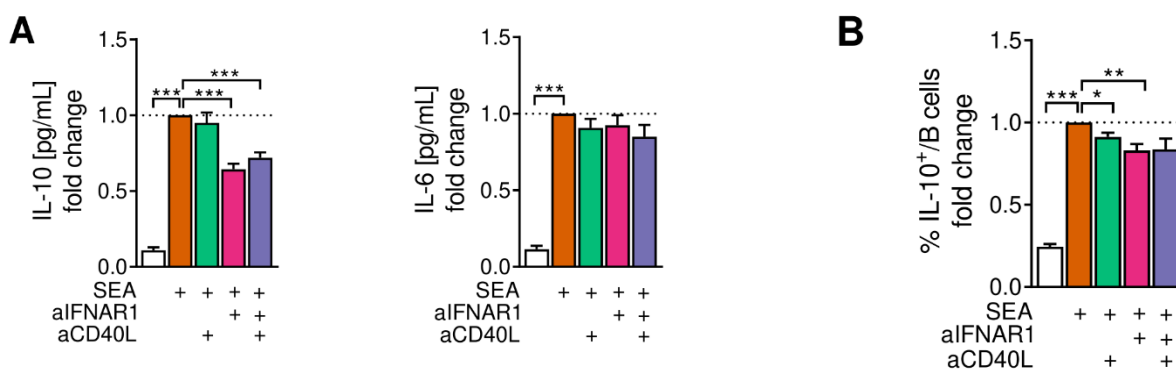


Figure 3. Ex vivo block of IFNAR1 reduces B cell IL-10 production. Splenocytes from SEA-injected mice (100µg SEA i.p. on d0 & d7; section d14) were re-stimulated *ex vivo* with SEA (20ug/mL) for 2 days in the presence or absence of anti-CD40L (10µg/mL) and anti-IFNAR1 (10µg/mL) blocking antibodies as indicated. After 2 days of culture, supernatants were analysed for IL-10 and IL-6 concentration by ELISA (A), and the percentage of IL-10⁺ B cells assessed by flow cytometry (B). Summary of 2 experiments, n=10/group. Data are presented as mean ± SEM. Significant differences were determined by RM-One Way ANOVA & Dunnett's post test comparing all groups to the SEA-stimulated positive control. * p < 0.05, ** p < 0.01, *** p < 0.001.

IFNAR1 signalling is dispensable for Breg cell induction *in vivo*

To assess whether IFN-I signalling provides important signals for Breg cell development and IL-10 production in response to *S. mansoni* egg products not only *in vitro* but also *in vivo*, we induced Breg cell development by two doses of i.p. administered *S. mansoni* eggs (5000) in WT control or IFNAR1^{-/-} mice, a model we previously showed to be very suitable to demonstrate schistosome-induced splenic Breg cell development⁸. The absence of IFNAR1 did not affect the concentration of IL-10 in B cells and total splenocyte culture supernatants in response to restimulation with SEA and anti-CD40 (Figure 4A). In addition, the percentage of IL-10⁺ B cells seemed increased rather than

decreased in IFNAR1^{-/-} mice (Figure 4B). Additionally, no changes in IL-10 production could be observed when blocking IFNAR1 signalling by means of *in vivo* administration of anti-mouse IFNAR1 blocking antibody (suppl. Figure 5). Thus, IFNAR1 signalling seems to be dispensable for the induction of Breg cells to *S. mansoni* egg challenge *in vivo*.

