Immune modulation by schistosomes: mechanisms of regulatory B cell induction and inhibition of allergic asthma

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Chapter 5

TYPE I INTERFERONS PROVIDE ADDITIVE SIGNALS FOR REGULATORY B CELL INDUCTION BY *S. MANSONI IN VITRO*, BUT DO NOT SYNERGIZE WITH *S.MANSONI*-SPECIFIC SIGNALS *IN VIVO*

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ABSTRACT

The helminth *Schistosoma 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*. Chronic *S. mansoni* infection induced a systemic IFN-I signature. Recombinant IFNα 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 show that while IFN-I can serve as a co-activator for Breg cell IL-10 production, they are not central *in vivo* in response to *S. mansoni*.

INTRODUCTION

The helminth *Schistosoma mansoni* induces a network of regulatory immune cells during the chronic phase of infection [1]. The induction of B cells with regulatory properties, so called regulatory B (Breg) cells, by *S. mansoni* has been studied extensively [2-5]. Breg cells as part of the regulatory network play an important role in limiting immunopathology and attenuate responses to bystander antigens such as allergens [6]. Breg cell induction as observed during chronic infection can be replicated by soluble egg antigens (SEA) [7, 8] and even the single, egg-derived molecule IPSE/alpha-1 [8] 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 B cell receptor (BCR [9-12]), CD40 [9, 13-16] and the toll-like receptors (TLR) TLR2/4 [17-19], TLR7 [20] and TLR9 [17]. Moreover, different cytokines including IL-21 [21], IL-35 [22, 23], BAFF [24, 25], APRIL [26] 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 IFNα subtypes and a single IFNβ, central in the immune response to viral infections [28]. 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 antigen 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 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 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 brasiliensis* induces IFN-I in skin DCs [34]. 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* eggs [33].

B cells express the IFNα/β receptor (IFNAR) and respond to IFN-I [35-37]. 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 pathology [38]. 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 IFNα 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 IFNα production, SLE patients have hyper-activated pDCs that fail to induce Breg cells, possibly due to Breg cells being less responsive to supra-optimal concentrations of IFNα [39]. In patients with certain types of multiple sclerosis (MS) IFNβ therapy is a commonly applied treatment option. It has been reported that IFNβ therapy not only increased IL-10 production by monocytes and T cells [40, 41], but also B cells and plasmablasts [42].

Whereas Breg cells can be induced by *S.mansoni*-derived antigens *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 *S. mansoni* infection induced a systemic IFN-I signature *in vivo*. Recombinant IFNα 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*.

RESULTS

S. mansoni induces a systemic IFN-I 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 IFNα3 in the majority of animals (**Figure 1**), while lower doses of 20-40 cercariae did not (data not shown). Systemic levels of IL-5 and IL-12/23p40 were similarly increased, while IFNβ, IL-10, and IL-17 were only elevated in a minority of animals (**Figure 1**). 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.

Recombinant IFNα 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 development [8], while others have reported a synergistic effect of IFNα and CD40 ligation on the development of IL-10-producing human B cells [39]. We therefore tested the effect of simultaneous stimulation of splenic B cells with SEA, agonistic anti-CD40 antibody (aCD40) and recombinant IFNα *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 IFNα, whereas IFNα alone had no effect (**Figure 2A**). The strongest induction of B cell IL-10 production could be observed when cells were co-stimulated with SEA and aCD40, compared to SEA alone (**Figure 2A**). IFNα 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+aCD40, whereas IL-10 production seemed to plateau at 10^5 U/mL IFNα (**Figure 2A**). IL-10 production after co-stimulation with IFNα increased up to 4-fold compared to the control condition without addition of IFNα. IFNα also enhanced IL-6 production, a pro-inflammatory cytokine known to be produced by B cells, in response to SEA and aCD40, 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+aCD40 in the presence of IFNα did not increase (**Figure 2B**). This suggests that the peak of the stimulatory activity of IFNα occurs earlier, possibly because of a decline in the IFNα concentration in culture supernatant due to consumption, and had already passed 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 IFNα. Already a low concentration of 10^3 U/mL IFNα strongly amplified the CpG ODN1826-induced cytokine production (**suppl. Figure 1A**) and the percentage of 10-producing B cells (**suppl. Figure 1B**). These data show that IFNα 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.