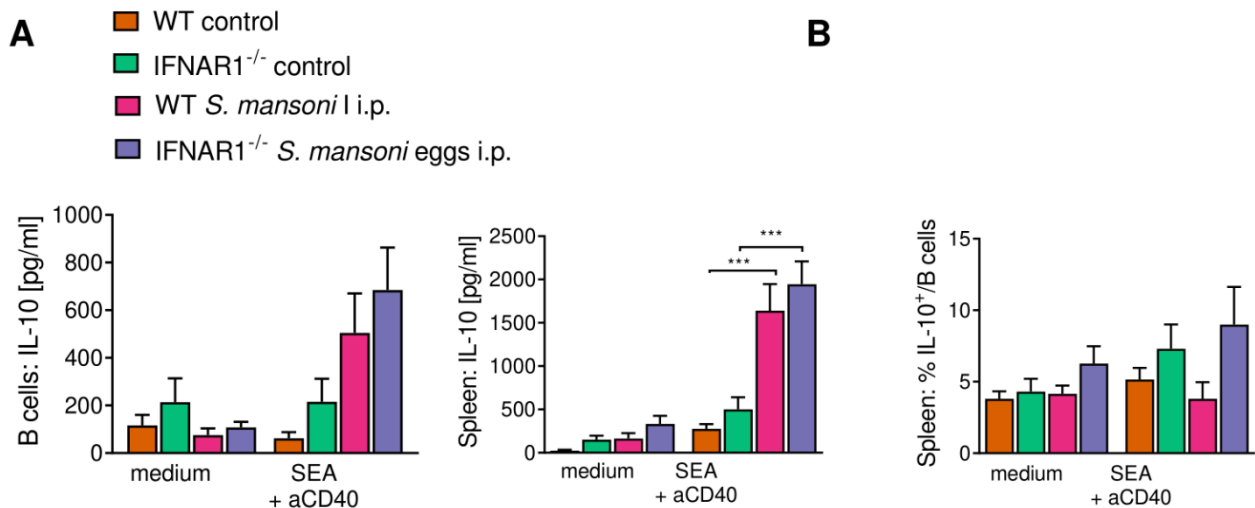


Figure 4. IFNAR1 signalling is dispensable for Breg cell induction in vivo. Splenocytes and MACS-isolated CD19⁺ B cells from *S. mansoni* egg-injected mice (5000 *S. mansoni* eggs i.p. on d0 & d7; section d14) were re-stimulated ex vivo with SEA (20μg/mL) and anti-CD40 (2μg/mL) for 2 days. After 2 days of culture, supernatants of isolated B cell and total splenocyte cultures were analysed for IL-10 concentration by ELISA (A), and the percentage of IL-10⁺ B cells within splenocyte cultures assessed by flow cytometry (B). Summary of 2 experiments, n=8-10/group. Data are presented as mean ± SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. ** p < 0.01, *** p < 0.001

DISCUSSION

In this study we sought to address whether IFN-I might provide the 'missing link', synergizing with *S. mansoni*-derived signals for the induction of Breg cell IL-10 production. We show that, although *S. mansoni* infections and injections with egg Ags induce systemic IFN-I signature, and IFN-I signalling enhances *in vitro* IL-10 production by Breg cells exposed to *S. mansoni* Ags, IFN-I responsiveness is ultimately dispensable for Breg cell induction by *S. mansoni* eggs *in vivo*.

We and others have previously shown that chronic *S. mansoni* infection induces Breg^{3, 4, 43, 44} and that this Breg cell-inducing effect can be replicated by isolated eggs, SEA and even the single, egg-derived molecule IPSE/alpha-1 in the absence of adult worms and a natural infection^{7, 8}. Components of SEA directly bind to splenic B cells⁸, but the receptors ligated and signalling pathways activated by these Ags remain to be identified. Moreover, SEA immunization is less potent than chronic infection at Breg cell induction *in vivo*, and the induction of Breg cells by IPSE/alpha-1 has only been demonstrated *in vitro*⁸. Helminth infections trigger a multitude of different immune responses in the host *in vivo*, and it is likely that additional signals, in addition to helminth molecules, are required for optimal Breg cell induction.

We found an increased concentration of IFN-I in serum of mice actively infected with *S. mansoni*, and enhanced expression of ISGs in the spleen of SEA-injected mice. These data are in line with previous reports on the capacity of *S. mansoni* eggs or egg Ags, *H. polygus* infection and *N. brasiliensis* Ags to induce IFN-I³¹⁻⁴³. pDCs are considered an important source of IFN-I⁴⁵. IFN-I were however produced by conventional DCs (cDCs) rather than pDCs after SEA-stimulation of BMDCs *in vitro*³³. We have not addressed the cellular source of IFN-I in our study, therefore both pDCs and cDCs remain possible sources. Notably, little is reported to date regarding IFN-I production by human DCs in response to helminths but work of our own group suggests that *S. mansoni* egg Ags do not induce IFN-I in human monocyte-derived DCs (Everts, personal communication).

We show here that recombinant IFN α , while having no measurable effect on its own, significantly, and dose-dependently increased IL-10 production by B cells in response to *in vitro* stimulation with SEA alone or SEA+aCD40. Additionally, in harmony with previous mechanistic studies⁸ and models of chronic *S. mansoni* infection⁴⁶, we provide evidence that MZ B cells, as opposed to FO B cells, are responsible for this increase in IL-10. IFN α was also shown to have a synergistic effect on SEA+aCD40-induced IL-6 production, albeit to lesser extent. Conversely, the percentage of IL-10⁺ B cells was unchanged or slightly reduced after 3 days of culture in the presence of increasing amounts of IFN α , suggesting that IFN-I may change the dynamics and timing of IL-10 production. Menon et

al. observe an optimal IL-10 induction in naïve TLR9-stimulated B cells at 50×10^5 U/mL IFN α and a less effective stimulation at higher concentrations³⁹, whereas we find an additive effect even at 1×10^6 U/mL on both SEA- and TLR9-stimulated B cells on IL-10 concentration in culture supernatants. The fact that IFN α has no effect at all on IL-10 or IL-6 expression by itself underpins that IFN-I signalling modulates responses in pre-activated B cells rather than providing an activation signal to B cells by itself, which has been similarly reported by others^{27,42}. In this context, it is plausible that stimulation with *S. mansoni*-derived Ags *in vitro* provides this pre-activation signal, rather than SEA- and IFN-I-specific signalling pathways synergizing to promote B cell IL-10 production. This is in line with previous reports describing IFN-I signalling to regulate B cell responses to other pre-activating stimuli such as BCR or TLR7 ligation^{35,36}. In this context, Braun et al. show that murine, mature splenic B cells get partially activated by treatment with IFN α/β , characterized by the upregulation of activation markers and increased survival in the absence of proliferation or terminal differentiation, and display enhanced response to BCR ligation³⁵. Poovassery and colleagues report that both BCR and IFNAR signalling restore TLR7-induced B cell hyporesponsiveness³⁶. That the percentage IL-10⁺ B cells tends to decrease at the end of culture might suggest that the peak of IFN α stimulatory activity has occurred earlier and that after 3 days of culture the IFN-I concentration in culture supernatant has already declined, making an earlier time point for the assessment of IL-10⁺ B cells preferable.

Arguably, *in vitro* stimulation of isolated B cells with recombinant IFN α does not mimic the natural situation very well. We therefore also assessed the role of IFN-I signalling on Breg cell recall responses *ex vivo*. Blocking IFNAR1 upon *ex vivo* restimulation of *in vivo* SEA-induced Breg cells significantly reduced IL-10, but not IL-6 production. Adding blocking antibody against CD40L to the cultures, and thereby preventing the ligation of CD40 expressed on B cells by accessory cells present in whole splenocyte cultures, had only negligible effects. This might indicate that, while CD40 ligation has previously been shown to enhance B cell IL-10 expression^{8,9,15}, it does not provide additional signals for B cell IL-10 production in this restimulation setting. This might point at a difference in the contribution of CD40 signalling to Breg cell induction upon concurrent priming of B cells with an Ag and agonistic anti-CD40⁸ and upon *ex vivo* restimulation as performed in this study. Alternatively, it is also possible that insufficient CD40 ligation was occurring in our chosen culture conditions, and so explains why blocking CD40 had a negligible effect. Finally, although injections with *S. mansoni* egg-products can effectively drive both systemic IFN-I responses and Breg development⁸, we found B cell IL-10 production to be unaltered in IFNAR1^{-/-} mice upon egg i.p. administration, suggesting that IFN-I signalling is dispensable in this setting. However, it is unknown