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.

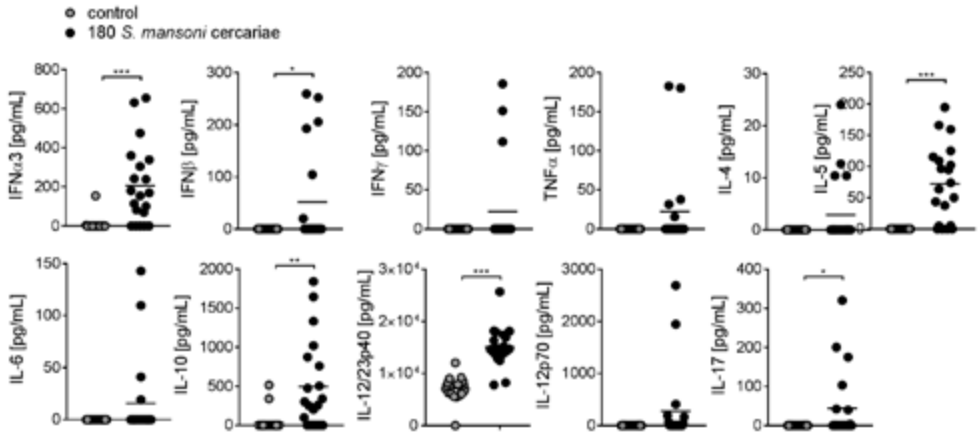

Figure 1. *S. mansoni* induces a systemic type I IFN signature. Mice were infected with 180 *S. mansoni* cercariae and serum samples taken at d49 of infection for assessment of cytokine levels by ELISA/CBA. Pooled data from 2 experiments, n=20/group. Significant differences were determined by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

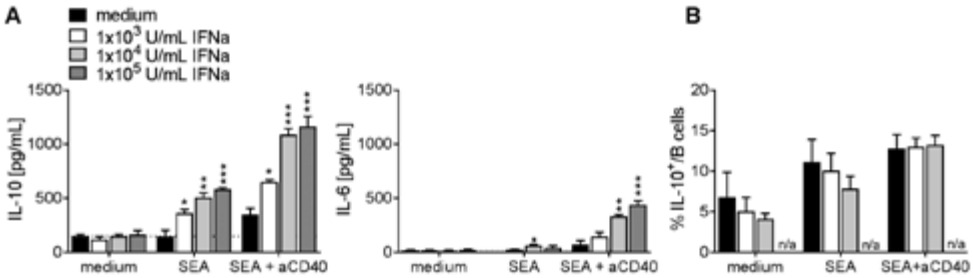

Figure 2. Recombinant IFNα enhances SEA/aCD40-induced B cell IL-10 production. B cells were isolated from the spleen of naïve mice and stimulated *in vitro* with SEA (20µg/mL), aCD40 (0.5 µg/mL) and IFNα (10^3-10^5U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and IL-6 concentration by ELISA (**A**), and the percentage of IL-10^+ B cells assessed by flow cytometry (**B**). Summary of 3 (A) or 2 (B) experiments, each data point is the mean of two technical replicates. Data are presented as mean ± SEM. Significant differences were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