whether other signals have compensated for the lack of IFN-I, and so, a full blown Breg response is still driven in IFNAR^{-/-} mice. In addition, we have not tested the role of IFN-I during schistosome infections in IFNAR^{-/-} mice and therefore cannot exclude that IFN-I produced in response to repetitive stimulations and chronic inflammation may still contribute to Breg cell development during schistosomiasis. Combined, our data suggests that *in vivo*, where multiple pathways are activated simultaneously and potentially act synergistically, IFN-I signalling is not essential for the development and activation of Breg cells against *S. mansoni* eggs.

The physiological role of IFN-I in helminth infections has not been extensively studied to date. Enteric *H. polygyrus*-induced IFN-I protects from RSV co-infection³¹. SEA-stimulated BMDCs induce IFN-I³², and SEA-stimulated cDCs as well as skin DCs exposed to *N. brasiliensis* were shown to be dependent on IFN-I signalling for their effective induction of Th2 response^{33,34}. Therefore, more research is needed to fully understand the role of IFN-I in helminth, and more specifically in *S. mansoni* infections.

Collectively, the data presented here show that, while IFN-I can enhance IL-10 production by *S. mansoni*-activated Breg cells both *in vitro* and *ex vivo*, IFN-I signalling is dispensable for the formation and activation of *S. mansoni*-induced Breg cells *in vivo*. A better understanding of the signals for optimal Breg cell development and activation is required to develop novel therapies around Breg cells.

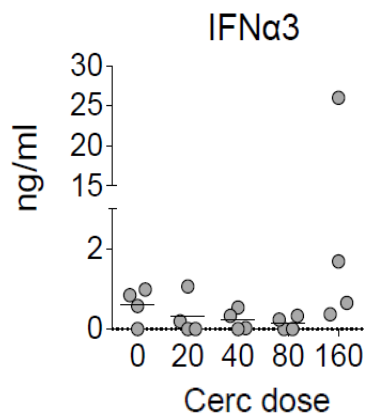
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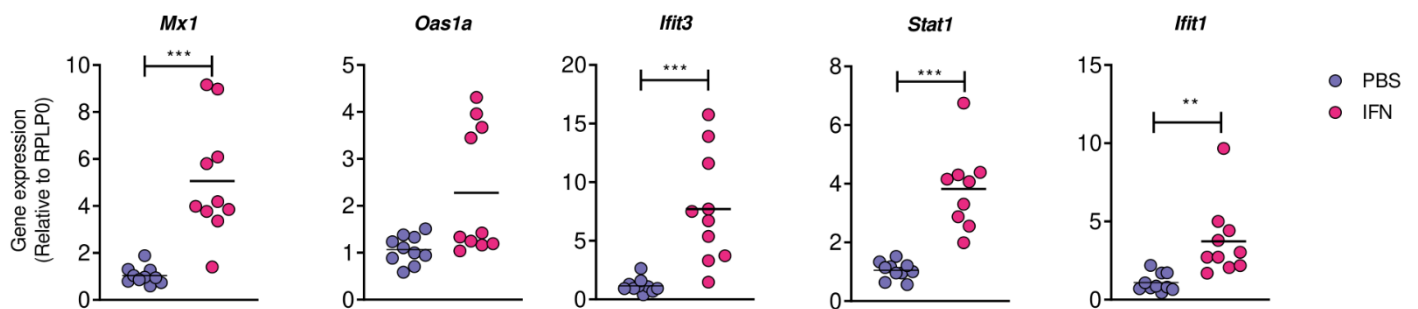
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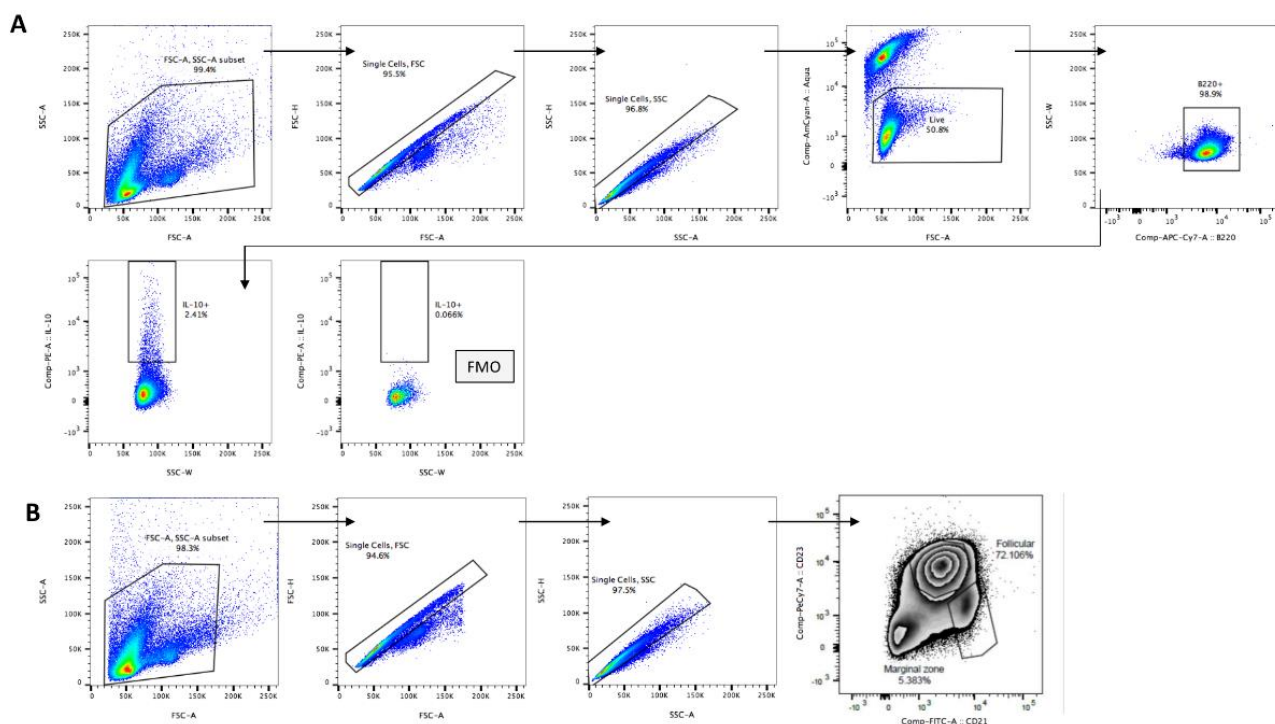
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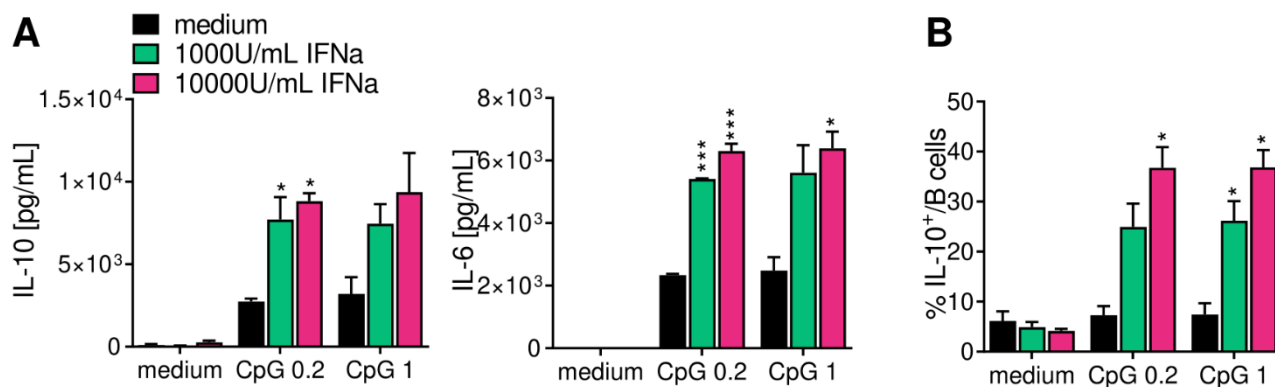
Supplementary Figure 1. A trend towards enhanced IFN α 3 levels in the serum of mice infected with 20-160 cercariae. Mice were infected with 20-160 cercariae and serum samples were taken at d56 of infection for assessment of cytokines by ELISA. Data from 1 experiment, with 4 mice per group.