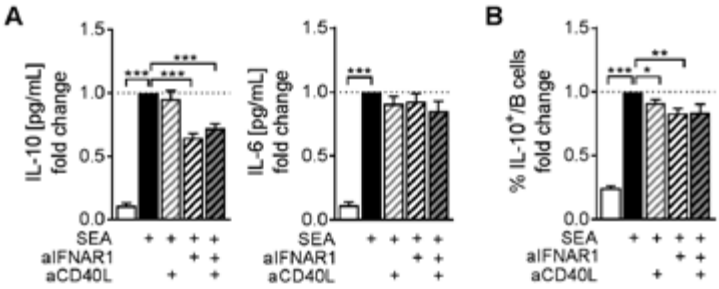

Figure 3. *Ex vivo* block of IFNAR1 reduces B cell IL-10 production. Splenocytes from SEA-injected mice (100µg SEA i.p. on d0 & d7; section d14) were re-stimulated *ex vivo* with SEA (20ug/mL) for 2 days in the presence or absence of anti-CD40L (aCD40L; 10µg/mL) and anti-IFNAR1 (aIFNAR1; 10µg/mL) blocking antibodies as indicated. After 2 days of culture, supernatants were analyzed 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 development [8]. 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 aCD40 (**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 2**). Thus IFNAR1 signalling seems to be dispensable for the induction of Breg cells to *S. mansoni* egg challenge *in vivo*.

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* infection induces a systemic IFN-I signature, and IFN-I signalling enhances *in vitro* IL-10 production by Breg cells exposed to *S. mansoni* antigens, 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 cells [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 cells [8], but the receptors ligated and signalling pathways activated by these antigens 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.

Our data showing an increased concentration of IFN-I in serum of mice actively infected with *S. mansoni* are in line with previous reports on the capacity of *S. mansoni* eggs or egg antigens,

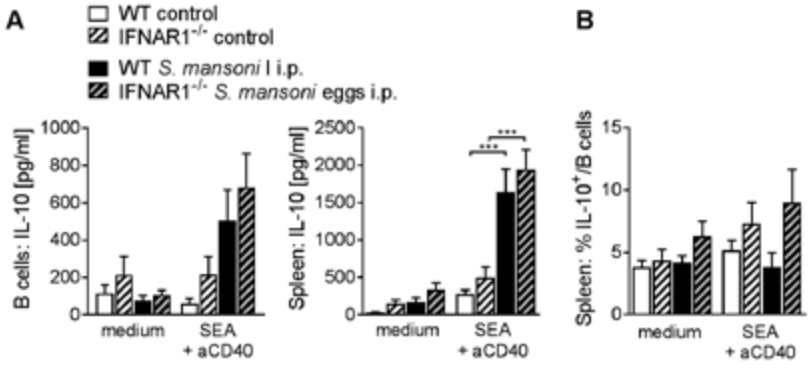

Figure 4. IFNAR1 signalling is dispensable for Breg cell induction *in* vivo. Splenocytes and MACS-isolated CD19$^+$ B cells from *S. mansoni* egg-injected mice (5000 *S. mansoni* eggs i.p. on d0 & d7; section d14) were re-stimulated *ex vivo* with SEA (20µg/mL) and aCD40 (2µg/mL) for 2 days. After 2 days of culture, supernatants of isolated B cell and total spenocyte cultures were analyzed 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. ** $p < 0.01$, *** $p < 0.001$.

H. polygrus infection and *N. brasiliensis* antigens to induce IFN-I [31-34]. pDCs are considered an important source of IFN-I [45]. 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 antigens do not induce IFN-I in human monocyte-derived DCs (Everts, personal communication).

We here show that recombinant IFNα, 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. IFNα also had 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 IFNα, suggesting that IFN-I may change the dynamics and timing of IL-10 production. Menon et al. observed an optimal IL-10 induction in naïve TLR9-stimulated B cells at $50x10^5$ U/mL IFNα and a less effective stimulation at higher concentrations [39], whereas we find an additive effect even at $1x10^6$ U/mL on both SEA- and TLR9-stimulated B cells on IL-10 concentration in culture supernatants. The fact that IFNα 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 antigens *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 IFNα/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 ligation [35]. Poovassery and colleagues report that both BCR and IFNAR signalling restore TLR7-induced B cell hyporesposiveness [36]. That the percentage IL-10$^+$ B cells tends to decrease at the end of culture might suggest that the peak of IFNα 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 IFNα 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 antibodies 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 antigen and agonistic aCD40 [8] and upon *ex vivo* restimulation as performed in this study. Finally, 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. This strongly suggests that *in vivo*, where multiple pathways are activated simultaneously and potentially act synergistically, IFN-I signalling does not play a major additive role for the development and activation of Breg cells in response to *S. mansoni*.