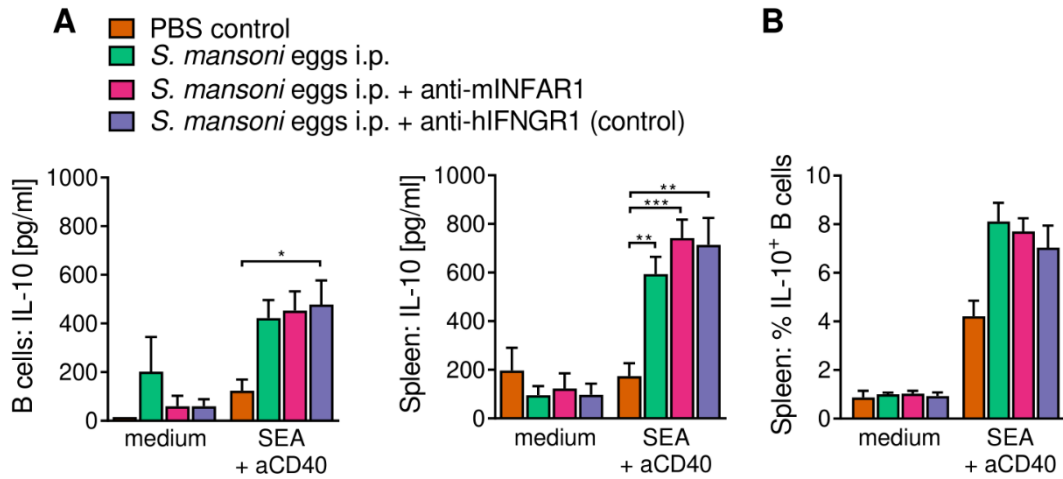
Supplementary Figure 2: Expression of interferon stimulated genes in IFN α injected mice. 12 hours post-injection, splenocytes were isolated from PBS- and IFN α -treated mice. The mRNA expression of interferon-stimulated genes (ISGs) was assessed by qPCR (relative to RPLP0). Data from 2 experiments, n=2-8 per group. Significant differences were determined by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplementary Figure 3. Flow cytometry gating schemes. (A) Identification of IL-10 production from 3-day cultured B cells. (B) Cell sorting scheme for follicular and marginal zone B cell subsets from MACs isolated B cell.



Supplementary Figure 4. Recombinant IFN α enhances CpG-induced B cell IL-10 and IL-6 production. B cells were isolated from the spleen of naïve mice and stimulated in vitro with CpG ODN1826 (class B; 0.2-1 μ M) and IFN α (103-104 U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 concentration by ELISA (A), and % IL-10 B cells assessed by flow cytometry (B). Summary of 2-3 experiments, each data point is the mean of two technical replicates. Data are presented as mean $\pm$ SEM. Significant differences were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$.



Supplementary Figure 5. IFNAR1 signalling is dispensable for Breg cell induction in vivo. Mice were treated as depicted in A. On day 14, spleens were harvested and total splenocyte cell suspensions and isolated CD19⁺ B cells restimulated with SEA (20ug/ml) and anti-CD40 (2ug/ml) for 2 days. Supernatants were analyzed for IL-10 and IL-6 concentration by ELISA (B), and the percentage of IL-10⁺ B cells assessed by flow cytometry (C). Data from one experiment, n=5/group. Data are presented as mean $\pm$ SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.