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-infection [31]. SEA-stimulated BMDCs induce IFN-I [32], 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.

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.

S. mansoni infection & preparation of SEA

Routinely, mice were infected percutaneously with approximately 40 cercariae, and all readouts were performed during the chronic phase of infection (14-16 weeks p.i.). For the high dose infection model, mice were infected with approximately 180 cercariae and serum collected on day 49 after infection. *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 described [46]. 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.

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.

***In vitro* stimulation**

Splenic $CD19^+$ B cells ($1.5x10^6$/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 ($5x10^{-5}$ 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 IFNα (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).

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). FcγR-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) according to the manufacturer's instructions. The concentration of cytokines in serum of chronically infected mice was assessed by BD cytometric bead array (CBA) Flex-set kits (BD Biosciences), except for IFNα3 and IFNβ which were measured by ELISA (PBL).

Statistical analysis

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

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SUPPLEMENTARY MATERIAL

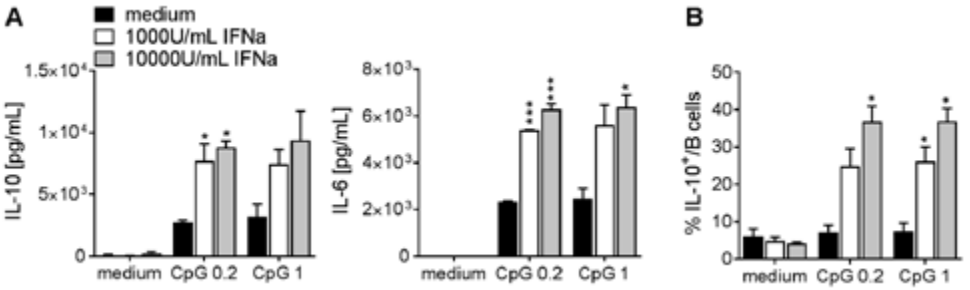

Figure S1. Recombinant IFNα enhances CpG-induced B cell IL-10 and IL-6 production. B cells were isolated from the spleen of naïve mice and stimulated *in vitro* with CpG ODN1826 (class B; 0.2-1μM) and IFNα (10^3-10^4 U/mL) as indicated. After 3 days of culture, supernatants were analyzed for IL-10 and 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 ± SEM. Significant differences were determined by one-way ANOVA followed by Dunnett's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$.

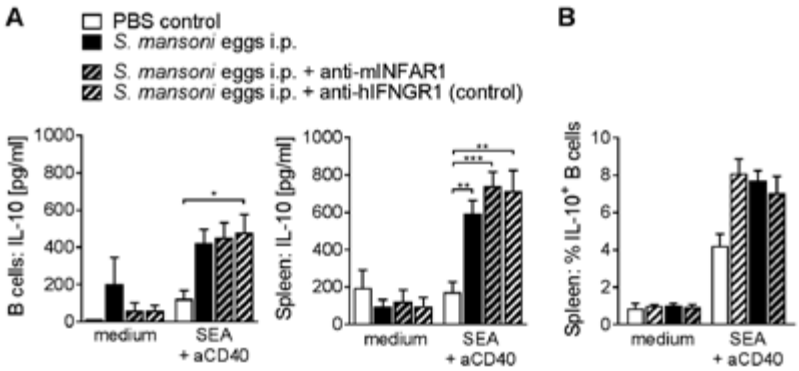

Figure S2. IFNAR1 signaling is dispensable for Breg cell induction *in vivo*. Mice were treated as depicted in **A.** On day 14, spleens were harvested and total splenocyte cell suspensions and isolated $CD19^+$ B cells restimulated with SEA (20ug/ml) and aCD40 (2ug/ml) for 2 days. Supernatants were 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 ± SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.